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ORIGINAL ARTICLE

Prospecting endophytes from different Fe or Zn accumulating wheat genotypes for their influence as inoculants on plant growth, yield, and micronutrient content

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Abstract

The plant microbiome is known to play a significant role in improving plant productivity and quality of produce, and the endophytic component was explored toward developing inoculants for enhancing micronutrient concentration in grains. A set of 213 endophytes (201 bacteria and 12 fungi) were isolated, from a set of 13 wheat genotypes, identified through preliminary screening as low and high iron and zinc accumulating genotypes. A pot experiment, with two low accumulator genotypes and eight selected endophytes, was undertaken, followed by field evaluation with both high and low Zn or Fe accumulator genotypes. Screening of these endophytes identified 11 bacteria and 2 fungi as being efficient for zinc solubilization, while 10 bacteria and 2 fungi were promising siderophore producers. Zinc and iron uptake were enhanced by one to several folds over the recommended dose of NPK (RDF) in the pot experiment. Two sets of promising endophytes, identified as *Bacillus subtilis* (DS-178) and *Arthrobacter* sp. (DS-179) for zinc accumulation, and *Arthrobacter sulfonivorans* (DS-68) and *Enterococcus hirae* (DS-163) for iron acquisition in grains, were selected. Significant increase of 14–20% in plant growth and yield was recorded in field experiment, with 75% increase, over RDF, in terms of Fe or Zn accumulation in wheat grains. Phytic acid, an anti-nutritional factor, was significantly lower in grains from endophyte inoculated treatments in the wheat genotypes evaluated. This illustrated the promise of these endophytes in improving both the translocation of micronutrients and enhancing the quality of wheat grains.

Keywords Endophytes · Micronutrients · Phytic acid · Siderophore · Translocation

Introduction

Agricultural practices are increasingly focusing on innovative strategies not only to enhance productivity but also tackle micronutrient malnutrition (hidden hunger). Most food grains show the deficiency of zinc and iron micronutrients which affects more than 2 billion human beings globally (White and Broadley 2009). Worldwide, this is known to be mainly due to the deficiency of various bioavailable micronutrients in staple crops (Govindaraj et al. 2011), resulting from intensive agricultural practices eroding its availability in agricultural soils. As high as 47 and 13% of Indian soils respectively are considered as deficient for zinc and iron (Singh 2009); in addition, arable land with low pH is categorized as poor in terms of availability of Zn and Fe (Welch et al. 1991). Plants also exhibit differential potential for the uptake of nutrients, which depends on the genetic make-up of the plant, besides the growth environment.

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The malnutrition problem can be overcome by the fortification of grains of staple crops consumed by human beings with micronutrients like Fe and Zn. Two major research foci can help to provide solutions to this problem: (1) increasing the availability of micronutrients in soils and (2) increasing their uptake and translocation by crop plants from soil, and their storage in bioavailable form, in the edible parts of the plants. There are several reports on chemical fertilization of micronutrients in soil or the use of foliar sprays in crops to improve the quality and yield of crops. However, the high reactive potential of free ions (Zn^{2+} and Fe^{2+}) raises several questions regarding the effectiveness of the soil-based application of chemical fertilizers and their availability in form(s) useful to the plant.

Endophytes can be a more effective option for improving plant growth and nutrient mobilization than rhizospheric microorganisms, as they are better linked with the metabolic activities of the plants, having originated from the internal microbiome. With the advances in modern sequencing approaches facilitating the analyses of the unculturable microbiome of plants, it has been shown that endophytes mainly belong to Phylum Proteobacteria, followed by Firmicutes and Actinobacteria (Santoyo et al. 2016), which represents an overlap with those of the rhizosphere microflora. Commonly observed genera include *Pseudomonas*, *Bacillus*, *Burkholderia*, *Stenotrophomonas*, etc. However, only scanty information is available on the role of endophytes, both bacterial and fungal endophytes, in the biofortification of cereal grains, with Fe or Zn, mainly in rice or wheat (Ramesh et al. 2014; Abaid-Ullah et al. 2015). *Piriformaspora indica*, an endophytic fungus, has been reported to improve plant growth and enhance uptake of different micronutrients (Gosal et al. 2010). Wang et al. (2014) utilized endophytes recovered from Zn hyper-accumulator *Sedum alfredii* to improve the accumulation of Zn in the grains of rice. The level of accumulation of micronutrients in the grains depends mainly on the genotype of crop plant and also on the availability of zinc and iron in the soil matrix.

However, comparative analyses of wheat genotypes and their categorization as low/high accumulators, their behavior in soils with low/high micronutrient availability, and isolation of endophytes from such characterized genotypes have not been undertaken. Although our ongoing investigations in this area have shown the potential of these endophytes in improving the translocation of zinc to the grains, in both hydroponic and soil-based pot experiments (Singh et al. 2017a, b), details regarding the preliminary aspects, particularly related to their isolation and characterization, of these isolates have not been published. The novelty of the present investigation is the use of differential accumulation of Zn^{2+} and Fe^{2+} in grains as a screen to identify high or low accumulating wheat genotypes, by growing them in two types of soil (with low/high Fe–Zn availability). This was followed by the isolation, characterization, and identification of efficient endophytes from the selected genotypes. The present investigation was also carried out with the hypothesis that inoculation with potent microbial endophyte(s) can help the plant realize its full genetic potential, particularly pertaining to the accumulation of zinc and iron in grains. To prove this hypothesis, a series of experiments were carried out that included screening of eight endophytes in low Fe/Zn accumulating wheat genotypes in pot experiment, selection of promising endophytes which enhanced zinc and iron in grains, and their evaluation under field condition, with both low/high Fe and Zn accumulating genotypes. As phytic acid in grains is an anti-nutritional factor which reduces the availability of Fe, Zn, Ca, and Mg, the grains were also analyzed for this attribute.

Materials and methods

Physico-chemical characterization of soil

The two soils were collected from farms of IARI, New Delhi and Krishi Vigyan Kendra (KVK), Hisar. Major macronutrients in soils like nitrogen, phosphorus, and potassium were determined by using alkali potassium permanganate method (Subbiah and Asija 1956), Olsen method (Olsen 1954), and flame photometer method (Standfold and English 1949), respectively. Two major micronutrients, zinc and iron, were analyzed by the DTPA extraction method (Lindsay and Norvell 1978). The pH (Singh et al. 1999a) and EC (Singh et al. 1999b) were measured and organic carbon evaluated by the method of Walkley and Black (1934).

Wheat genotypes and their characterization

A set of 13 different wheat genotypes, namely, CIM 412, WSM 24, 4HPYT 433, 4HPYT404, HD 2967, DPW 621-50, HPW B1, T-297/4HPYT 415, 4HPYT 414, GW-07-112, HD 3086, and T-311/4HPYT 429, were collected from the Division of Genetics, ICAR–Indian Agricultural Research Institute (IARI), New Delhi.

A pot study was carried out using soil from the experimental fields of IARI and KVK, Hisar. Each pot was filled with 6 kg soil, in which the NPK (120, 60, 40 kg ha^{-1}) were added at the recommended doses (RDF). Thirteen genotypes were screened for the uptake of zinc and iron in root, shoot, and grain. Eight seeds of each of the genotypes were sown in individual pots and after germination, four plants were maintained in each pot. Each treatment was taken in four replicates. Samples were collected from three replications at maturity for determination of Fe and Zn. A fine powder was made from plant parts, followed by acid digestion using HNO_3 and $HClO_4$ (4:1 ratio). Iron and zinc were analyzed using atomic emission spectrophotometry (AAS), 4100-MP AES

spectrometer Agilent Technology (Lindsay and Norvell 1978). One replicate (30-day-old plant) from each treatment was used for the isolation of bacterial and fungal endophytes from root and shoot.

Isolation of endophytes from wheat genotypes

Shoot and root samples were collected from different wheat genotypes for the isolation of endophytes. Fourteen different nutrient combinations (nutrient agar media, T_3 media, Jensen agar media, King's B agar media, soil extract agar media, trypticase soya agar media, R2A agar media, semi-solid nitrogen free bromothymol malate media, N-free Okon's media, Kenknight agar media, Pikovskaya media, potato dextrose agar media, Rose Bengal agar media, Czapek Dox agar media) were utilized for the isolation of bacterial and fungal endophytes. The fresh plant samples (1 g shoot or root) were surface sterilized sequentially using 0.1% $HgCl_2$ for 3 min and 70% alcohol for 30 s, washed with six changes of sterile distilled water, and macerated using mortar–pestle under aseptic conditions. The crushed samples were serially diluted and appropriate dilutions spread plated individually. The plates were incubated at 30 °C until growth was observed. Morphologically distinct colonies were purified by subculturing on respective plates and stored on slants at 4 °C for further studies.

Characterization of endophytes for zinc solubilization and siderophore production

Bacterial and fungal endophytes were further screened for solubilization of various insoluble zinc salts (zinc oxide, zinc carbonate, and zinc phosphate) and for siderophore production (Saravanan et al. 2007). Zinc solubilizing potential of endophytes was determined by using different concentrations (5 and 15 mM) of various zinc salts amended to glucose–tryptone agar medium containing (g/L): 5.0 tryptone, 10.0 glucose, 5 mM or 15 mM zinc salt, and 20.0 agar; pH 7.2 ± 0.2. The isolates were spot inoculated (6 μL) on the respective media and following incubation for 5 days at 30 °C, and the zone of solubilization, if any, was measured. The ability to produce siderophores was analyzed by spot inoculation of each isolate on nutrient agar medium supplemented with chrome azurol S (CAS) dye solution (Milagres et al. 1999). Plates were incubated at 30 °C for 5 days for halo zone formation and the presence of orange halo zone around the colonies was taken as a positive test.

Quantitative estimation of Zn solubilized and siderophore production

Based on qualitative analysis, 11 bacterial and 2 fungal endophytes were selected, which were efficient for zinc solubilization, and 10 bacterial and 2 fungal endophytes for siderophore production. Quantitative analysis of zinc solubilized in liquid medium amended with 5 mM of zinc salts (zinc oxide, zinc carbonate, and zinc phosphate) was further undertaken. The amount of zinc solubilized was analyzed by inoculating 1% culture of each endophytes in liquid medium and incubating at 30 °C, 150 rpm for 5 days. Un-inoculated medium for each zinc salt served as control separately. Siderophore production among selected isolates was carried out in nutrient broth with 1% inoculum followed by incubation at 30 °C, 150 rpm for 5 days. After incubation, the culture supernatant was collected by centrifugation at 10,000 rpm for 10 min. Available zinc in culture supernatant was analyzed using AAS (Lindsay and Norvell 1978). For estimation of siderophore production, 0.5 mL of dye solution containing CAS, $FeCl_3$, and cetyl trimethyl ammonium bromide (CTAB) was added to 0.5 mL of supernatant and incubated for 10 min at room temperature. The absorbance was read at 630 nm (Payne 1994). The amount of siderophores produced was calculated by estimation of Fe chelated in reaction mixture. Various concentrations of zinc (0–3 ppm) and chrome azurol S dye reagent (100–800 μL) were used for standard curve preparation and quantitative estimation of zinc and iron, respectively.

Selection of efficient endophytes for Zn and Fe acquisition and translocation in wheat grain

Six bacterial and two fungal endophytes, each efficient for Zn solubilization and siderophore production, were taken, which were isolated from 13 wheat genotypes based on preliminary experiments (Singh 2016) set up in factorial design. These endophytes were used to inoculate two different wheat genotypes, 4HPYT-404 and CIM-412, which had shown lesser accumulation of zinc or iron in grains, respectively. A pot experiment using Hisar soil (low zinc and iron availability) was carried out in National Phytotron Facility at ICAR–IARI, New Delhi. The soil was sterilized by tyndallization/intermittent sterilization procedure, using an autoclave, and 6 kg soil was filled in each pot. The wheat seeds were coated with inoculum by soaking the seeds in log phase broth cultures (containing10^9 CFU/mL) for 30 min, the seeds coated with only nutrient broth were used for the uninoculated RDF treatments. Recommended dose of NPK (120:60:40 kg/ha) was applied in all the treatments. All treatments were taken in triplicate and randomized. The pot experiment was repeated to confirm the results.

Identification of selected efficient endophytes

Four most promising isolates were taken up for 16S rRNA-based identification. Genomic DNA was extracted by using ZR fungal/bacterial DNA Mini Prep™ genomic DNA

isolation kit (ZYMO Research Corporation, USA) and amplified 16S rRNA gene was amplified using PA and PH universal primer (Edwards et al. 1989). Amplified products of 16S rRNA gene were purified using Qiagen purification Kit. Sequencing of the purified 16S rRNA gene was done by Sci-Genome Pvt. Ltd., Bangalore. The aligned sequences were analyzed for maximum homogeneity with available 16S rRNA gene sequence in NCBI database through BLASTn tools and the similarity index was used for their identification. The sequences were aligned using CLUSTAL W program and phylogenetic tree constructed using Mega7 software (Kumar et al. 2016). These sequences have been submitted to NCBI, with the accession numbers MH204206–204209, respectively, for the promising bacterial strains—*Arthrobacter sulfonivorans* DS-68, *Enterococcus hirae* DS-163, *Bacillus subtilis* DS-178, and *Arthrobacter* sp. DS-179.

Plant growth-promoting activities of selected efficient endophytes

IAA production ability of endophytes was determined using the method of Patten and Glick (2002), and phosphate solubilization ability of endophytes was determined by spotting on tricalcium phosphate containing Pikovskaya (1948) agar plates. HCN production ability of endophytes was determined using Castric's method (Castric 1975). Detection of ammonia production was done using Dye's method (Dye 1962). The ability to produce siderophore was analyzed by spot inoculation of each isolate on nutrient agar medium supplemented with chrome azurol S (CAS) dye solution (Milagres et al. 1999). ACC deaminase enzyme activity was assayed according to method of Glick et al. (1998). Acetylene reduction assay (ARA) was used for examination of nitrogenase activity of the endophytes (Lee and Yoshida 1997).

Field experiment

To evaluate the performance of the promising endophytes, a field experiment was conducted during Rabi season of 2015–2016 in the experimental field of ICAR–IARI, New Delhi. Two endophytes *A. sulfonivorans* DS-68 and *E. hirae* DS-163, selected as being most promising for the biofortification of Fe and two endophytes *B. subtilis* DS-178 and *Arthrobacter* sp. DS-179, efficient for the biofortification of Zn in low accumulator wheat genotype, identified based on pot experiment were used. Inocula were prepared by amending nutrient broth (50 mL) and incubating at 30 °C, 160 rpm for 24 h, to maintain 10^9 CFU/ mL. The wheat seeds were soaked in culture broth for 30 min. The seeds used for the RDF treatments (Control) were treated with blank nutrient broth. Recommended doses of fertilizers (RDF—NPK 120:60:40 kg/ha) was uniformly given to all the plots. This experiment was repeated again in Rabi season of 2016–2017. Details of physico-chemical characteristics given represent the mean of the 2 years (Supplementary Table 1).

The field experiment was set up in factorial design with 16 treatments, arranged in a randomized mode, in triplicate. (1) Low or high Fe accumulating genotypes (4HPYT-414 and 4HPYT-433, respectively) were inoculated with (i) RDF + *A. sulfonivorans* DS-68, (ii) RDF + *E. hirae* DS-163, (iii) RDF + $FeSO_4$, and (iv) only RDF; and (2) low or high Zn accumulating genotypes (4HPYT-414 and K-65, respectively) were inoculated with (i) RDF + *B. subtilis* DS-178, (ii) RDF + *Arthrobacter* sp. DS-179, (iii) RDF + $ZnSO_4$, and (iv) only RDF. The plot size was 12 m^2 and row to row distance was 30 cm. Bunds were made around the plots to avoid mixing of inocula. The plants were watered as and when required.

Analyses of plant growth, biomass, and yield

Five plants were harvested from each treatment replicate after 30 and 60 days of sowing and data on shoot length, and root and shoot fresh weight was recorded. The plant parts were oven dried at 80 °C for 3 days and the dry weight recorded. At harvest, the data was recorded for number of spikes/m^2, number of grains/spike, 1000-grain seed weight, and grain yield (kg/ha). All the samples were collected in triplicate.

Analysis of grain quality

Zinc and iron accumulation in harvested plants of each treatment were analyzed by following the method of Lindsay and Norvell (1978). Wheat grain quality was determined by estimation of proteins, carbohydrates, phytic acid, and phosphorus. The total protein content of the wheat grains was estimated by Bradford method (1976). Total carbohydrate content was estimated by anthrone method (Hodge et al. 1962). Phytic acid content was determined using the protocol given by Wade and Morgan (1955). Phosphorus content was estimated by the method of Jackson (1967).

Statistical analysis

The experimental data was used for generation of mean values of three replications of each individual treatment in an experiment, and then statistically analyzed by Minitab 17 statistical software using one-way analysis of variance (ANOVA). Comparisons between mean values of obtained data of each experiment were carried out by Tukey's test ($P < 0.05$).

Results

Characterization and classification of wheat genotypes, and analyses of soil characteristics

The 13 genotypes of wheat showed a differential behavior when grown in soil containing low or high available zinc/iron content. Three genotypes (4HPYT-404, GW-07-112, HD-3086) accumulating < 20 mg Zn/kg grains were designated as low accumulators. The rest of the 10 genotypes accumulating > 20 mg Zn/kg grains in soils with low available zinc–iron content were designated as high accumulators of zinc (Table 1). Similarly, one genotype (GW-07-112) accumulating > 45 mg Fe/kg grains was categorized as a high accumulator and the remaining 12 genotypes were categorized as low accumulators of iron. Genotype CIM-412 exhibited highest accumulation of zinc (31.17 mg Zn/kg grains), whereas genotype GW-07-112 could accumulate the highest amount of iron in grains (52.93 mg Fe/kg grains) when grown in soil with low availability of these micronutrients, respectively (Table 2). However, the genotypes behaved differently in soils with high iron and zinc content, although in general, the accumulation of iron and zinc was higher for all genotypes evaluated, when availability was higher.

The genotypes accumulating > 35 mg Zn/kg dry weight of grain or 45 mg Fe/kg dry weight of grains, when grown in soils with high availability of these respective micronutrients, were classified as high accumulators. Only two genotypes 4HPYT-414 and CIM-412 showed < 45 mg Fe/kg grains and were categorized as low accumulators for iron (Table 2), while the remaining 11 genotypes were designated as high accumulators of iron. In the case of zinc accumulation in grains, only one genotype 4HPYT-414 was classified as low, while the remaining 12 genotypes were classified as high zinc accumulators.

Both the soils IARI and Hisar soils show contrasting physicochemical characteristics (Supplementary Table 1). The available zinc content in IARI and Hisar soil was 1.43 and 0.15 mg/kg of soil, and available iron content was 4.75 and 1.34 mg/kg of soil, respectively. Based on the measures of availability of zinc and iron in the soil, IARI soil was characterized as high available zinc and iron content soil, whereas Hisar soil was characterized as low available zinc and iron content soil.

Isolation of endophytes from wheat genotypes

Isolation of endophytes was carried out from roots and shoots of 13 different genotypes resulting in a total of 201 bacteria and 12 fungi, with distinct morphology, using 14 different media (Supplementary Table 2). Among the 213 morphotypes, 201 (94.42%) bacterial and 12 (5.58%) fungal morphotypes were obtained from shoot and root of various genotypes of wheat. The comparative distribution of bacterial and fungal isolates in shoot was 93.20 and 6.80%, respectively. Likewise, in root it was 95.54 and 4.46%, respectively. The distribution of bacterial and

Table 1 Accumulation of zinc in root, shoot, and grain of different wheat genotypes grown in low/high available zinc–iron content soils*

Genotypes	Zinc concentration (mg/kg)					
	IARI soil			Hisar soil		
	Grain	Shoot	Root	Grain	Shoot	Root
CIM-412	41.30 ± 0.97^{fg}	145.63 ± 6.72^{e}	221.07 ± 5.92^{e}	31.17 ± 1.26^{a}	90.00 ± 4.00^{a}	168.67 ± 9.02^{a}
WSM-24	40.13 ± 0.15^{fgh}	175.67 ± 10.50^{cd}	245.67 ± 6.66^{de}	25.27 ± 0.25^{bc}	60.33 ± 4.51^{c}	140.33 ± 4.51^{bcd}
4HPYT-433	46.70 ± 1.47^{d}	194.33 ± 7.37^{bcd}	280.00 ± 7.94^{bc}	21.30 ± 0.20^{de}	39.33 ± 0.58^{def}	145.00 ± 9.00^{bc}
4HPYT-404	50.17 ± 0.12^{c}	200.00 ± 10.0^{abc}	306.70 ± 11.60^{a}	13.80 ± 0.61^{h}	32.00 ± 1.73^{f}	97.00 ± 7.26^{g}
K-65	69.70 ± 1.57^{a}	186.67 ± 6.51^{bcd}	275.07 ± 10.10^{bc}	22.40 ± 0.10^{d}	43.67 ± 3.06^{d}	122.40 ± 0.10^{def}
HD-2967	39.30 ± 0.20^{gh}	177.00 ± 14.18^{cd}	269.30 ± 10.10^{cd}	20.53 ± 0.06^{e}	38.33 ± 2.08^{def}	135.00 ± 10.00^{cd}
DPW 621-50	45.60 ± 1.64^{de}	182.67 ± 5.69^{bcd}	280.40 ± 5.39^{bc}	26.37 ± 0.12^{bc}	54.67 ± 2.52^{c}	155.00 ± 10.00^{ab}
HPW B1	43.03 ± 0.12^{ef}	174.67 ± 8.39^{d}	274.67 ± 10.79^{bc}	20.10 ± 0.10^{ef}	35.33 ± 2.52^{ef}	122.37 ± 2.47^{def}
T-297/4HPYT-415	48.00 ± 0.17^{cd}	223.00 ± 2.65^{a}	310.00 ± 10^{a}	25.13 ± 0.12^{c}	45.33 ± 2.08^{d}	126.73 ± 2.83^{cde}
4HPYT-414	31.20 ± 1.82^{i}	126.67 ± 6.43^{e}	194.00 ± 4.58^{f}	22.30 ± 0.20^{d}	40.67 ± 2.08^{de}	145.00 ± 9.00^{bc}
GW-07-112	38.10 ± 0.20^{h}	183.33 ± 11.06^{bcd}	244.77 ± 11.55^{de}	17.43 ± 0.06^{g}	37.67 ± 2.52^{def}	104.67 ± 5.03^{fg}
HD-3086	59.00 ± 1.0^{b}	204.00 ± 3.61^{ab}	294.60 ± 4.80^{ab}	19.17 ± 0.15^{f}	39.67 ± 1.53^{def}	108.00 ± 3.00^{efg}
T-311/4HPYT-429	41.00 ± 1.0^{fgh}	185.33 ± 8.62^{bcd}	231.83 ± 6.29^{e}	26.50 ± 0.50^{b}	69.67 ± 4.16^{b}	126.50 ± 0.50^{cde}

*The experimental data are the average of three replicates ± SD. Mean with different letters in the same column differ significantly at $P \leq 0.05$ (Tukey's test)

Table 2 Accumulation of iron in root, shoot, and grain of different wheat genotypes grown in low/high available zinc–iron content soils*

Genotypes	Fe concentration (mg/kg)					
	IARI soil			Hisar soil		
	Grain	Shoot	Root	Grain	Shoot	Root
CIM-412	43.32 ± 2.65^{f}	376.33 ± 6.51^{b}	802.67 ± 6.43^{a}	22.00 ± 1.80^{h}	152.67 ± 9.45^{g}	201.00 ± 3.61^{g}
WSM-24	53.67 ± 1.15^{de}	177.00 ± 6.93^{f}	328.33 ± 20.98^{fg}	27.83 ± 1.26^{fg}	238.67 ± 11.37^{abc}	251.67 ± 5.86^{cdef}
4HPYT-433	86.30 ± 0.97^{a}	367.00 ± 13.00^{b}	691.00 ± 7.21^{b}	31.53 ± 2.15^{def}	208.00 ± 11.36^{def}	257.33 ± 8.08^{cde}
4HPYT-404	49.29 ± 2.59^{ef}	473.00 ± 25.94^{a}	814.33 ± 10.02^{a}	38.17 ± 0.15^{b}	233.00 ± 11.00^{abcd}	261.67 ± 7.09^{cd}
K-65	57.75 ± 3.05^{bcd}	364.00 ± 20.42^{b}	794.00 ± 11.27^{a}	31.67 ± 2.08^{def}	198.00 ± 8.00^{ef}	309.33 ± 10.07^{b}
HD-2967	65.83 ± 0.76^{b}	285.83 ± 10.28^{de}	365.83 ± 0.76^{e}	26.00 ± 2.00^{g}	218.33 ± 9.45^{cde}	246.00 ± 6.00^{def}
DPW 621-50	60.33 ± 0.29^{bcd}	273.67 ± 5.92^{de}	359.67 ± 5.51^{ef}	36.97 ± 0.15^{bc}	180.00 ± 5.00^{fg}	250.30 ± 5.89^{cdef}
HPW B1	57.17 ± 0.76^{cde}	312.33 ± 2.52^{cd}	612.67 ± 12.06^{c}	33.43 ± 0.06^{cde}	234.00 ± 10.00^{abcd}	293.33 ± 5.03^{b}
T-297/4HPYT-415	52.43 ± 1.86^{de}	349.33 ± 5.97^{bc}	705.83 ± 5.39^{b}	33.70 ± 1.15^{cde}	248.00 ± 10.00^{ab}	267.33 ± 10.50^{c}
4HPYT-414	33.80 ± 3.32^{g}	151.50 ± 9.50^{f}	316.33 ± 7.77^{g}	34.57 ± 0.21^{bcd}	185.33 ± 12.06^{f}	249.10 ± 7.71^{cdef}
GW-07-112	65.17 ± 0.29^{bc}	252.17 ± 15.61^{e}	368.50 ± 5.63^{e}	52.93 ± 1.43^{a}	251.00 ± 8.19^{a}	353.00 ± 2.65^{a}
HD-3086	63.50 ± 6.19^{bc}	305.67 ± 17.21^{d}	589.00 ± 5.20^{cd}	30.07 ± 0.06^{ef}	197.67 ± 8.39^{ef}	239.37 ± 5.04^{ef}
T-311/4HPYT-429	60.33 ± 4.75^{bcd}	288.50 ± 11.69^{de}	571.33 ± 27.15^{d}	33.33 ± 0.85^{cde}	221.67 ± 10.41^{bcde}	232.67 ± 8.08^{f}

*The experimental data are the average of three replicates ± SD. Mean with different letters in the same column differ significantly at $P \leq 0.05$ (Tukey's test)

fungal endophytes among various genotypes of wheat varied widely (Supplementary Fig. 1). Bacterial endophytes were frequently found in both shoot and root in all genotypes (Fig. 1), but the frequency of morphotypes in root was higher. Fungal endophytes were found only in CIM-412, WSM-24, DPW621-50, T-297/4HPYT-415, and 4HPYT-414 genotypes and more abundant in root (Fig. 1).

Characterization of endophytes for zinc solubilization and siderophore production

The endophytes were screened for solubilization of different insoluble sources of zinc and for siderophore production in plate assays (Supplementary Fig. 2). The qualitative analysis showed that out of 213 endophytes, 108 (100 bacteria and 8

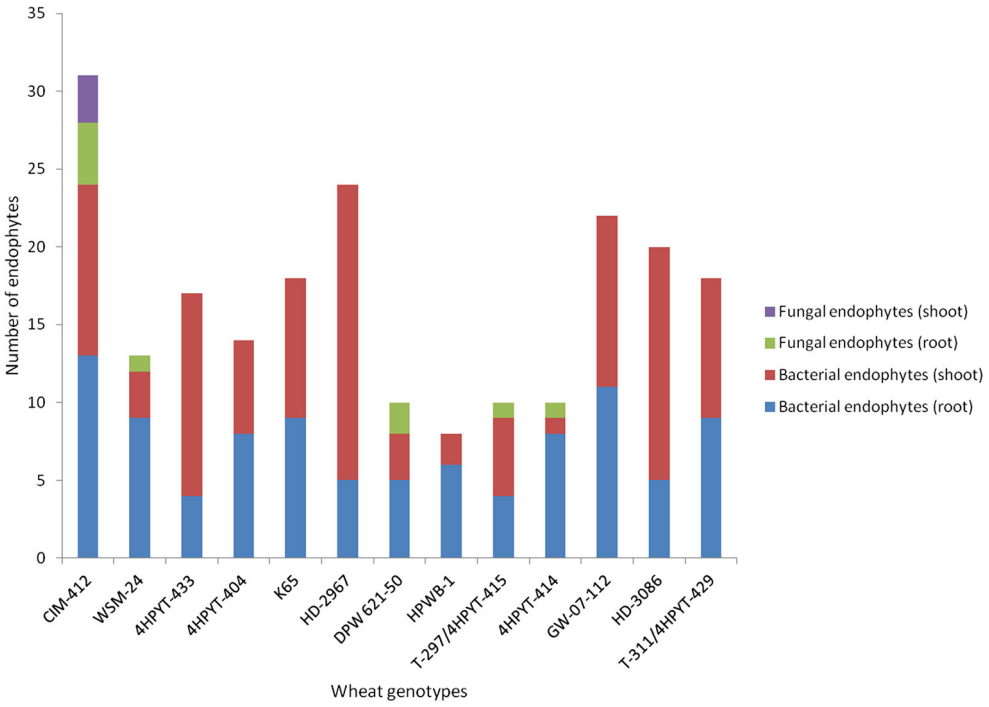

Fig. 1 Relative distribution of bacterial and fungal endophytes in root and shoot tissues of different wheat genotypes

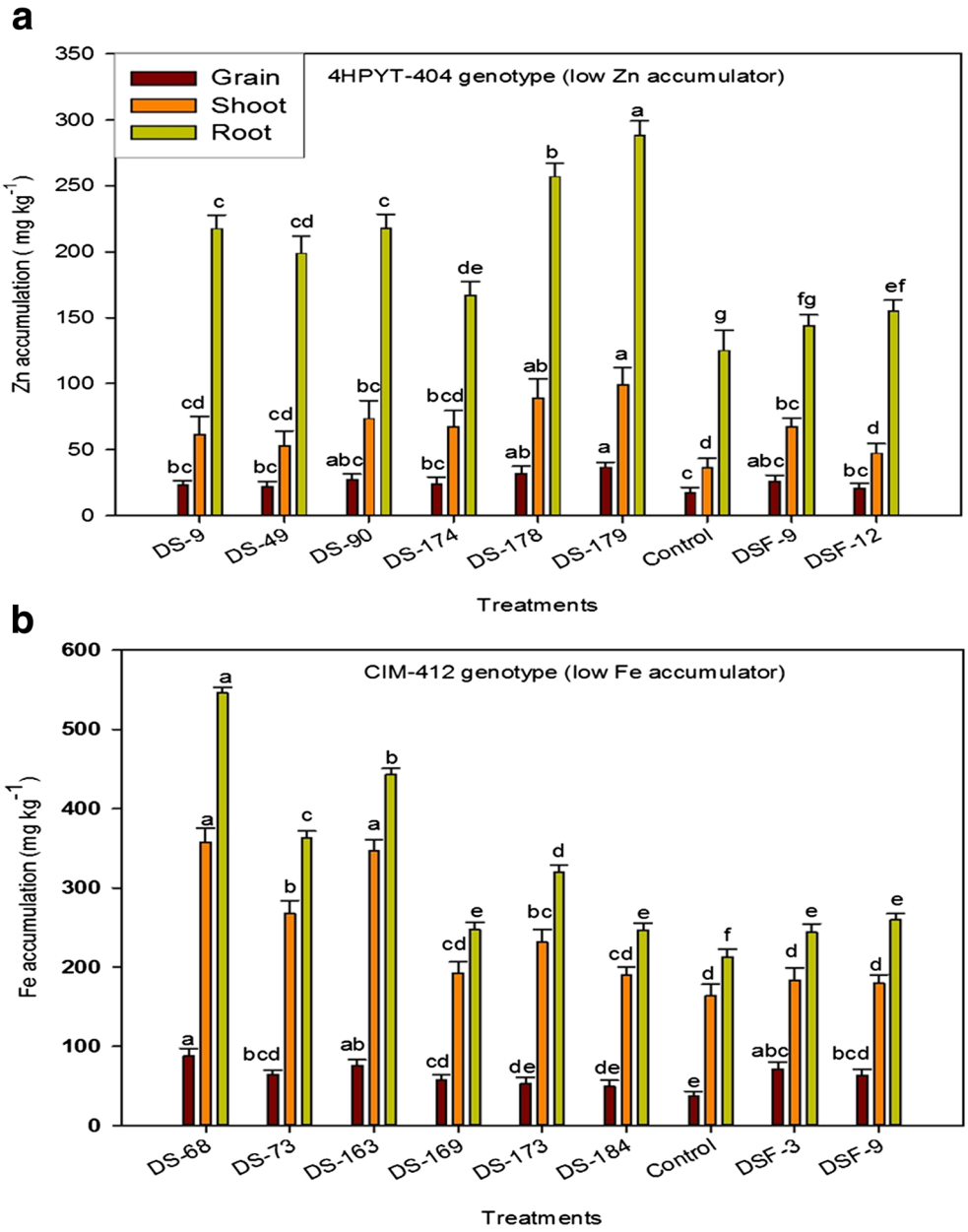

Fig. 2 Influence of inoculation of endophytes on zinc and iron uptake in root, shoot, and grain of low zinc and iron accumulating genotype of wheat grown under low available zinc–iron soils: **a** 4HPYT-404 genotype; **b** CIM-412 genotype

fungi) and 51 (49 bacteria and 2 fungi) isolates were able to solubilize different insoluble sources of zinc or produce siderophore, respectively (Supplementary Tables 3 and 4). Among the different salts of zinc, zinc oxide was solubilized by maximum number of isolates both at 5 and 15 mM concentration, while zinc phosphate was solubilized by only 30% of the isolates.

Quantitative estimation of zinc solubilized and siderophore production

Based on qualitative analysis, 11 bacterial and 2 fungal isolates were selected for solubilization of zinc salts (zinc oxide, zinc carbonate, and zinc phosphate) in liquid medium, whereas 10 bacterial and 2 fungal isolates were used for quantitative estimation of siderophore production. Among the bacterial and fungal isolates, solubilization of zinc in liquid medium ranged from 1.46 to 8.33 μg/mL, 2.33–7.77 μg/mL, and 1.20–5.83 μg/mL when zinc oxide, zinc carbonate, and zinc phosphate were used as insoluble sources of zinc, respectively (Supplementary Fig. 3A). The siderophore production measured in terms of CAS dye chelated Fe per minute ranged from 12.95 to 46.41 μg among the 12 isolates screened; highest values were recorded in DS-68 (Supplementary Fig. 3B).

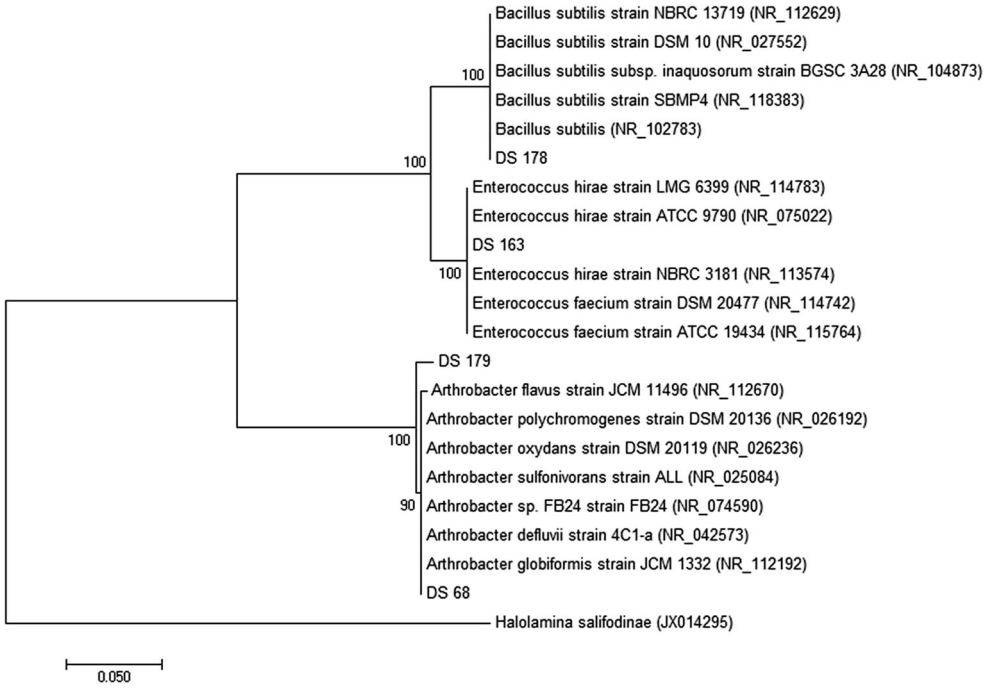

Fig. 3 Phylogenetic tree showing the relationship among the four endophytes, 16S rRNA gene sequences with reference sequence obtained through BLAST analysis. The sequence alignment was performed using CLUSTAL W program and tree constructed tree using maximum likelihood method using Mega7 software (Kumar et al. 2016)

Selection of efficient endophytes for Zn and Fe acquisition and translocation

Preliminary studies were carried out in soil with low availability of zinc–iron using wheat genotypes identified as low accumulators for zinc or iron, with eight endophytes (six bacteria and two fungi), efficient for zinc solubilization or siderophore production, as inoculants. In general, all the endophytes significantly improved the accumulation of iron and zinc in the grains, as compared to uninoculated RDF. The percent increase in zinc uptake ranged from 15% to more than 2-fold and iron uptake ranged from 15% to almost 3-fold, respectively, due to inoculation of endophytes. Among the endophytes, the isolates DS-178 and DS-179 were more potent for zinc acquisition (Fig. 2a), whereas endophytes DS-68 and DS-163 were efficient for iron acquisition in grains (Fig. 2b). In general, for all the treatments, the content of zinc and iron was more in roots followed by shoots and grain. On an average, the zinc content in shoot was 32.71% of that in root, while in grains, it was 41.75% of shoots in RDF. Endophyte inoculation that led to average values of Zn in shoot and grain were 32.8% of that in root and 39.25% of that in shoot, respectively. Similarly for iron concentration, the percentage value in shoots to that of root was 77.01% and in grains it was 22.83% of shoots in the uninoculated RDF. Due to endophyte inoculation, the average values of Fe in shoot and grain were 73.06 and 26.79% of the values recorded for root and shoot, respectively (Supplementary Table 5).

Table 3 Response of low and high Zn/Fe accumulating wheat genotypes to inoculation of bacterial endophytes in high available zinc–iron content soils, in terms of yield and yield component*

Treatments	4HPYT-414 genotype (low Fe accumulator)		4HPYT-433 genotype (high Fe accumulator)	
	Number of spikes (m^2)	Yield (kg/ha)	Number of spikes (m^2)	Yield (kg/ha)
RDF	171.0 ± 22.0^{b}	2106.0 ± 110.0^{b}	196.0 ± 15.0^{b}	2653.0 ± 115.0^{b}
RDF + $FeSO_4$	252.0 ± 12.0^{a}	2416.0 ± 76.0^{a}	264.0 ± 35.0^{a}	2950.0 ± 100.0^{a}
RDF + *A. sulfonivorans* DS-68	244.0 ± 13.0^{a}	2641.01 ± 99.0^{a}	273.0 ± 25.0^{a}	3100.0 ± 100.0^{a}
RDF + *E. hirae* DS-163	225.0 ± 16.0^{a}	2416.0 ± 125.0^{a}	238.0 ± 15.0^{ab}	2960.0 ± 125.0^{a}
	4HPYT-414 genotype (low Zn accumulator)		K-65 genotype (high Zn accumulator)	
RDF	171.0 ± 22.0^{b}	2106.0 ± 110.0^{b}	211.0 ± 11.0^{c}	2726.0 ± 87.0^{c}
RDF + $ZnSO_4$	265.0 ± 16.0^{a}	2410.0 ± 116.0^{a}	268.0 ± 17.0^{b}	3190.0 ± 65.0^{b}
RDF + *B. subtilis* DS-178	260.0 ± 20.0^{a}	2650.0 ± 110.0^{a}	301.0 ± 7.0^{a}	3503.0 ± 55.0^{a}
RDF + *Arthrobacter* sp. DS-179	226.0 ± 15.0^{a}	2453.0 ± 60.0^{a}	253.0 ± 12.0^{b}	3025.0 ± 125.0^{b}

*The experimental data are the average of three replicates ± SD. Mean with different letters in the same column differ significantly at $P \leq 0.05$ (Tukey's test)

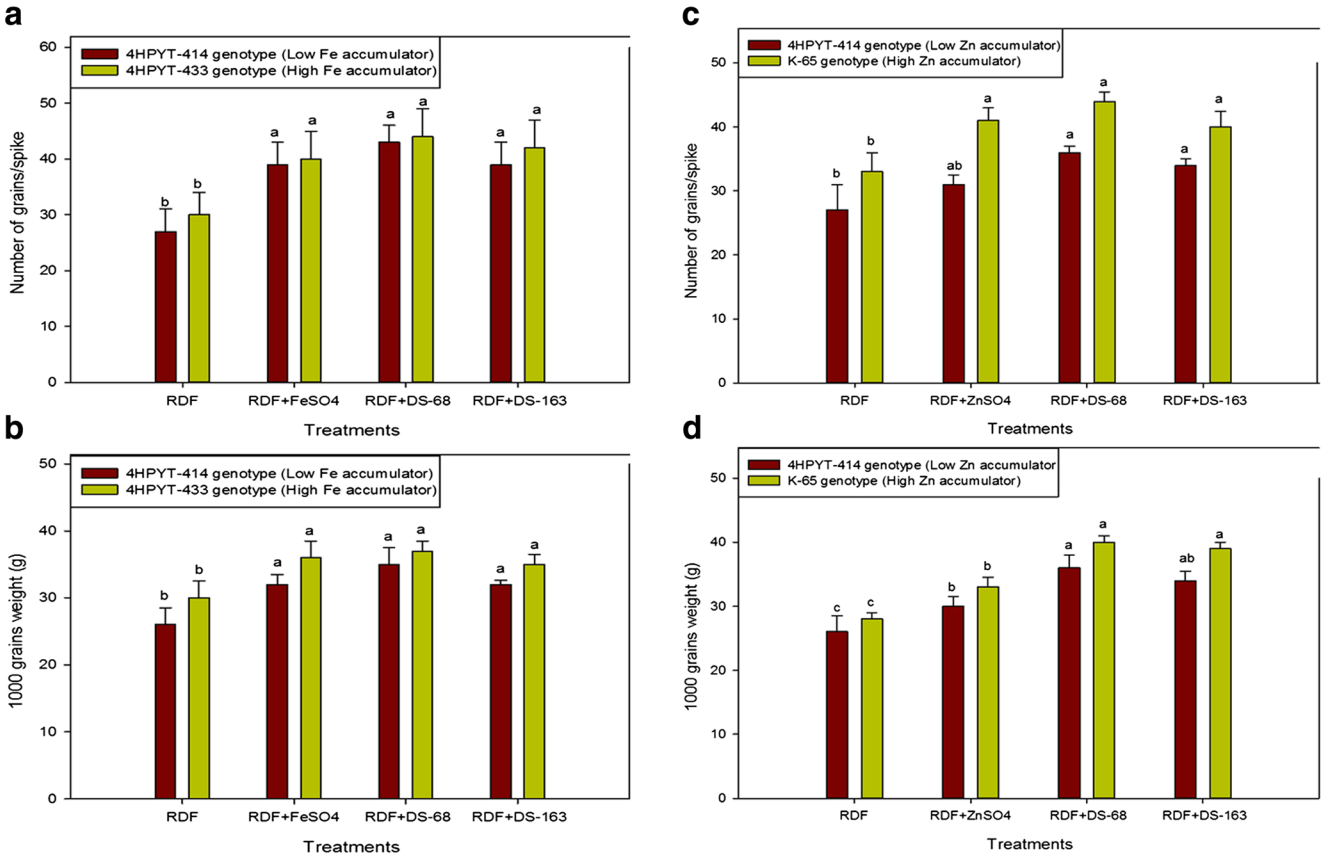

Fig. 4 Response of low and high Zn accumulating wheat genotypes to inoculation of bacterial endophytes in high available zinc–iron soils, in terms of number of grains/spike and 1000-grain weight

Phylogenetic characterization of the efficient endophytes

Based on the 16S rRNA gene sequencing, in the four most efficient isolates, two for zinc accumulation in grains were identified as *B. subtilis* (DS-178) and *Arthrobacter* sp. (DS-179), and two for iron acquisition were identified as *Arthrobacter sulfonivorans* (DS-68) and *Enterococcus hirae* (DS-163) (Fig. 3). These sequences have been submitted to NCBI, with the accession numbers MH204206–204209.

Plant growth-promoting activities of endophytes

All the four endophytes showed IAA production, siderophore production, phosphorus solubilization, and ammonia production ability. Among the endophytes, *A. sulfonivorans* DS-68 produced the highest amount of IAA (28 μg/mL) and *Arthrobacter* sp. DS-179 exhibited greater ammonia production ability. *Arthrobacter sulfonivorans* (DS-68) and *E. hirae* (DS-163) were more efficient for phosphorus solubilization activity. Only *B. subtilis* tested positive for HCN production. None of the endophytes showed ACC deaminase activity or nitrogen-fixing potential (Supplementary Table 6).

Influence of endophytes on wheat genotypes in field experiment

Our results revealed that endophyte inoculation significantly increased the fresh weight and dry weight of root and shoot, whereas a non-significant effect on shoot length was recorded (Supplementary Table 7). In case of low Fe accumulating wheat genotype, siderophore producing endophytes increased the root fresh weight and dry weight by 2- to 2.3-fold, respectively, over the RDF. Likewise, in the case of high Fe accumulating wheat genotype, root/shoot fresh weight and dry weight were enhanced by more than 1–2-fold over the RDF. Inoculation of siderophore producing endophytes brought about 1.3- to 2.4-fold enhancement in terms of shoot length, shoot fresh weight, and shoot dry weight over the RDF treatment in the low Fe accumulating wheat genotype.

Inoculation of zinc solubilizing endophytes brought about a 2-fold increase in the root fresh weight and dry weight of both low and high zinc accumulating wheat genotypes, over the RDF. However, both low and high zinc accumulating wheat genotypes exhibited differential behavior for length, fresh weight, and dry weight of shoots, with respect to endophyte inoculation. In case of low zinc accumulating wheat genotype, shoot length, shoot fresh weight, and shoot dry

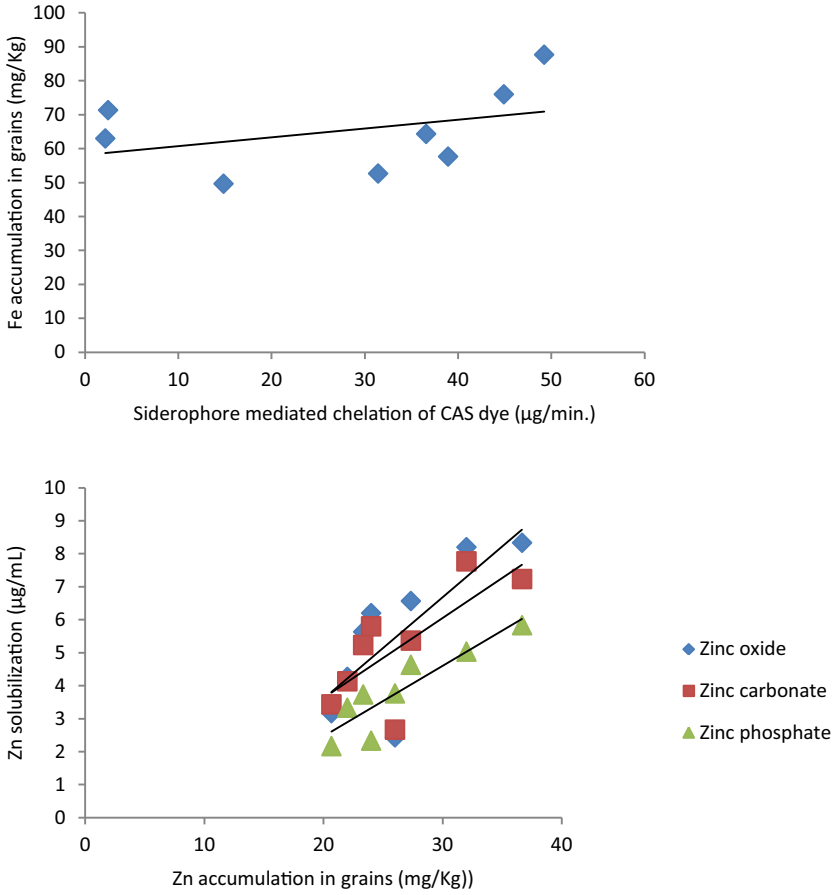

Fig. 5 Correlation between microbial phenotypes of Zn solubilization/siderophore production and the plant phenotype designated in terms of of mineral accumulation

weight enhanced by 1–2-fold over RDF, while 8%, 92%, and 61% increase was observed in the high zinc accumulating wheat genotype. It was evident that in the treatment RDF + $FeSO_4$ or $ZnSO_4$, only the high accumulator wheat genotypes showed significant increases in fresh and dry weight of root.

The yield and yield parameters were significantly influenced by application of $FeSO_4$ or $ZnSO_4$ and also by the inoculation of endophytes (Table 3 and Fig. 4). Both the endophytes (*A. sulfonivorans* DS-68 and *E. hirae* DS-163) used were statistically significant in both low and high Fe accumulating genotypes with regard to for Fe fortification, yield, and yield parameters (number of spikes/m^2, number of grains/spike, 1000-grain seed weight). Inoculation of siderophore producing endophytes increased the grain yield of low Fe accumulating genotype by 20%, while the high Fe accumulating wheat genotype exhibited 14.2% more yield, as compared to RDF. *Arthrobacter sulfonivorans* DS-68 had more influence on yield in both low and high Fe accumulating genotypes with respect to yield, as compared to *E. hirae* DS-163 and $FeSO_4$ treatments.

Inoculation of zinc solubilizing endophytes increased the grain yield of both low and high zinc accumulating wheat genotypes by almost 20%. However, among the endophytes or treatments, no significant differences were recorded in both low and high Fe accumulating genotypes, in terms of yield and yield parameters. Application of $FeSO_4$ could significantly influence yield and yield parameters for all treatments.

The low and high Zn accumulating genotypes also gave a similar trend in terms of yield, when fertilized with $ZnSO_4$, or inoculated with endophytes (Table 3 and Fig. 4). The performance of both endophytes was statistically significant in terms of yield and other yield-related parameters analyzed. The grain yield and other yield parameters also responded significantly to amendment of $ZnSO_4$, particularly in the low accumulator. However, in the high accumulator genotype K-65, a significant difference was observed with regard to yield and yield parameters with *B. subtilis* DS-178.

Response of endophyte inoculation on accumulation of Zn and Fe in wheat under field condition

Our results revealed that Fe and Zn accumulation was highest in roots, followed by shoots and grains. Biofortification of Fe and Zn in different plant tissues was significantly improved by endophyte inoculation (Table 4). In general, the amount of Fe and Zn in grains due to inoculation of endophytes was 1.5-fold higher as compared to uninoculated RDF (RDF), as compared

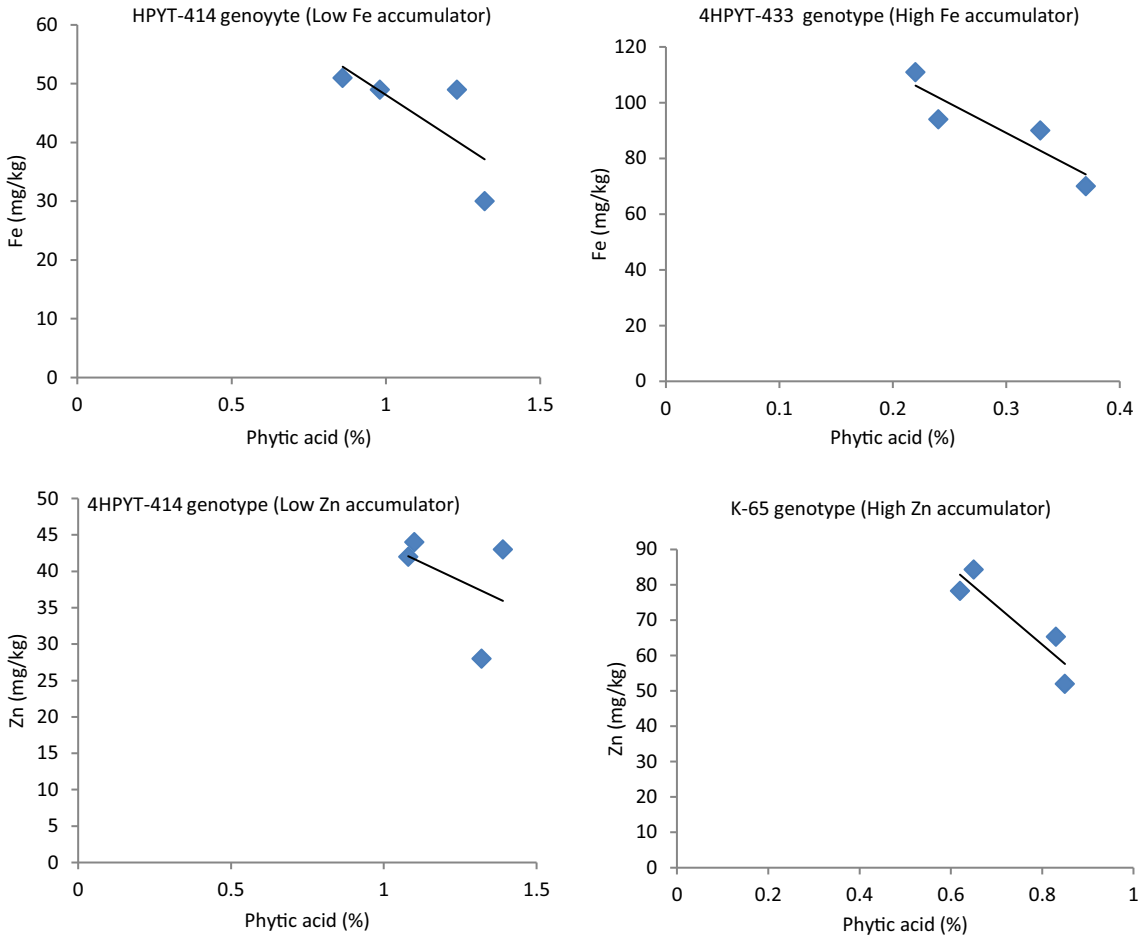

Fig. 6 Correlation between phytic acid and Fe/Zn content in wheat grains

to 1.4-fold higher values due to application of $FeSO_4$ and $ZnSO_4$.

Application of $FeSO_4$ or $ZnSO_4$ also resulted in statistically significant increase in Fe or Zn content in grains. In case of Fe accumulation, all treatments increased the Fe accumulation in plant parts, but did not show significant differences among the treatments. A similar trend was found with respect to zinc accumulation, although numerically higher or statistically at

Table 4 Response of low and high Zn/Fe accumulating wheat genotypes to inoculation of bacterial endophytes in high available zinc–iron content soils (field trial) in terms of biofortification of Fe and Zn in roots, shoots, and grains*

Treatments	Fe concentration (mg/kg)					
	4HPYT-414 genotype (low Fe accumulator)			4HPYT-433 genotype (high Fe accumulator)		
	Root	Shoot	Grain	Root	Shoot	Grain
RDF	306.0 ± 16.0^b	160.0 ± 12.0^c	30.0 ± 2.0^b	780.0 ± 116.0^a	454.0 ± 26.0^b	70.0 ± 9.0^b
RDF + $FeSO_4$	403.0 ± 20.0^a	262.0 ± 15.0^b	49.0 ± 6.01^a	942.0 ± 123.0^a	646.0 ± 16.0^a	90.0 ± 4.0^a
RDF + *A. sulfonivorans* DS-68	453.0 ± 12.0^a	299.0 ± 10.0^a	51.0 ± 6.03^a	1033.0 ± 125.0^a	640.0 ± 37.0^a	111.0 ± 8.0^a
RDF + *E. hirae* DS-163	428.0 ± 25.0^a	253.0 ± 7.0^b	49.0 ± 5.05^a	933.0 ± 119.0^a	654.0 ± 21.0^a	94.0 ± 10.0^a
	Zn concentration (mg/kg)					
	4HPYT-414 genotype (low Zn accumulator)			K-65 genotype (high Zn accumulator)		
RDF	194.0 ± 7.01^b	135.0 ± 14.0^b	28.0 ± 4.01^b	313.3 ± 12.0^b	253.0 ± 16.01^a	52.0 ± 3.01^c
RDF + $ZnSO_4$	310.0 ± 10.12^a	216.3 ± 15.3^a	43.0 ± 6.1^a	460.3 ± 26.2^a	303.0 ± 15.2^a	65.3 ± 5.1^b
RDF + *B. subtilis* DS-178	311.0 ± 7.01^a	198.3 ± 18.12^a	42.0 ± 4.2^a	395.1 ± 14.4^a	281.0 ± 27.3^a	78.3 ± 5.04^a
RDF + *Arthrobacter* sp. DS-179	325.0 ± 25.2^a	230.3 ± 19.0^a	44.0 ± 4.03^a	446.0 ± 41.13^a	305.0 ± 23.3^a	84.3 ± 5.02^a

*The experimental data are the average of three replicates ± SD. Mean with different letters in the same column differ significantly at $P \leq 0.05$ (Tukey's test)

Table 5 Response of low and high Zn/Fe accumulating wheat genotypes to inoculation of bacterial endophytes in high available zinc–iron content soil, in terms of grain quality*

Treatments	4HPYT-414 genotype (low Fe accumulator)				4HPYT-433 genotype (high Fe accumulator)			
	Phytic acid (%)	Protein (%)	Carbohydrate (%)	Phosphorus (%)	Phytic acid (%)	Protein (%)	Carbohydrate (%)	Phosphorus (%)
RDF	1.32 ± 0.10^{a}	9.36 ± 1.16^{b}	61.00 ± 3.00^{a}	0.37 ± 0.03^{b}	0.37 ± 0.03^{a}	9.6 ± 0.66^{b}	64.33 ± 4.50^{a}	0.33 ± 0.02^{b}
RDF + $FeSO_4$	1.23 ± 0.07^{a}	10.70 ± 0.47^{ab}	65.00 ± 5.00^{a}	0.38 ± 0.03^{b}	0.33 ± 0.03^{a}	10.21 ± 3.21^{ab}	67.67 ± 3.21^{a}	0.38 ± 0.04^{b}
RDF + *A. sulfonivorans* DS-68	0.86 ± 0.03^{b}	12.21 ± 0.30^{a}	69.33 ± 4.04^{a}	0.49 ± 0.02^{a}	0.22 ± 0.01^{b}	11.87 ± 0.42^{a}	70.33 ± 2.08^{a}	0.50 ± 0.02^{a}
RDF + *E. hirae* DS-163	0.98 ± 0.03^{b}	11.67 ± 0.04^{a}	69.33 ± 2.51^{a}	0.47 ± 0.02^{a}	0.24 ± 0.03^{b}	11.36 ± 1.04^{ab}	70.00 ± 4.00^{a}	0.55 ± 0.05^{a}
	4HPYT-414 genotype (low Zn accumulator)				K-65 genotype (high Zn accumulator)			
RDF	1.32 ± 0.10^{a}	9.36 ± 1.16^{b}	61.00 ± 3.00^{b}	0.37 ± 0.03^{b}	0.85 ± 0.05^{a}	10.03 ± 0.50^{c}	65.00 ± 4.00^{a}	0.36 ± 0.03^{a}
RDF + $ZnSO_4$	1.39 ± 0.04^{a}	10.67 ± 0.51^{ab}	65.00 ± 2.00^{a}	0.39 ± 0.03^{b}	0.83 ± 0.04^{a}	10.56 ± 0.33^{bc}	67.00 ± 3.00^{a}	0.40 ± 0.03^{a}
RDF + *B. subtilis* DS-178	1.08 ± 0.09^{b}	11.50 ± 0.88^{a}	68.67 ± 3.51^{a}	0.50 ± 0.03^{a}	0.62 ± 0.02^{b}	12.19 ± 1.05^{ab}	70.33 ± 1.53^{a}	0.48 ± 0.08^{a}
RDF + *Arthrobacter* sp. DS-179	1.10 ± 0.08^{b}	11.65 ± 0.48^{a}	66.33 ± 3.51^{a}	0.49 ± 0.03^{a}	0.65 ± 0.04^{b}	12.20 ± 0.31^{a}	67.67 ± 3.51^{a}	0.47 ± 0.06^{a}

*The experimental data are the average of three replicates ± SD. Mean with different letters in the same column differ significantly at $P \leq 0.05$ (Tukey's test)

par values with the $FeSO_4$/$ZnSO_4$ treatments were recorded with the inoculation of endophytes.

Influence of endophyte inoculation on the nutritional quality of wheat grains

Phytic acid was significantly lower in grains due to endophyte inoculation, irrespective of wheat genotype used. Phytic acid reduced by 30% in low Fe accumulating wheat genotypes while in high Fe accumulating wheat genotype, the decrease was 28%, over the RDF. The zinc solubilizing endophytes decreased the phytic acid almost by 24% in both low and high zinc accumulating wheat genotypes, which was significantly lower (2–4 g/kg), as compared to RDF and RDF + $FeSO_4$ (Supplementary Table 8).

Both siderophore producing (*A. sulfonivorans* and *E. hirae* DS-163) and zinc solubilizing endophytes (*B. subtilis* DS-178 and *Arthrobacter* sp. DS-179) increased the protein concentration in grains of the wheat genotypes tested, which was significantly higher than the $FeSO_4$ or $ZnSO_4$ + RDF treatment for the low Fe accumulating genotype 4HPYT-414, but not for the others (Table 5). Carbohydrate content of grains ranged from 600 to 700 g/kg, but did not exhibit significant differences among the inoculated or uninoculated treatments, except with the RDF treatment involving low Zn accumulator (Supplementary Table 8). Phosphorus content in grains was significantly enhanced due to inoculation with endophytes in the low Zn/Fe accumulator and high Fe accumulator genotypes. High Zn accumulator K-65 was statistically equal to uninoculated RDF in terms of phosphorus content in grains (Table 5; Supplementary Table 8).

Discussion

Rice and wheat are the two staple cereal crops, which represent important sources of nutrition to millions of people around the world. The low availability of zinc and iron, the two most important micronutrients, is a global concern; hence, concerted efforts are required to fortify rice and wheat grains with these micronutrients.

Survey of published literature revealed that rhizospheric microorganisms are important agents for biofortification of crop grains with Fe or Zn (Abaid-Ullah et al. 2015; Adak et al. 2016; Rana et al. 2012). However, in recent years, the focus has shifted the use of endophytes based on interesting information regarding the role of physiological and metabolic activities of endophytes in the growth, health, and development of crop plants (Gaiero et al. 2013; Hardoim et al. 2008; Sturz et al. 2000). Endophytic microorganisms are more promising than rhizospheric microorganisms in promoting plant growth, as they have close proximity with internal tissues of plant (Reiter et al. 2002; Weyens et al. 2013). Similar to PGPR, direct growth promotion mechanisms of endophytes include the production of phytohormones, nitrogen fixation, phosphate solubilization, and ACC deaminase activities, while indirect mechanisms of plant growth promotion include disease suppression by the production of antimicrobial compounds like antibiotic compounds, HCN, ammonia, and siderophore production (Bashan et al. 1991; Fan et al. 2017; Glick et al. 1998; Leong, 1986; Xie et al. 1996; Yang et al. 2009) which also increases plant growth and vigor.

Zn is an essential mineral for IAA production and internode elongation. Interveinal chlorosis in mid leaves, small size of leaves, and abnormal grain formation are some major crop-specific symptoms in soils with low zinc availability (Wiese

1993; McCauley et al. 2009). Fe is a major component of chlorophyll and cytochrome, and interveinal chlorosis in newly emerging leaves is a major symptom of Fe deficiency (Follett and Westfall 1992; Soetan et al. 2010). The synthesis and release of Zn-mobilizing phytosiderophores (PS) by the roots and uptake of Zn–PS complex and Fe acquisition by extrusion of both protons and reducing substances (phenols) from the roots are common strategies of plants to overcome these deficiencies (Tagliavini and Rombolà 2001; Marschner et al. 1986). Microbe-mediated strategies include chelation of iron by siderophores, production of organic acids in the root exudates or proton extrusion leading to changes in the soil pH, as well as production of phytohormones which are implicated in the enhanced uptake of iron and zinc (Hindt and Guerinot 2012; Chen et al. 2014).

Significant variations, reflective of the inherent genetic constitution of the wheat genotypes, were observed in the accumulation of iron and zinc grown in low or high availability of these micronutrients. In literature, there are reports of significant genotypic variations in zinc concentration in seeds of cereals and legume crops (Raboy et al. 1984) and in finger millets (Yamunarani et al. 2016). In a study with 81 cultivars of bread wheat, large variations were observed in the concentration of grain iron and zinc ranging from 41.4 to 67.7 mg/kg and 36.4 to 73.8 mg/kg, respectively (Badakhshan et al. 2013). Similar results were reported in wheat accessions of diverse origin (Velu et al. 2011) and also in a set of high yielding lines of wheat (Oury et al. 2006).

Based on the amount of iron and zinc accumulated in the grains, the genotypes were classified as low and high accumulators for iron or zinc. In order to understand the significance of soil characteristics in the accumulation of iron and zinc in the grains of wheat, two soils with contrasting values of available iron and zinc were used. The soil obtained from KVK, Hisar was calcareous with low levels of iron and zinc—1.34 and 0.15 ppm was designated low available zinc–iron soils, whereas the sandy-clay-loam soil of IARI farm having high values of 4.75 and 1.43 ppm of iron and zinc, respectively, was designated as high available zinc–iron soils. The threshold levels in soils for designating as low iron availability is 4.5 ppm and for zinc is 0.6 ppm (Sillanpää 1982).

The lower critical content of Zn in whole plant is 32 mg Zn/kg in spring wheat, 15 mg Zn/kg in rice, 15–22 mg Zn/kg in maize, 8 mg Zn/kg in sorghum, 25 mg Zn/kg in winter wheat, 22 mg Zn/kg in groundnut, and 25 mg Zn/kg in chickpea (Brennan and Bolland 2002; Srivastava and Gupta 1996; Takkar 1991). However, differences can also occur between different varieties of these crops (Alloway 2001). Frossard et al. (2000) reported that critical content of Fe and Zn in wheat grains are 45 and 35 mg/kg, respectively. The Zn sufficiency range is 15–70 mg Zn/kg in spring wheat and 20–70 mg Zn/kg in corn (Rosen and Eliason 2005), while the Fe sufficiency range in wheat, rice, and pearl millet were recorded between 28.8 and 56.5 mg/kg, 7.5–24.4 mg/kg, and 32–111 mg/kg, respectively (Graham et al. 1999, 2001; Welch and Graham 2004; Anuradha et al. 2017). Perusal of the variations in the concentration of iron and zinc in root, shoot, and grains of 13 genotypes grown in low/high available zinc–iron soils clearly reflects that the soil type has a limited bearing on the classification of the genotypes into low and high accumulators. In terms of the accumulation of zinc in grains, a majority of genotypes tested were high accumulators, both in soils with low (10 out of 13) and high (12 out of 13) availability of these micronutrients. Interestingly, the iron content of soil was instrumental in the classification of the genotypes into low and high accumulators; in soils designated as low availability, 12 genotypes were identified as low accumulators whereas in the soil designated as high availability, only two genotypes were designated as low accumulators. These results indicate that the level of iron in soil is more critical as compared to zinc.

In addition to looking at the concentration of micronutrients in plant genotype and levels of nutrients in soil, the third dimension of contribution through microbial endophytes in the accumulation of iron and zinc was also investigated. There are several reports on the isolation of bacteria and fungi from the different parts of crop plants (Pereira et al. 2016; Pisarska and Pietr 2012; Xu et al. 2016), but most of these endophytes have been implicated in plant growth promotion or disease suppression (Goudjal et al. 2014; Melnick et al. 2011). Bacterial endophytes were frequently found in all wheat genotypes, but fungal endophytes were isolated only from selected genotypes. The abundance of bacterial and fungal endophytes was more pronounced in root as compared to shoot.

With a view to select potential endophytes that can enhance the accumulation of zinc and iron in grains of wheat, the ability to solubilize different insoluble sources of zinc and to produce siderophores was used as the primary screening filter. In earlier studies, it has been reported that a major part of iron is accumulated in the root system (greater than 95% of the total per plant content) of olive cultivars. Although zinc content was higher in roots, the amount accumulated was much less than 95% (Chatzistathis et al. 2009). Differences were observed in the distribution of iron and zinc in roots, shoots, and grain indicative of the partitioning of iron and zinc following different patterns in plant.

The percent Zn and Fe present in shoots to that of root was 32.71 and 77.01%, respectively; likewise, the percent zinc and iron present in grains to that of shoot was 41.75 and 22.83%, respectively. The results clearly indicate that although a lower amount of zinc is translocated from root to shoot, its partitioning to grain is better as compared to iron. The concentration of Cu, Fe, Mn, and Zn in the seeds might depend on the amount taken up by roots during grain maturity and the amount translocated to the grain from vegetative tissue (shoots, leaves, etc.) via the phloem. Earlier studies suggested

that Zn showed good phloem mobility than iron (Pearson and Rengel 1994; Kochian 1991). These studies support our remobilization results. It can be hypothesized that endophytic microbiome may be mediating better translocation and our recent published work supports these conclusions (Singh et al. 2017a, b). The endophytes isolated from zinc hyperaccumulator *Sedum alfredii* enhance the zinc concentration in shoots and roots under hydroponic conditions and also in grains, when grown in soil (Wang et al. 2014). Sirohi et al. (2015) also reported *Pseudomonas fluorescens* mediated zinc fortification in wheat. Inoculation of *Providencia* sp. PW5 resulted in an increase of 105.3% in iron content in wheat (Rana et al. 2012). Similarly, there are some reports for enhanced uptake of zinc in maize and rice due to inoculation of rhizospheric microorganisms (Prasanna et al. 2015; Tariq et al. 2007).

In earlier reports, it has been emphasized that siderophore producing microorganisms play an important role in iron acquisition and improving plant growth. Inoculation of siderophore producing fluorescent *Pseudomonas* contributed to biofortification of iron in chickpea plant (Khalid et al. 2015). The potential of zinc solubilizing bacteria in zinc fortification of soybean grains has been reported (Sharma et al. 2012). The ability to solubilize zinc appears to be prevalent among different groups of bacteria (Mäder et al. 2011; Sharma et al. 2013), and our results also support these observations. In our study, almost 50% of the endophytes (108 out of 213) were able to solubilize one or the other sources of insoluble zinc. Based on the initial screening, 11 bacteria and 2 fungi were identified as efficient for zinc solubilization, whereas 10 bacteria and 2 fungi were efficient for siderophore production. There was a positive correlation ($r = 0.383$) between siderophore production and Fe accumulation in grains by endophytes. Similarly, Zn solubilization activity of endophytes positively correlates ($r_{oxide} = 0.76$, $r_{carbonate} = 0.74$, $r_{phosphate} = 0.90$) with the accumulation of Zn in grains (Fig. 5).

The four most efficient isolates, identified for their potential in enriching zinc/iron in the grains, were identified based on 16S rRNA gene sequencing. They were identified as *B. subtilis* (DS-178), *Arthrobacter* sp. (DS-179) for Zn, and two for iron acquisition identified as *A. sulfonivorans* (DS-68) and *E. hirae* (DS-163) and the sequences submitted to NCBI, with the accession numbers MH204206–204209. Busse (2016) reclassified selected *Arthrobacter* species based on phylogenetic grouping, 16S rRNA gene sequence similarities, homogeneity in peptidoglycan types, quinine systems, and polar lipid profiles. Hence, the basonym *A. sulfonivorans* is now given the name *Pseudarthrobacter sulfonivorans.*

Inoculation of the promising two zinc solubilizing (*B. subtilis* DS-178 and *Arthrobacter* sp. DS-179) and two siderophore producing endophytes (*A. sulfonivorans* DS-68 and *E. hirae* DS-163) brought about significant enhancement in plant growth, biomass, yield, and micronutrient uptake. Endophyte inoculation increased the fresh weight and dry weight of both root and shoot by 2-fold over the RDF in low accumulating genotypes; in high accumulating genotypes, 2.4-fold higher values over the RDF were recorded after 30 days of sowing. These results are consistent with those of previous studies in which the endophytic microorganisms enhance plant growth significantly. Khan et al. (2015) observed that endophytic fungus culture filtrates increases the plant (Dongjin rice) growth attributes (root and shoot length, total biomass, and chlorophyll content) in comparison to RDF. *Cadophora malorum* Cs-8-1 endophytic fungus was also shown to promote the growth of rice plants (You et al. 2013). In our present study, two zinc solubilizing (*B. subtilis* DS-178 and *Arthrobacter* sp. DS-179) and two siderophore producing endophytes (*A. sulfonivorans* DS-68 and *E. hirae* DS-163) showed phosphate solubilization activity, IAA production ability, and siderophore production ability, which can be correlated with plant growth promotion observed. Recent reports demonstrated that endophyte inoculation enhances the plant growth and biomass through the phytohormone production, such as indole-3-acetic acid (IAA), gibberellins (GAs), and cytokinins (Khan et al. 2014a, b; You et al. 2013). Ahemad and Kibret (2014) reported that IAA produced by fungi promotes the root growth and nutrient uptake, leading to increased plant biomass. Phytohormones, especially IAA, produced by endophytic bacteria may increase the plant growth and this can trigger plant protection against adverse environmental conditions by enhancing cellular defense systems (Bianco et al. 2006).

Phosphate solubilizing endophytes increase the phosphorus availability to plants and increase plant growth and development (Richardson et al. 2001). Endophytes have two types of mechanisms to convert the insoluble phosphorus to soluble form of phosphorus: (1) organic acid production (Taurian et al. 2010) and (2) phytase or phosphatase enzymatic activity (Idriss et al. 2002). Siderophore producing and zinc solubilization activity of endophytes also promote plant growth (Berg et al. 2005; Li et al. 2008; Yang et al. 2009). These plant growth-promoting endophytes also enhanced wheat grain yield and yield-related components. Endophyte inoculation was observed to significantly increase the number of spikes/m^2, number of grains/spike, 1000-grain seed weight, and grain yield by 15–39% over RDF, with respect to low or high Fe or Zn accumulating wheat genotypes. Earlier studies have illustrated the potential of *Bacillus cereus* GS6 in improving the symbiotic efficiency of soybean through enhanced P mobilization (Arif et al. 2017).

Enhancement in micronutrients in the plant can lead to efficient metabolic activities. Endophytes or rhizospheric microorganisms possess several mechanisms to enhance the uptake of micronutrients in plant tissues and stimulation of plant growth, and these include secretion of phenolics-like substances, siderophores, organic acids in the root exudates,

proton extrusion, eliciting modification in root morphology and anatomy of crop plants, and the production of signaling molecules such as auxin and gibberelic acid (Chen et al. 2014; Desai and Archana 2011; Fasim et al. 2002; Hayat et al. 2012; Hindt and Guerinot 2012; Ivanov et al. 2012; Kobayashi and Nishizawa 2012; Li et al. 2010). *Neotyphodium coenophialumcan* stimulated exudation of phenolic compounds in the rhizosphere of continental tall fescue (*Lolium arundinaceum*) with chelating characteristics; this was implicated in directly improving iron uptake (Malinowski et al. 2004; Malinowski and Belesky 2000). Our recent work using these endophytes illustrated that the siderophore producing and zinc solubilizing endophyte modulated the organic acid production, leading to anatomical changes in root (including root hair extensions or expansion of root cortex, endodermis, pericycle, xylem vessels, and vascular bundles) and resulted in the overexpression of *TaZIP3* and *TaZIP7* genes in roots and shoots. This facilitated Fe and Zn translocation and the higher values of Fe and Zn in roots and shoots (Singh et al. 2017b).

Analyses of the results from the present experiment revealed that the low and high Fe or Zn accumulating genotypes respond positively to endophyte inoculation. This illustrates that the genetic potential of any genotype for biofortification of grains with Fe or Zn can be fully realized by inoculating with siderophore producing endophytes (for iron) or zinc solubilizing endophytes (for zinc). In our field trial, due to siderophore producing endophyte inoculation, Fe concentration in grains of low and high Fe accumulating wheat genotypes was enhanced by 67 and 46%, respectively, over the RDF. Zinc solubilizing endophytes increased the Zn concentration in grains of low and high Zn accumulating wheat genotypes by 53 and 55%, respectively, over the RDF. In case of RDF + $FeSO_4$, the amount of Fe in grains of low and high Fe accumulating wheat genotypes increased by 63 and 28%, respectively, over the RDF. In case of RDF + $ZnSO_4$, the amount of Zn in grains of low and high Zn accumulating wheat genotypes was increased by 53 and 25%, respectively, over the RDF. Although the percent increase of Fe or Zn concentration in grains of low accumulating wheat genotypes was found similar in plants receiving endophyte inoculation or $FeSO_4$/$ZnSO_4$ application, these values were much higher in genotypes designated as high accumulators. This is reflective of microbial inoculation facilitating the particular genotype to reach its true potential, in terms of genetic potential of the high and low accumulators, which is almost 2-fold higher in terms of Fe, but only 40% in terms of Zn. In our earlier investigation conducted with these two zinc solubilizing endophytes (*B. subtilis* DS-178 and *Arthrobacter* sp. DS-179) in pot experiments, 2-fold increase in the zinc concentration in wheat grains was recorded, as compared to RDF (Singh et al. 2017a); however, Fe-related analyses was not reported.

Another aspect relevant to biofortification strategies is the bioavailability of micronutrients in cereal and legume grains, which is often low because it is affected by antinutritional factors such as phytic acid (Liang et al. 2008). Phytic acid forms chelation complexes with Fe^{2+}/Zn^{2+} and decreases the bioavailability of these micronutrients in dietary food, thus acting as an antinutritional factor (Hunt 2003; Kumssa et al. 2015). Vaid et al. (2014) reported that inoculation of *Burkholderia* sp. SG1 + *Acinetobacter* sp. SG3 led to the reduction in phytate/Zn ratio in grains of the rice. Their results revealed that both the low and high Fe and Zn accumulating genotypes respond in an almost similar manner to endophyte inoculation, with respect to phytic acid reduction in grains. In our investigation, endophyte inoculation decreased phytic acid concentration in grains by approximately 26% over the RDF. This reduction of phytic acid concentration in grains may be correlated with increasing Fe or Zn concentration in grains ($r = -0.825$ for phytic acid vs. Fe content in grains; $r = -0.660$ between phytic acid and Zn content in grains), as depicted by the positive and significant correlations between phytic acid content in grains with low/high accumulators (Fig. 6). The increased available phosphorus concentration in grains, as a result of phosphorus solubilization activity of endophytes, correlates well with the enhanced uptake of phosphorus by plants. As these endophytes possess the ability to solubilize phosphorus, they may also possess phytase activity, which could have led to the reduction in phytic acid with simultaneous enhanced uptake of Zn/Fe due to stimulated expression of the associated genes.

One of the reasons put forth in support involves the role played by Zn as a co-factor for RNA polymerase, and increased Zn may stimulate gene expression (Cakmak et al. 2010; Marschner 1995; Price 1962). Aslam et al. (2010) found that inoculation of plant growth-promoting rhizobacteria like *Rhizobium* enhanced the protein content in chickpea grains, while Aamir et al. (2013) reported that inoculation/co-inoculation with rhizobium and PGPR improved the protein content in mung bean grains. It has been suggested that this may be due to higher expression of RNA polymerase involved in protein synthesis, in the presence of more Zn (Price 1962).

Conclusions

It can be surmised that the interesting inherent genetic variations in the wheat genotypes with regard to the uptake of Fe and Zn observed led to a differential behavior in relation to the accumulation of micronutrients when grown in soils with low/high availability of zinc and iron. This not only aided in the categorization of the wheat cultivars into low and high accumulators but also highlighted that the level of iron in soil is more critical, as compared to zinc in terms of its uptake by wheat genotypes. Endophytes isolated from such cultivars showed diversity in their zinc solubilizing and siderophore producing attributes, which facilitated in the identification of

promising isolates, for use as inoculants to improve micronutrient uptake and accumulation in grains. Such endophytes can be used to develop a microbe-based technology for biofortification of Fe and Zn and nutritional quality improvement of wheat grains. Evaluating these endophytes under varied agro-climatic conditions is proposed in our future studies.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals: N/a This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Consent was obtained from all the individual participants included in the study.

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