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Host Genotype and Edaphic Factors Cumulatively Influence the Occurrence of Siderophore-producing Bacteria Associated with Rice (*Oryza sativa* L.)

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Abstract

Seed-borne rice endophytes are capable of disseminating into host plant tissues as well as to their rhizosphere. Here, we investigated the occurrence of siderophore-producing bacteria (SPB) in the seed endospheres of two distinct rice (*Oryza sativa* L.) cultivars, TK8 (ssp. *japonica*) and TCN1 (ssp. *indica*), and their dissemination into the rhizospheres through culture-dependent methods. Their patterns of occurrence in the rhizospheres as well as in the root and shoot tissues of 30 day-old cultivars grown in three different kinds of soils were tested. The significance of SPB on Fe sequestration of TCN1 was studied using *Enterobacter* sp. LS-756. TK8 seeds were found to be not only abundant in endopsheric SPB (> 10-fold), but also exhibited enhanced SPB dissemination into the rhizosphere (1.3-fold) as compared to TCN1. The proportion of endophytic SPB was consistently higher in roots than in shoots, and it was found to decline with decreasing soil pH. A similar declining trend was further evident through the analysis of SPB composition in the rhizospheric and bulk soils. LS-756-inoculated TCN1 seedlings under low availability of Fe showed 32%, 178%, and 368% increases in Fe, chlorophyll, and chlorophyll b contents as compared to the uninoculated controls. Thus, the occurrence of seed-borne endophytic SPB and their dissemination into the rhizosphere vary significantly according to the rice genotype. Higher co-occurrence of SPB in the rhizosphere and internal root tissues of rice plants grown under Fe-limited conditions and the enhanced Fe uptake due to SPB inoculation substantiated their potential involvement in Fe sequestration.

Introduction

Iron (Fe) is a fundamental element required by plants for photosynthesis, mitochondrial respiration, nitrogen assimilation, and hormone biosynthesis, as well as pathogen defense (Mori et al., 1991; Hansch & Mendel, 2009; Yu et al., 2011). It is abundant in the mineral solid phase of soils (average 3.8%) (Lucena, 2006). However, it is one of the most growth-limiting nutrients due to its predominant existence in the form of ferric ions (Fe^{3+}), which are difficult to take up by plants and associated microorganisms (Rajkumar et al., 2010). A deficiency of Fe leads to inhibition of chlorophyll biosynthesis and declined plant growth and productivity (Hansch & Mendel, 2009).

Siderophore-producing bacteria (SPB) are known to facilitate Fe acquisition under Fe-limited conditions (Neilands, 1995; Miethke & Marahiel, 2007; Ahmed & Holmström, 2014). Siderophore production is one of the characteristic features of plant growth-promoting bacteria (Katiyar & Goel, 2004; Yu et al., 2011; Yasmin et al., 2012; Naureen et al., 2015). Siderophores can chelate Fe^{3+} and transport the resultant complexes into the cell cytoplasm through membrane receptors (Boopathi & Sankara, 1999). Plants inoculated with SPB can absorb iron from the secreted siderophores through various mechanisms including degradation, chelation and release of iron, direct uptake of siderophore-Fe complexes, or through ligand exchange reactions (Schmidt, 1999). Carvalhais et al. (2013) demonstrated that plants devoid of soil bacteria suffered from iron deficiency. Microbial siderophores may stimulate plant growth directly by increasing the bioavailability of iron in the soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens with less efficient iron-uptake systems (Joseph et al., 2007). Siderophores facilitate plant protection as they deprive phytopathogens of iron by swiftly binding to the bioavailable forms of iron (Verma et al., 2011; Aznar et al., 2015).

Mechanistic understanding of endophytic bacterial colonization and plant-microbe interaction are important for the possible manipulation of endophytic bacteria/microbiome for sustainable agriculture (Liu et al., 2017). Young rice plants are highly susceptible to iron deficiency due to their limited secretion of phytosiderophores that sequester Fe (Mori et al., 1991). Endophytic bacterial taxa of rice vary considerably according to the plant growth stage, tissue type, and host genotype as revealed through culture-dependent and-independent analyses (Elbeltagy et al., 2000; Engelhard et al., 2000; Okunishi et al., 2005; Mano et al., 2006, 2007; Sun et al., 2008; Mano & Morisaki, 2008; Kaga et al., 2009; Hardoim et al., 2012; Hameed et al., 2015). Some of the rice endophytes exhibit plant probiotic attributes (Barraquio et al., 1997; Chaudhary et al., 2012; Rungin et al., 2012; Sessitsch et al., 2012; Hameed et al., 2015). Interestingly, rice seeds themselves act as reservoirs for a diverse set of endophytic bacteria (Mano et al., 2006; Kaga et al., 2009; Hardoim et al., 2012; Hameed et al., 2015). Hardoim et al. (2012) demonstrated remarkable dissemination of seed-borne endophytes into the shoot and root tissues of rice using aseptic soil. Similarly, gnotobiotic experiments performed by Hameed et al. (2015) showed a significant discharge of seed-inhabiting endophytes into the rice rhizosphere during the early stages of seedling development. However, the influence of host genotype and edaphic factors on the occurrence of SPB associated with rice plants is not well understood.

Thus, the objectives of the present work were to estimate the occurrence of SPB in the seed endospheres of two distinct rice (Oryza sativa L.) cultivars, TK8 (ssp. japonica) and TCN1 (ssp. indica), as well as in three kinds of bulk soils having contrasting pH and varying Fe contents. The proportion of SPB inhabiting the rhizospheres as well as the surfaces of the sterilized root and shoot tissues of 30 day-old TCN1 and TK8 grown in three different kinds of soils, and the effects of SPB on Fe utilization and growth of rice plants by an inoculation assay were also investigated. The study will provide evidence for the amelioration of Fe deficiency by SPB and contribute to the development of suitable strategies to improve crop growth in Fe-deprived soil.

Materials and Methods

Materials

Rice genotypes

The authentic TCN1 and TK8 seed stocks were obtained from the Taichung District Agricultural Research & Extension Station and the Taiwan Agricultural Research Institute, Council of Agriculture, Executive Yuan, respectively.

Soil types and their physicochemical parameters

Near-neutral, acidic, and red soils were collected respectively from the paddy fields of Huwei (23°41'53.3"N 120°25'48.5"E), Nantou (24°03'33.5"N 120°52'33.5"E), and Taoyuan (25°01'37.4"N 121°06'43.1"E), Taiwan. Soil samples were dried under sunlight and sieved through 2-mm mesh. Soil samples (10g) were immersed in distilled water (1:5, w/v) for 24h at room temperature for the determination of pH and electrical conductivity. The total content of the soil elements was analyzed. Total nitrogen was determined using the Kjeldahl method (Bremner & Mulvaney, 1982). P, K, Ca, Mg, Fe, and Mn were analyzed using the double acid ($HClO_4:HNO_3$ = 1:4, v/v) analysis (Jones, 2001). Heavy metals (Cu and Zn) were extracted according to the method of Jones (2001) and analyzed using an inductively coupled plasma spectrophotometer (JY 138 Ultrace ICP-AES, AST Instruments Corporation) (**Table 1**).

Methods

Seed endospheric bacterial counts and rhizospheric dissemination assay

Twenty seeds of the respective rice cultivars were surface sterilized with 3% sodium hypochlorite by gently shaking for 3m. After removing the sodium hypochlorite, seeds were washed with double distilled water three to four times. The efficacy of surface sterilization was verified by spread-plating the final rinsate on nutrient agar (NA, Himedia). The surface-sterilized seeds were aseptically crushed using a mortar and pestle with 5 ml Hoagland solution. Aliquots were spread-plated on NA after serial dilution for the total bacterial count. Simultaneously, aliquots were also spread-plated on chrome azurol S (CAS) media with hexadecyltrimethylammonium bromide (HDTMA) as an indicator for the corresponding SPB count (Schwyn & Neiland, 1987; Yu *et al.*, 2011). The NA and CAS plates were incubated for 3 days at 25°C under darkness. Colonies forming an orange halo were counted as positive for siderophore-production. NA and CAS cultures were done in triplicate. The ratio of SPB was determined by the formula: colony-forming units (CFU) of SPB/total bacterial CFU × 100%. Surface sterilized rice seeds of the respective cultivars were allowed to germinate in culture plates containing sterile Hoagland solution (250 ml plate^{-1}) for 10 days at 25°C under darkness. The rice seedlings were aseptically removed and the rhizospheric fluid was spread-plated after serial dilution on CAS media in triplicate for SPB counts, respectively.

Total bacteria and SPB count in bulk soils

Ten grams of respective non-sterile bulk soil samples were soaked in 95mL of 0.1% (w/v) sodium pyrophosphate in distilled water and mixed thoroughly by agitation (Vieire & Nahas, 2005). The homogenate was spread-plated on NA and CAS after serial dilution in triplicate for bulk soil bacteria and SPB counts, respectively.

Table 1. Physicochemical properties of the three soil-types used in the experiment

Soil-type	pH	EC (µS cm^{-1})	Total N (g kg^{-1})	P	K	Ca	Mg	Fe	Mn	Cu	Zn
				mg kg^{-1}							
Red	4.1	93	0.8	248.9	85.0	563.0	103.4	2176	352.0	10.8	21.7
Acidic	5.5	337	0.9	150.8	112.8	1156.3	129.7	652.8	488.2	3.4	15.0
Near-neutral	7.7	278	1.2	385.8	69.8	6485.5	389.0	882.0	338.0	6.0	22.9

Greenhouse pot experiments

Surface sterilized seeds (n = 30) of the respective cultivars were sown in pots containing 600g of non-sterile near-neutral, acid, or red soils in triplicate. Pots were placed in a greenhouse with an average temperature of 25-28°C. Seven days after germination, 20mL of Hoagland's solution was added to each pot twice a week. Plants were harvested after 30 days of growth and excessive rhizospheric soils were removed by gentle knocking. Root-bound soil was carefully collected, weighed, and spread-plated on NA and CAS after serial dilution in replicates for rhizospheric total bacteria and SPB counts, respectively.

The roots were washed thoroughly with running tap water to clear the soil particles. Root and shoot sections were separated, weighed, and surface sterilized with 3% sodium hypochlorite. The efficacy of surface sterilization was verified by spread-plating the final rinsate on NA and CAS plates. The surface sterilized tissues were aseptically macerated using a mortar and pestle with identical amounts of Hoagland's solution, and the homogenate was spread-plated on NA and CAS media after serial dilution in triplicate for endophytic total bacteria and SPB counts, respectively.

Identification of SPB

Colonies of interest were picked up from the CAS agar, purified, and preserved at –80°C as glycerol suspensions. The genomic DNA was extracted as previously described by Hameed *et al.* (2015). Bacteria were identified using the 1F (5'-GAG TTT GAT CAT GGC TCAG-3') and 9R (5'-AAG GAG GTG ATC CAA CCG CA-3') universal primers specific for a 16S rRNA gene fragment (Edwards *et al.*, 1989).

Inoculation assay

The surface sterilized seeds (n = 50) of TCN1 were treated with 15mL of freshly prepared cells (5×10^8 CFU ml^{-1}) of LS-756 for 5 days at room temperature. Fifteen healthy emerged seedlings were sown in pots containing 150 g of non-sterile near-neutral soil in triplicate. Pots were incubated in a growth chamber equipped with fluorescent lamps (T5-14W/AQ and FL40-EX/T5 6500k; approx. 801.5 ± 15.5 LUX) with a 12h photoperiod day^{-1} at 25°C. Fourteen days after planting, a fresh overnight culture of LS-756 (10 mL pot^{-1}) was supplied to the rice plants at 10^8 CFU ml^{-1} while the same amount of NB was introduced to the controls. Iron-deficient Hoagland's solution (10 mL pot^{-1}) was supplied 20 days post-planting. After 30 days of growth, the rice plants were harvested, cleaned, and processed for the estimation of root/shoot lengths, fresh/dry weights, and chlorophyll contents. Plants were dried at 65°C until they reached a constant weight for dry weight determination. Mineral (N, P, K, Ca, Mg, Fe, Mn, Cu, and Zn) compositions were estimated from the dried tissues as described above.

Fresh leaves (0.02g) were crushed in liquid nitrogen and the chlorophylls were extracted with 1.2mL dimethylformamide. The absorbance of crude extracts was measured at 662nm (A662) and 643nm (A643) by a spectrophotometer (GeneQuant 1300, GE Cambridge UK) and chlorophyll contents were determined using the below formulae:

Chlorophyll *a* content (µg mL^{-1}) = 12.70A662 - 2.93A643
Chlorophyll *b* content (µg mL^{-1}) = 20.70A643 - 4.62A662
Total chlorophyll content (µg mL^{-1}) = 17.90A643 + 8.08 A662

Statistical analysis

Data processing and statistical analyses were performed using Microsoft Excel version 2012 and IRRISTAT software with a one-way analysis of variance (One-way ANOVA). Differences between treatments were tested by an unpaired t-test and Duncan's multiple range tests. The figures are shown as mean ± standard deviation (SD).

Results and Discussion

Total and siderephore-producing endophytes in rice seeds

Detection of co-occurring SPB and their variations by rice genotype, tissue-, and soil-type is significant since previous investigations have

focused largely on counting total bacteria associated with rice tissues. The seed endospheres of TK8 and TCN1 respectively harbored (CFU g^{-1}) 2.8×10^6 and 1.5×10^5 total endophytes and 8.8×10^5 and 4.5×10^3 SPB (**Figure 1**). The percentage compositions of endospheric SPB in TK8 and TCN1 were estimated to be 32 and 3, respectively. A higher abundance of total bacteria in the seed endosphere of TK8 than that of TCN1 corroborated a previous study on these two rice genotypes (Hameed *et al.*, 2015). Earlier studies reported 10^2 to 10^6 and 3.5×10^5 CFU of endophytic bacteria g^{-1} seed weight of Kinuhikari (Mano *et al.*, 2006) and APO (Hardoim *et al.*, 2012) rice cultivars, respectively. The seed-borne SPB detected in the TK8 and TCN1 cultivars were in line with an earlier report (Loaces *et al.*, 2011). Nevertheless, our comparative analysis revealed a striking difference in the seed-borne SPB composition in the rice genotypes TK8 and TCN1. Rice seeds as plant microbiomes present both an opportunity and a challenge to the bacterial endophyte community. Endophytic bacteria have roles in plant germination and growth such as hormone modulation, nitrogen fixation, siderophore production, and phosphate solubilization that may allow them to be selectively chosen by plants (Walitang *et al.*, 2017).

Seed-borne endophytic bacteria have been demonstrated to disseminate into the rhizosphere (Hameed *et al.*, 2015). A possible deployment of SPB inhabiting the seed endosphere into the rice rhizosphere under Fe-limited conditions and their involvement in Fe utilization was presumed and investigated. We found substantial dissemination of SPB into the rhizosphere between the tested rice cultivars under gnotobiotic conditions. Interestingly, TK8 disseminated approximately 29.5% more SPB than the TCN1 cultivar (2.59×10^6 as compared to 2.00×10^6 CFU of SPB mL^{-1} rhizospheric fluid). Thus, TK8 inherently harbored a high number of total endophytes as well as SPB, and the percentage composition of siderophore producers was >10-fold as compared to that of
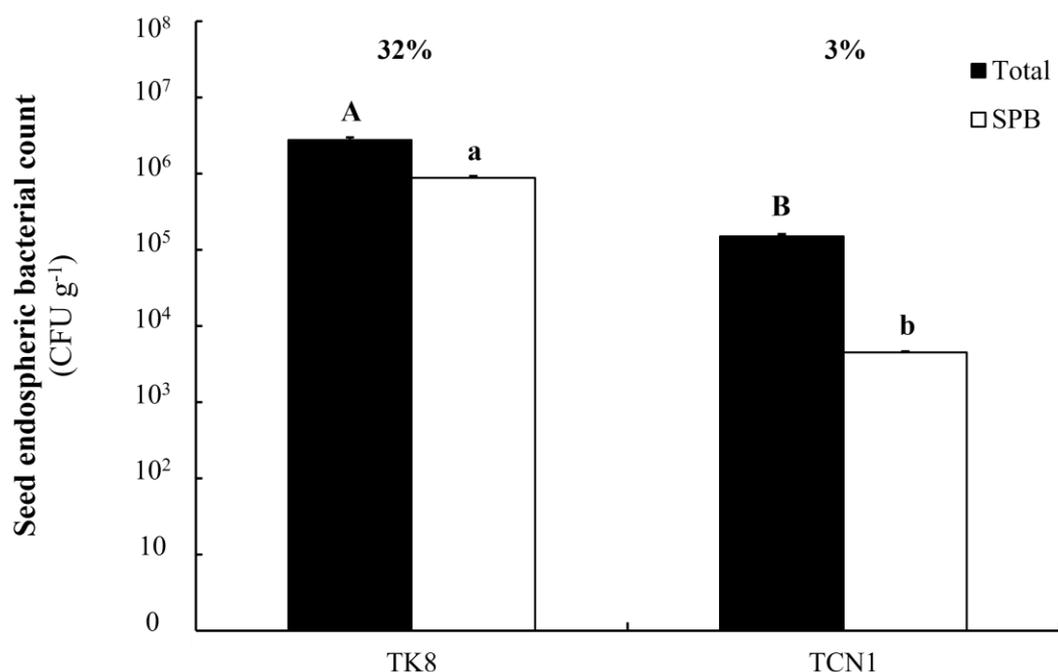

Figure 1. Colony-forming units (CFU) of endophytic total bacteria (filled-column) and siderophore-producing bacteria (SPB) (open-column) were retrieved per gram of rice seeds. The ratio of SPB (shown above the columns) was determined by the formula: total SPB CFU/total bacterial CFU × 100%. Data are mean ± SD (n = 3). Bars with the same letter are not significantly different at a 5% probability level by Duncan's multiple range test. TK8, *Oryza sativa* ssp. *japonica* cv. Taikeng 8; TCN1, *O. sativa* ssp. *indica* cv. Taichung Native 1.

TCN1. The higher occurrence of seed-borne SPB in the rhizospheric fluid of TK8 suggested the significant involvement of the host genotype in recruiting siderophore producers at the rhizosphere of rice seedlings. Our results corroborated a previous observation related to seed endospheric P-solubilizers (Hameed *et al.*, 2015).

Total and siderophore-producing bacteria in bulk soils

The physicochemical parameters that distinguish near-neutral (pH 7.7), acidic (pH 5.5), and red (pH 4.1) bulk soils employed for subsequent rice cultivation are listed in **Table 1**. Fe content was determined to be highest in the red soil (2176.0 mg kg^{-1}) followed by the near-neutral (882.0 mg kg^{-1}) and acidic soils (652.8 mg kg^{-1}). The total bacterial counts in the near-neutral, acidic, and red bulk soils (CFU g^{-1}) were estimated to be 3.8×10^6, 1.0×10^7, and $7.6 \times$

10^6, respectively. SPB counts in the respective samples were 2.1×10^5, 3.6×10^5, and 0.21×10^5 CFU g^{-1} (**Figure 2**). The proportions of SPB were 5.5, 3.6, and 0.3% in the near-neutral, acidic, and red soils, respectively. Thus, the total bacteria and SPB counts were high in the acidic soil, while the SPB composition was found to decrease with decreasing soil pH.

Soil harbors diverse microbial flora in which some exhibit plant growth-promoting attributes. Ahmad & Ahmad (2013) demonstrated that the bulk soils accommodated (CFU g^{-1}) 1.35×10^5 to 1.03×10^7 heterotrophic bacteria, corresponding to our results. Some bacteria can synthesize siderophores that maximize Fe utilization efficiency under Fe deficient conditions (Boopathi & Sankara, 1999). The soil pH may affect the population proportion of microbes that possess siderophore-producing activity (Lynch, 1995) since it determines the solubility and
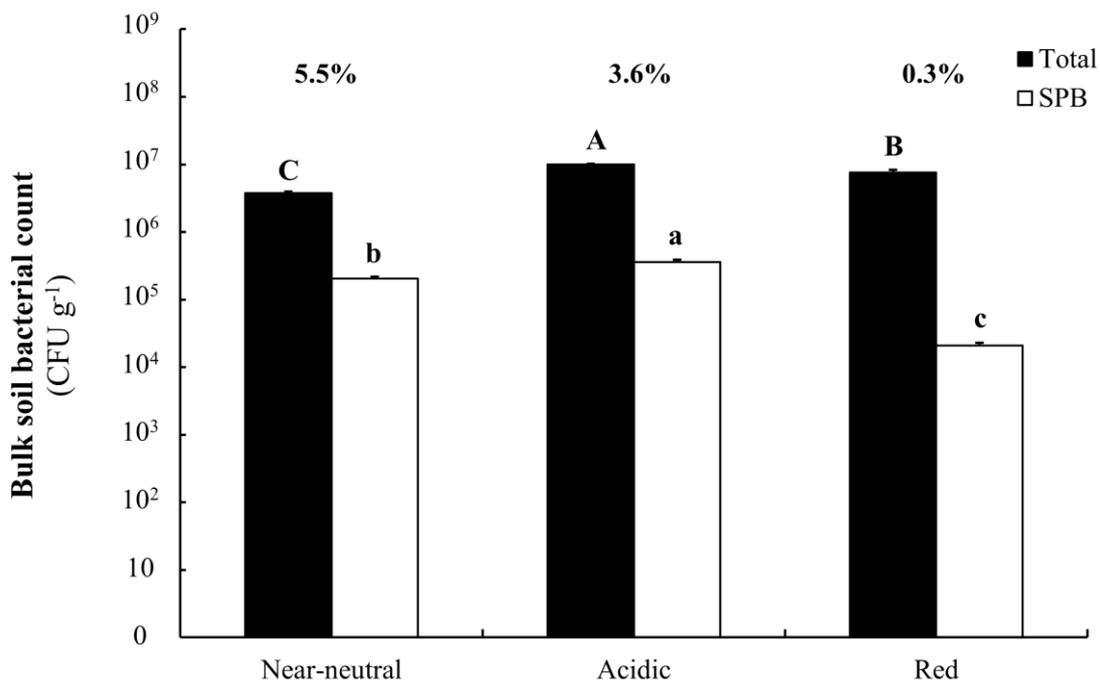

Figure 2. Colony-forming units (CFU) of total bacteria (filled-column) and siderophore-producing bacteria (SPB) (open-column) were found per gram of bulk soils. The ratio of SPB (shown above the columns) was determined by the formula: total SPB CFU/total bacterial CFU × 100%. Data are mean ± SD (n = 3). Bars with the same letter are not significantly different at the 5% probability level by Duncan's multiple range test.

availability of iron for organism growth. Iron bioavailability in an acidic pH environment (0.1 mol at pH 2.0) is higher than in a neutral one (10^{-18} mol at pH 7.0) (Sullivan *et al.*, 2012). The previous study had shown that SPB was more prevalent in alkaline pH soils as ferrous ions under this condition were promptly immobilized by metal ions in the soil (Prasad, 2003). Yu *et al.* (2017) reported that the optimum siderophore production was obtained at pH 6.0, which was significantly different from those obtained at other pH values ($P < 0.05$). In this study, we found a high occurrence of SPB in the near-neutral bulk soil followed by the acidic bulk soil and red soil that respectively accommodated 882.0, 652.8, and 2176.0 mg kg^{-1} Fe. Interestingly, the total bacterial count in the red bulk soil was well within the range of the near-neutral and acidic soils. Thus, while near-neutral soils inherently harbor higher levels of Fe, the increased Fe bioavailability and decreased pH most likely lowered the abundance of SPB in the bulk acidic and red soils.

Effects of genetic background and edaphic factors on the total and siderophore-producing rhizobacteria and endophytes

The phenotypes of the tested cultivars after 30 days of growth in their respective soil types are shown in **Figure 3a**. The total bacterial counts (CFU g^{-1}) in the rhizospheres of TK8 and TCN1 were respectively 1.6×10^4 and 1.4×10^4 in near-neutral soil, 3.0×10^4 and 2.3×10^4 in acidic soil, and 2.3×10^4 and 1.8×10^4 CFU g^{-1} in red soil (**Figure 3b**). The rhizosphere is a narrow layer of soil that is directly influenced by the roots and is well known to host a variety of plant growth-enhancing microorganisms. The population of the rhizospheric bacteria is not only influenced by soil type and culture conditions, but also by rice genotype since they vary in their chemical composition of root exudates (Costa *et al.*, 2006; Andreote *et al.*, 2014). Compant *et al.* (2010) reported that the plant rhizosphere harbors approximately 10^7-10^9 CFU of bacteria g^{-1} soil. Kumar *et al.* (2013) studied the diversity of the rhizobacterial community associated with basmati rice and showed that the total bacterial count (CFU g^{-1}) was the highest in the rhizospheric soil of Super Basmati (2.6×10^6),

followed by Pusa Sugandha 4 (1.5×10^6), Pusa Sugandha 5 (1.2×10^6), HBC 19 (2.4×10^5), and Panjab Basmati (7.8×10^4). Similarly, rhizobacterial counts were found to be 4.5×10^4 to 1.9×10^7 in Uttarkashi rice and 6.8×10^4 to 2.0×10^7 in Dehradun rice (Joshi *et al.*, 2011). Lower levels of total rhizospheric bacteria encountered in this study could be due to dissimilarity in terms of rice genotypes, soil types, and cultivation conditions.

Similarly, we further investigated the occurrence of siderophore producers in the rhizosphere of TK8 and TCN1 grown in near-neutral, acidic, and red soils. The rhizospheric SPB counts (CFU g^{-1}) in TK8 and TCN1 were respectively 1.4×10^2 and 0.09×10^2 in near-neutral, 9.8×10 and 8.5×10 in acidic, and 1.1×10 and 1.5×10 CFU g^{-1} in red soils. The proportions of SPB found in TK8 were 0.9, 0.3, and 0.1% of the total bacteria in the near-neutral, acidic, and red soils, respectively, whereas they were estimated to be 0.7, 0.4, and 0.1% for TCN1. Taken together, the total bacteria and SPB in the rhizospheric soils were comparatively fewer than in the corresponding bulk soils and the occurrence of SPB in the rice rhizospheres decreased with decreasing soil pH. The reduction was remarkable in the red soil irrespective of the tested rice genotypes. Most red soils have high contents of Fe oxides (Wilson *et al.*, 2004), and under acidic conditions, ferric complexes of catecholate siderophores are known to be unstable (Gaonkar & Bhosle, 2013). It is possible that the tested rice genotypes deployed fewer seed-borne SPB in the rhizosphere after sensing higher bioavailability of Fe in the red soil.

The occurrence of total endophytic bacteria in the surface sterilized root and shoot tissues of 30 day-old TK8 and TCN1 cultivars grown in near-neutral, acidic, and red soils were also investigated. We detected 1.8×10^4, 3.4×10^4, and 3.0×10^4 CFU g^{-1} total bacterial endophytes, and 8.6×10^3, 0.3×10^3, and 0.2×10^3 CFU g^{-1} SPB in the root tissues of TK8 cultivated in near-neutral, acidic, and red soils, respectively (**Figure 4**). Similarly, we found 5.9×10^4, 3.2×10^4, and 5.3×10^4 CFU g^{-1} bacterial endophytes, and 1.9×10^3, 0.3×10^3, and 0.4×10^3 CFU g^{-1} SPB in the root tissues of TCN1 cultivated in
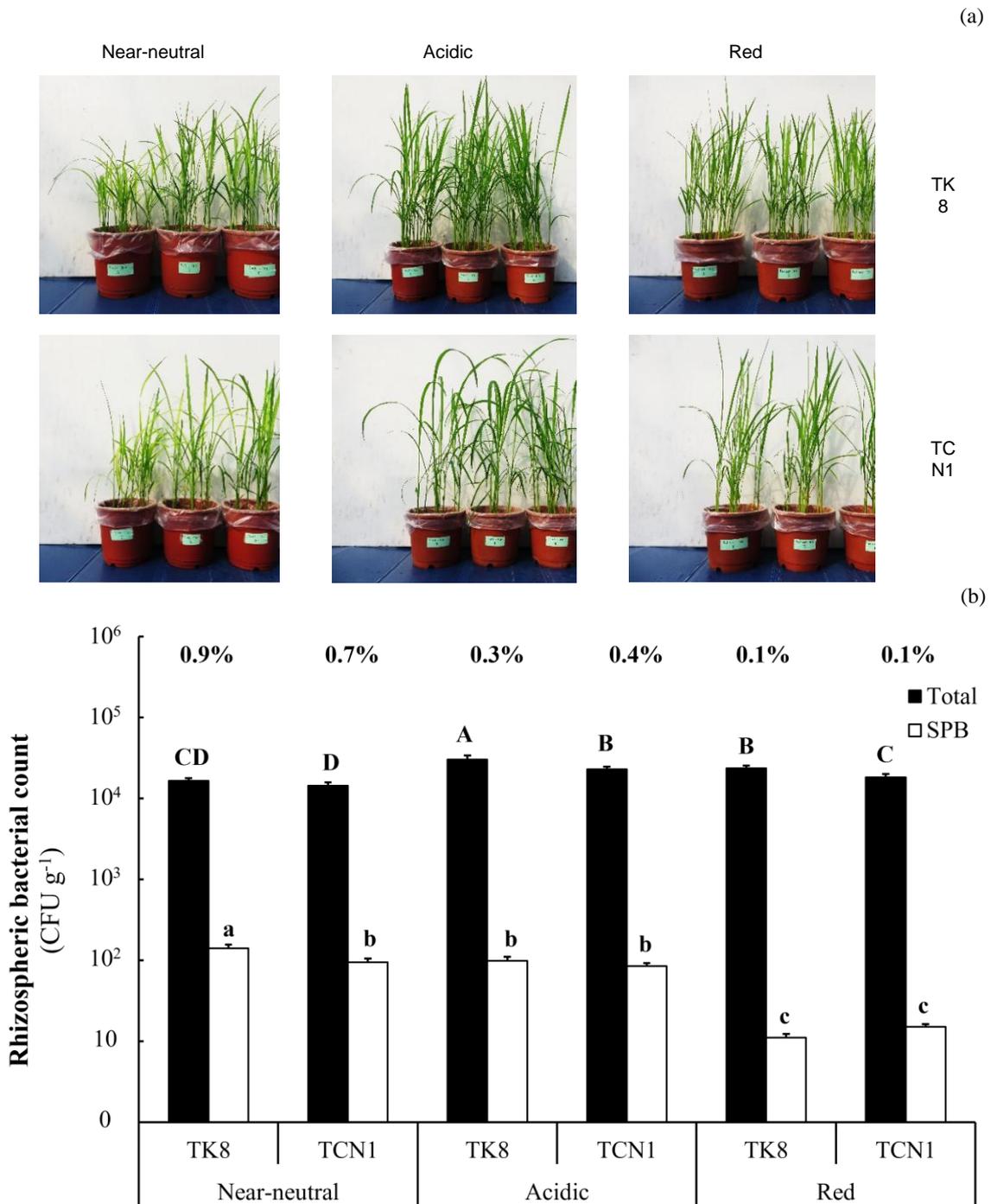
Figure 3. The phenotypes of 30 day-old rice cultivars of TK8 and TCN1 grown in tested soils under greenhouse conditions (a), and the colony-forming units (CFU) of total bacteria (filled-column) and siderophore-producing bacteria (SPB) (open-column) retrieved from the rhizosphere (b). The ratio of SPB (shown above the columns) was determined by the formula: total SPB CFU/total bacterial CFU × 100%. Data are mean ± SD (n = 3). Bars with the same letter are not significantly different at the 5% probability level by Duncan's multiple range test. TK8, *Oryza sativa* ssp. *japonica* cv. Taikeng 8; TCN1, *O. sativa* ssp. *indica* cv. Taichung Native 1.

near-neutral, acidic, and red soils, respectively. The percentage compositions of endophytic SPB in the root tissues of TK8 grown in near-neutral, acidic, and red soils were respectively 38, 9, and

1, whereas they were calculated to be 3, 22, and 3 in the respective shoot tissues. Similarly, the percentage compositions of endophytic SPB in the root tissues of TCN1 grown in near-neutral,

acidic, and red soils were respectively 15, 2, and 1, whereas they were calculated to be 5.5, 11, and 0 in the respective shoot tissues. Thus, the endophytic bacteria and SPB in the root and shoot tissues of TK8 and TCN1 grown in the tested soils were relatively fewer than those of the respective seed endospheres. The proportion of endophytic SPB inhabiting TK8 and TCN1 roots decreased with decreasing soil pH. In contrast, shoot tissues of TK8 and TCN1 grown in acidic soil showed a high abundance of SPB as compared to the remaining soil types.

We found that endophytic bacterial abundance was comparatively higher in root than in shoot tissues. The highest occurrence of endophytic bacteria (CFU g^{-1} fresh weight) was reported earlier in the root (2.0×10^5 to 3.0×10^6) followed by stem (4.0×10^3 to 4.0×10^5) and leaf (1.0×10^3 to 2.7×10^4 CFU g^{-1}) tissues irrespective of the different growth stages and soil types (Prakamhang *et al.*, 2009). Similarly, Loaces *et al.* (2011) detected a high abundance of heterotrophic bacteria (CFU g^{-1} fresh weight) in the roots (1.1×10^6) followed by leaves (8.9×10^4) during the tillering stage. The increased

abundance of endophytes in the roots may have been the result of active invasion and colonization of soil-borne bacteria (Botta *et al.*, 2013). In addition, dissemination of seed-borne endophytes likely contributed to the differential occurrence of total endophytes in the root and shoot tissues of TK8 and TCN1 under the tested soil regimes. The findings of Wang *et al.* (2020) support the hypothesis that the process of germination changes the microbial community inhabiting the seeds and partitions it into the root and shoot tissues. During transmission, phyto-beneficial bacteria and fungi can promote seedling growth as well as mitigate plant stress damage.

The presence of endophytic SPB was reported in grains, roots, and leaves of rice with densities ranging from 2.8×10^3 to 3.3×10^5 CFU g^{-1} fresh weight in different plant growth stages (Loaces *et al.*, 2011). Interestingly, endophytic SPB abundance declined with decreasing soil pH in the tested rice cultivars. The bioavailability of Fe decreases with increasing pH as ferrous ion gets readily immobilized by metal ions present in the soil (Prasad, 2003). A higher proportion of
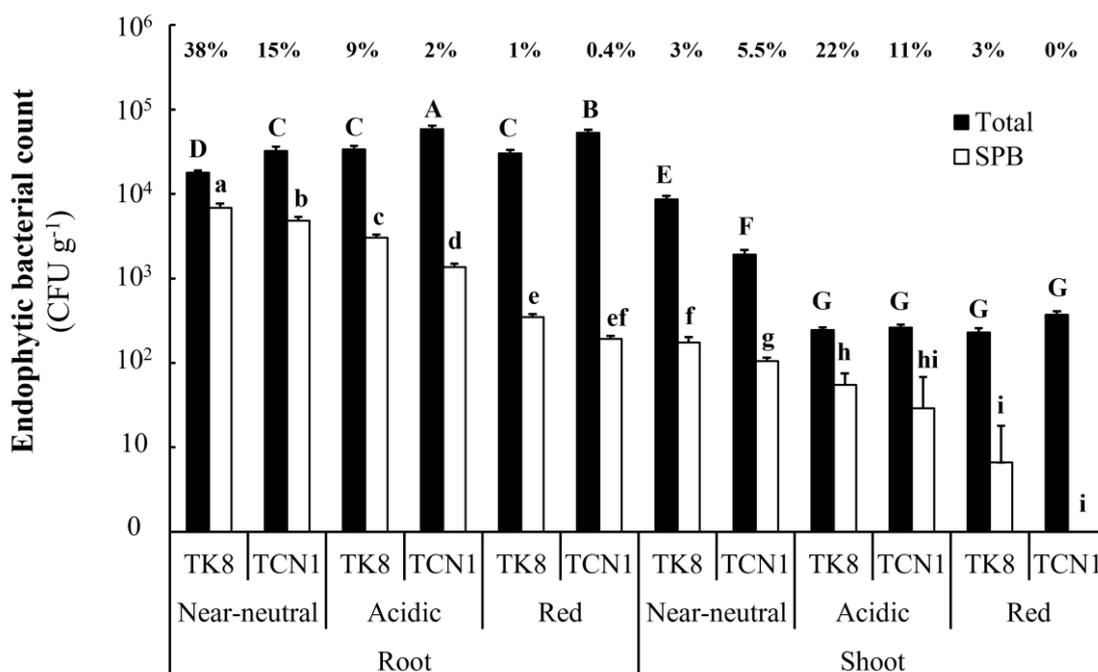

Figure 4. Colony-forming units (CFU) of endophytic total bacteria (filled-column) and siderophore-producing bacteria (SPB) (open-column) were retrieved per gram of root and shoot tissues of TK8 and TCN1 originating from three different soils. The ratio of SPB (shown above the columns) was determined by the formula: total SPB CFU/total bacterial CFU × 100%. Data are mean ± SD (n = 3). Bars with the same letter are not significantly different at the 5% probability level by Duncan's multiple range test. TK8, *Oryza sativa* ssp. *japonica* cv. Taikeng 8; TCN1, *O. sativa* ssp. *indica* cv. Taichung Native 1.

SPB in the near neutral soil reflects their possible involvement in Fe mobilization. In contrast, higher SPB abundances in the shoot tissues of TK8 and TCN1 were mainly due to declines in total endophytes.

Effects of siderophore-producing bacteria on rice growth

We investigated the participation of SPB in Fe sequestration *in planta* using strain LS-756, which was isolated from the shoot tissue of TCN1 cultivated in near-neutral soil. This strain was chosen as it exhibited the strongest siderophore-producing activity on CAS medium as compared to other strains. LS-756 was identified as a representative of the genus *Enterobacter* as it shared 100% 16S rRNA gene sequence similarity (0/531 bp) with *Enterobacter cloacae* subsp. *cloacae* ATCC 13047T (NCBI accession number was KX880385), a bacterium classified under biosafety level 1 (https://www.baua.de/). *Enterobacteriaceae* representatives such as *Enterobacter*, *Erwinia*, and *Klebsiella* have been identified as some of the most common rice-associated bacterial taxa (Hallmann *et al*., 1997). Some rice-associated *Enterobacter* strains reportedly possess siderophore-producing activity (Prakamhang *et al*., 2009; Loaces *et al*., 2011).

The impact of inoculation of the siderophore-producing strain *Enterobacter* sp. LS-756 under Fe deprived conditions on the Fe utilization and growth of TCN1 that inherently harbored lesser seed-borne SPB was evaluated. We found that seed germination and most of the growth characteristics in 30 day-old LS-756-inoculated TCN1 plants were on par with the uninoculated controls (**Table 2**). This could be due to the lack of complementary probiotic

features in LS-756. However, strikingly, the inoculation of LS-756 resulted in a significant (**$P \leq 0.01$) increase in chlorophyll *b* (21.9 ± 1.3 g mL^{-1}) and total chlorophylls (47.0 ± 4.3 g mL^{-1}) in the leaves, whereas the uninoculated control possessed 5.96 ± 0.78 and 26.42 ± 1.48 g mL^{-1} of the corresponding pigments. The loss of chlorophylls and carotenoids are the primary responses associated with the unavailability of Fe in plants (Belkhodja *et al*., 1998). Inoculation studies have been commonly used to elucidate the plant probiotic features of microbes (Fujii *et al*., 1987; Radzki *et al*., 2013; Young *et al*., 2013). Sharma & Johri (2003) proved that the siderophore-producing *Pseudomonas* strain GRP3 significantly increases chlorophyll contents and decreases chlorotic symptoms in *Vigna radiate*. According to Radzki *et al*. (2013), siderophores produced by strain *Chryseobacterium* sp. C138, isolated from the rhizosphere of *Oryza sativa*, significantly increased the plant yield, chlorophyll, and iron contents in tomato plants.

Elemental analyses revealed a 32% increase in Fe with a concurrent 64% decline in Zn in TCN1 tissues as a consequence of LS-756 inoculation when compared to the uninoculated control (**Table 3**). Thus, mineral nutrients, especially iron, induced a profound influence on chlorophyll production in TCN1. Meanwhile, the concurrent decline in the Zn content of rice plants was attributed to the synergism between Zn and Fe as reported in tomato plants (Gunes et al., 2008). Our inoculation study provided direct evidence for the involvement of SPB in Fe utilization, minimization of Zn uptake, and improvements of the chlorophyll contents in rice seedlings.

Table 2. Growth characteristics of 30 day-old rice plants

Treatment	Root length (cm)	Shoot length (cm)	Fresh weight (g 15 plants^{-1})	Dry weight (g 15 plants^{-1})	Chl *a* (µg mL^{-1})	Chl *b* (µg mL^{-1})	Total Chl (µg mL^{-1})
Control	14.2[a]±1.2	15.9[a]±1.0	1.4[a]±0.1	0.29[a]±0.04	20.37[a] ± 0.69	5.96[b]±0.78	26.42[b]±1.48
LS-756	16.0[a]±0.6	16.1[a]±1.4	1.5[a]±0.1	0.33[a]±0.02	24.89[a]± 3.59	21.92[a]±1.26	47.02[a]±4.29

Note:. The data reported here are expressed as mean ± SD, n = 15 in three replicates. Chl, chlorophyll. Within column, data followed by the same letter are not significantly different according to unpaired t-test (P <0.01).

Table 3. Elemental analyses of 30 day-old rice plants

Treatment	P	K	Ca	Mg	Fe	Mn	Cu	Zn
				(mg L^{-1})				
Control	14857.97	26981.31	8091.19	4997.63	4145.17	63.38	33.69	16.72
LS-756	13509.10	27132.42	8627.78	5047.03	5474.99	71.62	33.76	6.04

Conclusions

The present study documented a high co-occurrence of siderophore producers and other endophytic bacteria in the seed endosphere of two distinct rice genotypes, TK8 and TCN1, as compared to other proximal ecozones. We also demonstrated a significant co-dissemination of seed-borne total bacteria and SPB into the corresponding rhizospheres under Fe-limited conditions. Siderophore producers were encountered in all three kinds of bulk soils despite having contrasting pH and Fe compositions; however, their abundance was found to decrease with decreasing soil pH. Rhizospheric soil of 30 day-old TK8 and TCN1 grown in the tested soils, and also showed a decline in the abundance of siderophore producers at low soil pH. Similar soil pH-dependent waning was evident in siderophore producers inhabiting the internal root tissues of 30 day-old TK8 and TCN1. The inoculation assay of siderophore-producing *Enterobacter* sp. LS-756 provided direct evidence for its involvement in Fe sequestration and the enhancement of chlorophyll contents in TCN1. Taken together, the occurrence of siderophore-producers associated with rice varies with the soil type and host genotype. Furthermore, both soil-borne and seed-borne strains most likely contribute cumulatively to Fe sequestration in the early growth stages of rice plants.

Acknowledgments

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