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Exploring the rhizosphere of perennial wheat: potential for plant growth promotion and biocontrol applications

Gianluigi Giannelli[1], Lorenzo Del Vecchio[1], Martina Cirlini[1], Marco Gozzi[1], Laura Gazza[2], Gianni Galaverna[1], Silvia Potestio[3] & Giovanna Visioli[3]✉

Perennial grains, which remain productive for multiple years, rather than growing for only one season before harvest, have deep, dense root systems that can support a richness of beneficial microorganisms, which are mostly underexplored. In this work we isolated forty-three bacterial strains associated with the rhizosphere of the OK72 perennial wheat line, developed from a cross between winter common wheat and *Thinopyrum ponticum*. Identified using 16S rDNA sequencing, these bacteria were assessed for plant growth-promoting traits such as indole-3-acetic acid, siderophores and ACC-deaminase acid production, biofilm formation, and the ability to solubilize phosphate and proteins. Twenty-five strains exhibiting in vitro significant plant growth promoting traits, belong to wheat keystone genera *Pseudomonas*, *Microbacterium*, *Variovorax*, *Pedobacter*, *Dyadobacter*, *Plantibacter*, and *Flavobacterium*. Seven strains, including *Aeromicrobium* and *Okibacterium* genera, were able to promote root growth in a commercial annual wheat cultivar while strains from *Pseudomonas* genus inhibited the growth of *Aspergillus flavus* and *Fusarium* species, using direct antagonism assays. The same strains produced a high amount of 1-undecanol a volatile organic compound, which may aid in suppressing fungal growth. The study highlights the potential of these bacteria to form new commercial consortia, enhancing the health and productivity of annual wheat crops within sustainable agricultural practices.

Keywords Perennial wheats, PGPR, Microbial keystone taxa, VOCs, Biofertilizer, Biocontrol agents

Annual grains, including cereals, oilseeds, and legumes, play a crucial role in global food security, covering nearly 70% of cultivated lands and providing a significant portion of human caloric intake[1]. Wheat is the most widely grown cereal worldwide, with approximately 219 million hectares under cultivation by 2022, yielding an average of 3.6 tons per hectare, and a total production of 808 million tons[2]. However, being annual crops, their cultivation often leads to resource degradation, loss of biodiversity, and negative impacts on essential ecosystem services[3]. Moreover, the yields of conventional annual cropping systems are significantly affected by climate change[4]. Productivity losses and major setbacks are also caused by diseases induced by phytopathogens, particularly fungal species from the *Fusarium* and *Aspergillus* genera. *Fusarium* infections alone can affect various cereal crops, such as wheat, barley, corn, oats, and rice, resulting in yield losses ranging from 30–70%[5]. Besides threatening food security, these infections pose serious food safety concerns due to the production of harmful mycotoxins like fumonisins and aflatoxins[6,7]. To address the demands of modern agriculture, using beneficial microorganisms as biostimulants and biocontrol agents (BCAs) offers a promising, sustainable solution. These microorganisms enhance plant nutrition and growth while mitigating biotic and abiotic stresses. They also reduce the need for chemical fertilizers and pesticides[8,9]. Isolating strains with these combined benefits can streamline and optimize their agricultural application. Currently, the most common biofertilizers and BCAs are Plant Growth Promoting Rhizobacteria (PGPR)[10]. Among the different genera that include PGPR are *Azospirillum*, *Arthrobacter*, *Agrobacterium*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Flavobacterium*, *Micrococcus*, and *Pseudomonas*[11]. PGPR enhance plant growth through several mechanisms: biological nitrogen fixation, production of phytohormones like indole-3-acetic acid (IAA), mineral solubilization, and synthesis of ACC-deaminase. Indole 3-acetic acid,

[1]Department of Food and Drug, University of Parma, Parma, Italy. [2]Research Centre for Engineering and Agro-Food Processing, CREA, Rome, Italy. [3]Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parma, Italy. ✉email: giovanna.visioli@unipr.it

the most active phytohormone produced by PGPR, improves nutrient and water uptake, and helps plants cope with abiotic stresses such as salinity and drought[12,13]. ACC-deaminase reduces ethylene levels, supporting plant growth under stress[14,15]. PGPR also produce volatile organic compounds (VOCs), antimicrobials, lytic enzymes, and other metabolites that inhibit phytopathogenic fungi[8]. Additionally, siderophores increase iron uptake by plants and reduce iron availability to pathogens, aiding in biological control[16,17]. VOCs are small metabolites (< 300 Da) produced by microorganisms that can diffuse through soil, liquids, and air, serving as chemical messengers for microorganism-microorganism and microorganism-plant communication[18,19]. PGPR produce a variety of VOCs that enhance plant growth, stress tolerance, and biocontrol activity[20]. For example, *Bacillus mojavensis* I4 produced a blend of VOCs including acetoin, phenol, and 2,4-bis (1,1-dimethylethyl), which showed antagonistic activity against *Fusarium verticillioides*, *Fusarium graminearum*, and *Rhizoctonia solani*[21]. Mycelial growth of *Alternaria alternata* and *Sclerotium rolfsii* was inhibited by VOCs like n-octanol, decane, tetradecane, 1-(N-phenyl carbamyl)-2-morpholino cyclohexene, and tridecane produced by two strains of *Pseudomonas putida*[22].

To reduce the environmental impact of annual crop production, breeding perennial staple crops, such as perennial wheat, has emerged as a novel approach to sustainable agriculture[23]. Perennial hybrid lines regrow after harvesting and can be productive for up to four consecutive years without replanting. Although their yields are lower than annual crops, they offer expanded ecosystem services. Their deep root systems enhance soil carbon content, reduce nutrient leaching, and decrease the need for fertilizers and pesticides[24,25]. Additionally, the increased organic matter from their extensive root systems boosts the biomass, activity, diversity, and efficacy of rhizosphere microorganisms compared to annual crops[26–30]. Given these benefits, perennial grains could be a valuable source for isolating beneficial microorganisms and developing new microbial consortia for agriculture. A previous study using 16S rDNA next generation sequencing (NGS) approach analysed the rhizobiome of four perennial wheat lines, derived from crossing *Thinopyrum spp.* with common wheat[29]. The study found that rhizosphere microbial communities shifted over time: initially similar to annual wheat but during the following years resembling the perennial wheat parent line. Notably, the OK72 line stood out for its more developed root system and the increased presence of plant growth-promoting rhizobacteria (PGPR), with *Pseudomonas* representing over 15% of the rhizosphere taxa from the first year[29].

In this study, we isolated rhizosphere bacteria from the OK72 line after two years of being in the soil at CREA-IT's Montelibretti farm. Bacterial isolates were identified via 16S rDNA sequencing and tested for plant growth promotion in vitro and in vivo. We also assessed their potential as biocontrol agents against phytopathogenic fungi and identified VOCs linked to fungal inhibition. This research represents the first detailed isolation and characterization of culturable microbes from perennial wheat lines, potentially paving the way for their future use to enhance annual wheat and other crops' productivity under stress conditions. Further lab and field testing will be necessary to validate these findings.

Results

Isolation of rhizosphere bacteria isolates, 16S rDNA sequencing, phylogenetic analysis, and PGP traits

Based on morphology, forty-three bacterial strains were isolated from the roots of OK72 line. All bacterial isolates were subjected to PCR analysis for 16S rDNA gene amplification. A ~ 1500 bp fragment was successful amplified and sequenced in thirty-five out of forty-three strains. The 16S rDNA sequences obtained were aligned with the sequences available in the rRNA/ITS database by blast to obtain sequence homology (Supplementary Table S1). Sequence homology analysis assigned bacterial isolates to belong to twenty different genera, of which *Pseudomonas* and *Microbacterium* were the most represented. The forty-three bacterial strains were also analysed for some of the most common PGP traits such as IAA and siderophores production, biofilm formation, ACC-deaminase activity, phosphate solubilization and protease activity (Supplementary Table S1). Based on the results obtained by the in vitro analyses on PGP traits, twenty-five strains (Table 1) were selected for in vivo evaluation PGP traits on the commercial common wheat cultivar (*cv*) Bologna and for analyses of potential fungi inhibition and VOCs production. Table 1 shows the 16S rDNA-based homology of the selected strains and Fig. 1 shows the phylogenetic tree constructed using 16S rDNA sequences from the isolated strains and the reference sequences extracted from the NCBI database. The strains shared sequence homology with *Plantibacter*, *Okibacterium*, *Microbacterium*, *Frigoribacterium*, *Curtobacterium*, *Aeromicrobium*, *Rhodococcus*, *Flavobacterium*, *Pedobacter*, *Dyadobacter*, *Variovorax*, and *Pseudomonas* genera, and were grouped into seven different classes (Actinomycetes, Flavobacteria, Sphingobacteria, Cytophagia, Beta and Gammaproteobacteria) and into Actinomycetota, Bacteroidota, and Pseudomonadota phyla (Fig. 1).

The highest levels of IAA were found in OK_9, OK_18, and OK_54, with 99 µg mL^{-1}, 61 µg mL^{-1} and 88 µg mL^{-1}, respectively, while OK_1, OK_14, OK_15, OK_16, OK_26, OK_28, and OK_32, did not show any IAA production. For the remaining strains, the production of IAA ranges from 2 to 57 µg mL^{-1} (Table 1). High siderophores activity was detected in all bacterial strains, with values higher than 40 PSU (Table 1). In particular, the highest activity detected by the CAS assay was found for OK_30 (95.80 PSU) followed by strains OK_15, OK_16, OK_39, OK_42, OK_54 (more than 80 PSU). In vitro capability to form biofilm was detected for OK_2, OK_7, OK_8, OK_9, OK_14, OK_16, OK_17, OK_18, OK_22, OK_26, and OK_39. In addition, OK_1, OK_33, and OK_42 were able to degrade ACC and to grow under nitrogen-free medium. Phosphate solubilization ability and bacterial protease activity were also tested with qualitative test assays (Table 1): OK_4, OK_6, OK_7, OK_9, OK_18, OK_26, OK_27, OK_28, OK_32, and OK_45, showed protease activity, while OK_30, OK_39, OK_42, and OK_54 showed phosphate solubilization ability; OK_17 and OK_39 were found to be the only strains showing both protease activity and phosphate solubilization ability.

Strain	Homology (%)	GenBank accession no.	IAA (µg/mL)	PSU (%)	Biofilm formation (Abs units)	ACC-deaminase activity[a]	Protease activity (SI)[b]	Phosphate solubilization index (SI)[c]
OK_1	*Variovorax boronicumulans* (99.76)	PQ015127	0	66.98 ± 3.11	0.092 ± 0.033	+	–	–
OK_2	*Microbacterium yannicii* (100)	PQ015128	6.07 ± 2.89	58.07 ± 4.73	0.122 ± 0.043	–	–	–
OK_4	*Microbacterium proteolyticum* (100)	PQ015129	36.68 ± 7.95	44.93 ± 6.22	0.080 ± 0.011	–	2	–
OK_6	*Pedobacter petrophilus* (98.93)	PQ015131	10.96 ± 1.73	50.62 ± 9.45	0.084 ± 0.005	–	3.1 ± 0.1	–
OK_7	*Pedobacter borealis* (99.55)	PQ015132	15.38 ± 2.13	72.62 ± 12.36	0.258 ± 0.065	–	2.9 ± 0.1	–
OK_8	*Dyadobacter luteus* (97.21)	PQ015133	3.32 ± 0.34	50.94 ± 1.10	0.278 ± 0.165	–	–	–
OK_9	*Plantibacter auratus* (99.78)	PQ015134	99.39 ± 7.77	74.71 ± 12.38	0.504 ± 0.020	–	2.6 ± 0.2	–
OK_14	*Dyadobacter ginsengisoli* (98.24)	PQ015136	0	38.31 ± 4.15	0.177 ± 0.049	–	–	–
OK_15	*Flavobacterium bizetiae* (99.17)	PQ015137	0	88.94 ± 2.65	0.112 ± 0.023	–	–	–
OK_16	*Variovorax paradoxus (99.70)*	PQ015138	0	90.43 ± 2.48	0.373 ± 0.015	–	–	–
OK_17	*Pseudomonas azotoformans* (99.89)	PQ015139	0.92 ± 0.35	87.54 ± 4.25	0.838 ± 0.078	–	3.5 ± 0.9	2.7 ± 0.1
OK_18	*Microbacterium oleivorans* (100)	PQ015140	61.026 ± 10.49	58.64 ± 5.85	0.107 ± 0.029	–	2	–
OK_22	*Frigoribacterium endophyticum* (99.30)	PQ015143	48.22 ± 10.59	50.05 ± 5.30	0.255 ± 0.071	–	–	–
OK_26	*Aeromicrobium ginsengisoli* (97.99)	PQ015145	0	72.21 ± 10.52	0.831 ± 0.064	–	5.6 ± 0.6	–
OK_27	*Microbacterium aurantiacum* (99.86)	PQ015146	19.05 ± 5.70	38.18 ± 9.55	0.098 ± 0.008	–	2.3 ± 0.8	–
OK_28	*Curtobacterium herbarum* (99.75)	PQ015147	0	42.89 ± 8.56	0.085 ± 0.021	–	3.1 ± 0.5	–
OK_30	*Pseudomonas brassicacearum* (98.84)	PQ015148	2.78 ± 1.49	95.80 ± 6.09	0.086 ± 0.016	–	–	2.8 ± 0.1
OK_32	*Flavobacterium olei* (99.53)	PQ015150	0	47.05 ± 5.10	0.067 ± 0.016	–	2.7 ± 0.1	–
OK_33	*Rhodococcus qingshengii* (100)	PQ015151	44.18 ± 6.88	42.91 ± 12.99	0.086 ± 0.013	+	–	–
OK_35	*Okibacterium fritillariae* (99.82)	PQ015152	57.52 ± 6.12	65.71 ± 7.06	0.045 ± 0.003	–	–	–
OK_39	*Pseudomonas lactis* (100)	PQ015153	7.9 ± 0.74	85.65 ± 4.27	0.554 ± 0.033	–	+	2.5 ± 0.1
OK_42	*Pseudomonas canavaninivorans* (100)	PQ015155	12.51 ± 3.68	89.91 ± 0.30	0.096 ± 0.014	+	–	4.1 ± 0.1
OK_45	*Pseudomonas lactis (99.58)*	PQ015157	17.02 ± 2.46	73.02 ± 0.25	n.d.	–	2.3 ± 0.4	–
OK_54	*Pseudomonas brassicacearum* (98.17)	PQ015160	88.58 ± 1.05	88.43 ± 1.44	0.078 ± 0.004	–	–	3.3 ± 0.1
OK_90	*Plantibacter flavus* (99.55)	PQ015164	44.43 ± 3.96	40.46 ± 6.17	0.055 ± 0.009	–	–	–

Table 1. Identification and plant growth promotion traits of the twenty-five selected bacterial strains isolated from the rhizosphere of the OK72 perennial wheat line. [a] ACC-deaminase activity: (–) no bacterial growth on medium containing 1-aminocyclopropane-1-carboxylate (ACC) as the only N source; (+) bacterial growth on medium containing 1-aminocyclopropane-1-carboxylate (ACC) as the only source of N. [b] (–) No protease activity detected; (+) indicates positive protease activity but the morphology of the colony did not allow the measurement to be made. [c] (–) No phosphate solubilization detected. n.d. = Not determined. Data are average of three independent experiments ± standard deviation (S.D.)

Evaluation of bacterial growth promoting ability in commercial wheat cultivar at germination stage

The twenty-five bacterial strains (Table 1) were evaluated for their in vivo ability to stimulate root length in seedlings of the commercial annual wheat *cv* Bologna. Among those, seven strains were found to positively influence wheat roots growth. OK_1 and OK_26 showed the highest increase in roots length, with an induction of around 60%, followed by OK_32, with an induction of 50%. Plants derived from seeds inoculated with OK_26 and OK_35 showed an induction of 45% and 30%, respectively, while OK_4 and OK_33 showed an induction of around 30% and 20%, respectively (Fig. 2). No significant increase in fresh weight was observed in wheat seedlings derived from bacteria inoculated seeds with respect to uninoculated ones at this early stage of growth, except for OK_1 (Supplementary Fig. S1).

Mycelia growth inhibition of *Aspergillus flavus*, *Fusarium proliferatum*, and *Fusarium verticillioides*

The twenty-five selected bacterial strains were tested against the phytopathogenic fungal species *A. flavus*, *F. proliferatum*, and *F. verticillioides*, in a dual culture antagonism assay in order to assess their antagonistic activity. Three bacterial strains, namely OK_17, OK_39, and OK_45, were found to be effective against the three fungi. The three strains showed comparable effects. In fact, they were able to produce 40% and 50% growth inhibition of *A. flavus* after 5 and 7 days, respectively. The same strains showed 30% and 40% growth inhibition of *F. proliferatum* after 5 days and 7 days, respectively. A lower effect was detected for both strains against *F. verticillioides*, which showed 20% and 30% growth inhibition after 5 and 7 days, respectively (Fig. 3).

The antifungal activity of isolated strains was also assessed by the co-inoculation of bacterial cells and fungal conidia (Fig. 4). The antifungal activity of OK_17, OK_35, and OK_45 strains was confirmed in this second test. In addition, their activity was significantly increased by co-inoculation, achieving 100% growth inhibition of the three fungal species. Inhibition of 100% was also found co-inoculating OK_30, OK_42, and OK_54 with

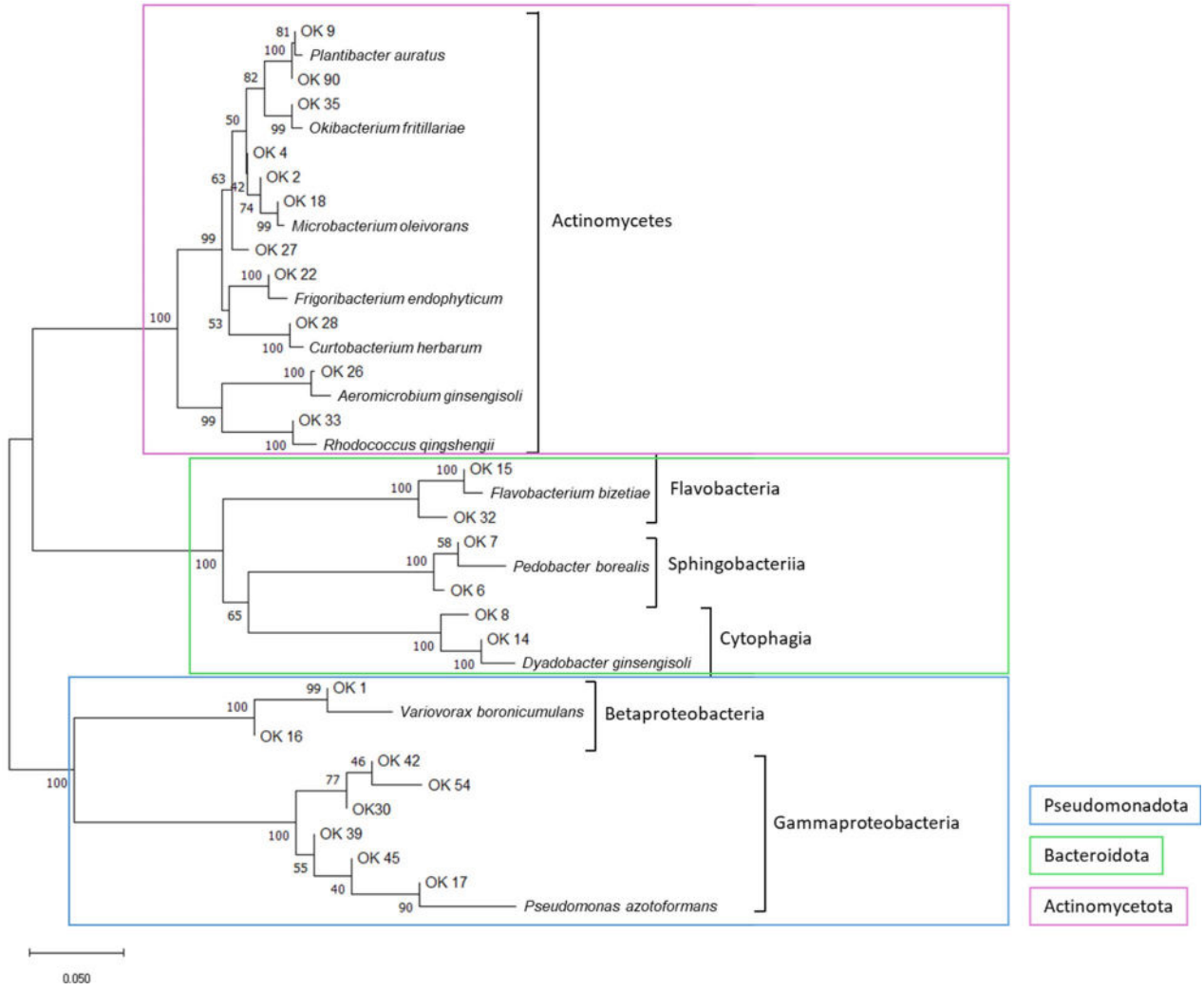

Fig. 1. Phylogenetic tree based on 16S rDNA sequences of twenty-five bacterial strains isolated from the rhizosphere of the OK72 perennial wheat line. Phylogenetic tree was constructed using neighbour-joining analysis between bacterial strains isolated from the rhizosphere of the OK72 perennial wheat line and selected bacteria reference 16S rDNA sequences. The isolated strains were selected based on their PGP traits and utilised for subsequent analyses. Bootstrap values are represented by numbers at the nodes based on 1000 replications. The open brackets group the strains according to their class, while the coloured frames group the isolates according to their phyla.

A. flavus and *F. proliferatum*. Around 50% and 20% inhibition was observed for OK_7 against *A. flavus* and *F. verticillioides*, respectively. *A. flavus* growth was 70% impaired by OK_33 and OK_90, althought they were found to be uneffective against both *Fusarium* species. A reduction in the growth of *F. verticillioides* of around 50% was observed in the co-inoculum with OK_30 and OK_54, and 60% in the co-inoculim with OK_35. For the other bacterial strains tested, the inhibition was lower than 40%.

Identification of VOCs

The bacteria listed in Table 1 and selected according to their PGP traits were also evaluated to produce VOCs in liquid PCA medium after one day of growth. The results showed that the isolated bacterial strains produce different VOCs (Table 2; Fig. 5). Eleven volatile compounds of various chemical classes were identified: 6 alcohols, 3 organic acids, 1 ketone, and 1 sulphur compound. Not all the VOCs were detected in all the tested

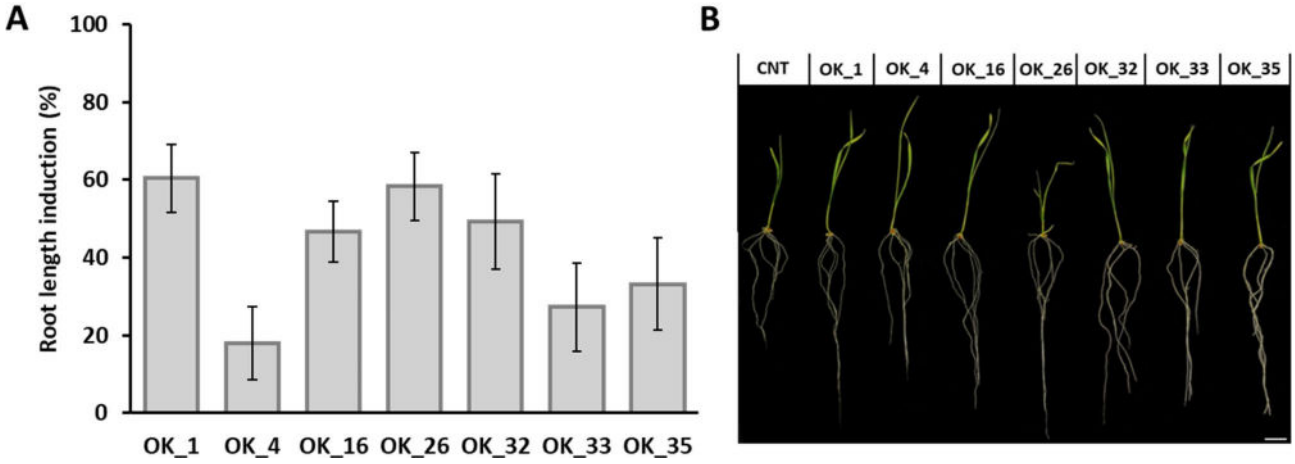

Fig. 2. Growth-promoting activity exerted by bacteria on the root length of a commercial wheat cultivar. (**A**) Effect of the inoculation of the commercial wheat *cv* Bologna seeds with bacterial strains on the seedlings' root length after ten days of growth. Data are presented as mean of thirty seedlings ± S.D. and are presented as percentage of roots length induction with respect to the control condition (uninoculated). Only data on bacterial strains that showed significant root length induction compared to control conditions according to ANOVA and Tukey's test ($p < 0.05$) are reported. (**B**) Representative images of control and bacterial inoculated single wheat seedlings. White bars in the photographs correspond to 2 cm.

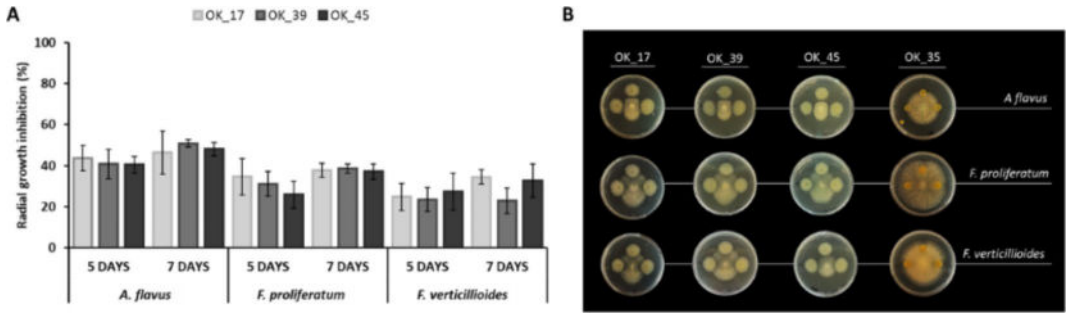

Fig. 3. Percentage of inhibition of radial mycelium growth of *Aspergillus flavus*, *Fusarium proliferatum*, and *Fusarium verticillioides* induced by bacterial strains in the dual cultures antagonistic assay. (**A**) Mycelia radial growth inhibition induced by OK_17, OK_39, and OK_45 against *A. flavus*, *F. proliferatum*, and *F. verticillioides* on PCA medium after 5 and 7 days of growth. Data presented are means of three independent replicates ± S.D. Only bacterial strains that showed significant fungal inhibition compared to control conditions according to ANOVA and Tukey's test ($p < 0.05$) are reported. (**B**) Representative pictures of the effects induced by OK_17, OK_39, and OK_45 after 7 days of growth. OK_35 which did not show any fungal inhibition was included in the figure to show the fungal growth in the presence of a negative strain.

samples; indeed, some samples showed the presence of only one VOC (OK_4, OK_17), while other samples presented a wider range of volatile components (Table 2).

Considering VOC concentrations, higher amounts of volatile compounds were found in OK_15, OK_17, OK_30, OK_32, OK_39, OK_45, and OK_54. The most representative compound was 1-undecanol (Table 2; Fig. 5). This component was detected in OK_17, OK_30, OK_39, OK_45, OK_54, and OK_63, but the highest amount was calculated in OK_45. Among all the other identified VOCs it is possible to observe significant differences ($p < 0.05$) based on the strains used in the experiment (Table 2). Dimethyl disulfide, 2-methyl-butanoic acid and 3-methyl-1-butanol were found in relevant amounts (Table 2; Fig. 5). Also 1-octanol was found in high concentrations, but it was detected only in two of the tested samples.

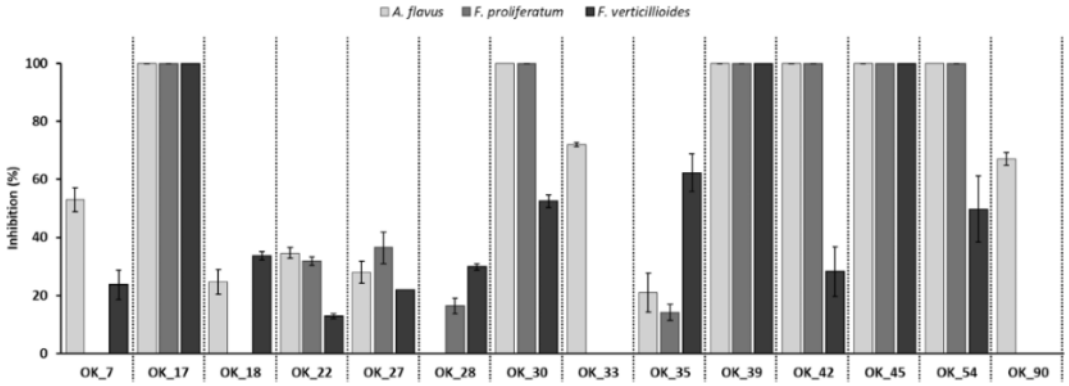

Fig. 4. Mycelia growth inhibition of bacteria co-inoculum against *Aspergillus flavus*, *Fusarium proliferatum*, and *Fusarium verticillioides*. Data are presented as percentage of inhibition with respect to the control (only fungal cultures) and are means of 6 biological replicates ± S.D. Only bacterial strains that showed significant fungal inhibition compared to control conditions according to ANOVA and Tukey's test ($p < 0.05$) are reported.

		Alchols					Ketones	Organic acids			Sulfur compounds
Sample	Benzyl alcohol	1-Butanol	2-Hexen-1-ol	3-Methyl-1-butanol	1-Octanol	1-Undecanol	2-Nonanone	Acetic acid	Butanoic acid	2-Methyl-butanoic acid	Dimethyl disulfide
OK_1			0.18 ± 0.01d								
OK_2	0.40 ± 0.03b	0.96 ± 0.02a		1.90 ± 0.13c				1.26 ± 0.10a.b	0.41 ± 0.03a	0.45 ± 0.01c	
OK_4				2.24 ± 0.13c							
OK_6	0.36 ± 0.06b	1.13 ± 0.02a		5.42 ± 0.16b				0.80 ± 0.01b		2.06 ± 0.17b	
OK_7	0.39 ± 0.01b			8.85 ± 0.46a				1.01 ± 0.06b		3.29 ± 0.20b	
OK_8	0.27 ± 0.08c	1.00 ± 0.03a		3.40 ± 0.09c						1.23 ± 0.11b.c	
OK_9		0.92 ± 0.04a		1.88 ± 0.17c				1.44 ± 0.07a	0.31 ± 0.04a	0.57 ± 0.02c	
OK_14	0.44 ± 0.10b			2.70 ± 0.49c						0.43 ± 0.11c	
OK_15	0.43 ± 0.01b			6.58 ± 0.71b				0.35 ± 0.02c		3.03 ± 0.43b	2.81 ± 0.06b
OK_16	0.18 ± 0.01d	0.13 ± 0.00b		0.50 ± 0.04e							6.03 ± 0.37a
OK_17						10.57 ± 0.11c					
OK_18		1.03 ± 0.04a		1.97 ± 0.18c				0.60 ± 0.05c	0.15 ± 0.02b	0.38 ± 0.00c	
OK_22	0.42 ± 0.08b	0.87 ± 0.08a	1.25 ± 0.00a	1.89 ± 0.08c				0.68 ± 0.01c		2.28 ± 0.20b	
OK_26	0.42 ± 0.08b			2.34 ± 0.05c				0.26 ± 0.02c			
OK_27	0.40 ± 0.07b	1.00 ± 0.11a		1.90 ± 0.28c						0.16 ± 0.00d	1.35 ± 0.03c
OK_28	0.54 ± 0.07a,b	1.06 ± 0.06a		2.55 ± 0.13c							
OK_30	0.28 ± 0.03c		0.47 ± 0.02c		7.87 ± 0.12a	12.53 ± 0.14c	1.23 ± 0.03b				
OK_32				6.78 ± 0.47b				1.60 ± 0.13a		8.08 ± 0.53a	1.53 ± 0.12b
OK_33	0.17 ± 0.02d		0.43 ± 0.04c							0.17 ± 0.00d	
OK_35	0.62 ± 0.10a			3.12 ± 0.23c			1.34 ± 0.05b	0.64 ± 0.08c	0.16 ± 0.00b	0.33 ± 0.02c	
OK_39			0.50 ± 0.06c			51.01 ± 1.51b					
OK_42			0.54 ± 0.02c	1.10 ± 0.12d							2.67 ± 0.07b
OK_45			0.85 ± 0.10b			88.44 ± 2.63a					
OK_54				2.03 ± 0.32c	9.50 ± 0.48a	71.80 ± 4.85a.b	2.87 ± 0.10a				
OK_90		1.43 ± 0.12a		2.26 ± 0.25c							

Table 2. Identified volatile compounds (VOCs), grouped on the basis of their chemical class, in the twenty-five bacterial strains and their concentration (ng mL^{-1}). Data are reported as mean ± standard deviation, derived from the analysis of three replicates per each sample. Letters highlight statistically significant differences among analysed samples, on the basis of one-way ANOVA test ($p < 0.05$).

Discussion

In agriculture, unsustainable practices like excessive fertilization and pesticide use often ignore the importance of soil biodiversity, disrupting beneficial soil-plant interactions in long-term fertilized fields[31,32]. Domestication has further altered the functional microbiome, compromising the plant-microbe relationship and reducing the

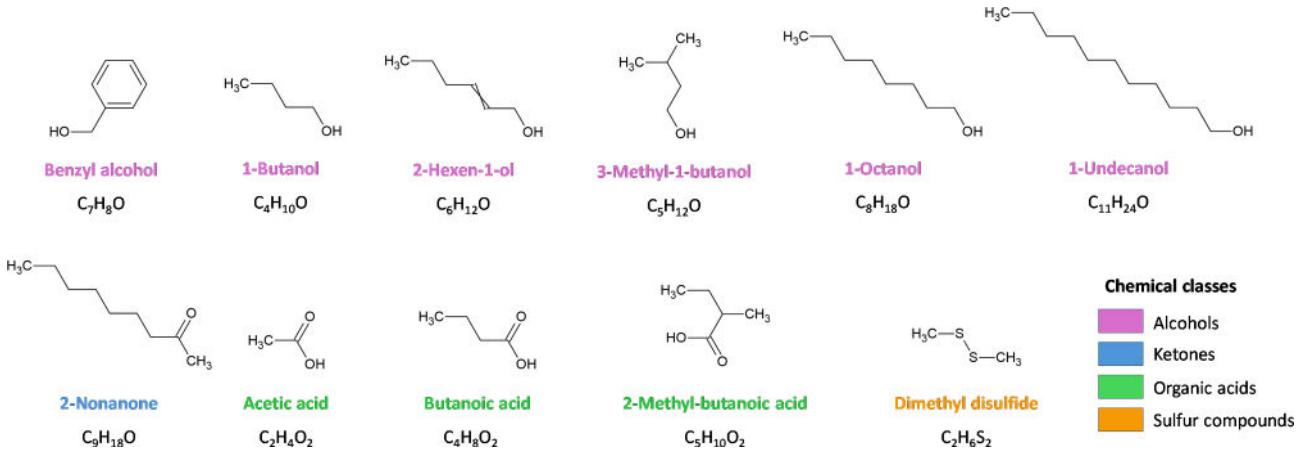

Fig. 5. Molecular formula and chemical structure of the identified volatile compounds. The chemical structure of each molecule was designed using ChemSketch v. 2023.1.2 (ACD/Labs, Canada).

natural biocontrol potential of rhizosphere bacteria against pathogenic fungi[33]. This loss of microbial complexity, especially in wheat, is exacerbated by both domestication and fertilization, leading to a decrease in beneficial microorganisms[34].

Perennial grains, with their deep root systems and no-tillage management, enhance soil microbial biodiversity and activity compared to annual grains[35]. They are valuable for selecting beneficial root-associated microorganisms. The microbial community in the rhizosphere of the perennial wheat line OK72, previously analysed using 16S rDNA NGS sequencing[29], showed a higher abundance of beneficial species, particularly from the *Pseudomonas* genus. This study isolates and characterizes rhizosphere bacterial strains from OK72 for their plant growth-promoting traits and antimicrobial properties against phytopathogenic fungi. The homology analysis revealed that the bacteria isolated belong to twenty different genera with the most represented *Pseudomonas* and *Microbacterium*, followed by the *Pedobacter*, *Dyadobacter*, *Plantibacter*, *Caulobacter*, and *Flavobacterium* (Supplementary Table S1) all of which are considered keystone taxa, playing a crucial role in wheat rhizobiome structure and function, enhancing nutrient availability, plant productivity, and pathogen control[36–38]. Finkel et al.[39] demonstrated that *Variovorax* can stabilize chemical conditions in complex communities, aiding *A. thaliana*'s development, and recruit beneficial bacteria even in established communities. Similarly, *Microbacterium* and *Rhodococcus* have been shown to affect community structure in *A. thaliana*'s phyllosphere[40]. Furthermore, Fan et al.[41] established the importance of keystone taxa in maintaining soil functioning and wheat production and in controlling plant pathogens in agricultural soil after thirty-five years of fertilization, providing the possibility to improve crop production by regulating microbial keystone taxa in agroecosystem. These findings suggest the potential of isolating beneficial bacterial strains from perennial grains to restore soil function and enhance productivity in intensive agroecosystems. In addition, in a more long-term perspective, it could be hypothesized the use of perennial wheats as cover crops in rotation with annual grains, to enrich the soil with key stone taxa able to improve their productivity and healthiness.

As shown in Table 1 and Supplementary Table S1, many bacterial strains from the OK72 rhizosphere exhibited high siderophore activity. Plants under iron limitation can adjust root exudates to favor siderophore-producing microbes[42]. The "Montelibretti" soil, with moderate alkalinity (Pucci et al., submitted), may cause iron deficiency[43]. The abundance of siderophore-active strains in OK72 suggests the plant encourages microbes that enhance iron solubilization. Genera such as *Variovorax*, *Pseudomonas*, and *Microbacterium* are known to promote wheat growth[44–46]. These beneficial effects of PGPR are reported across various plant species and conditions[47–50]. Preliminary data from our study showed that strains OK_1, OK_16, OK_26, OK_32, and OK_35 stimulated root growth in annual wheat, with OK_33 and OK_35 showing high in vitro production of IAA and ACC-deaminase activity (Table 1).

Another important feature of beneficial microorganisms present in the rhizosphere is the production of antimicrobial compounds for biocontrol against plant pathogenic species. The tests conducted against *A. flavus*, *F. proliferatum*, and *F. verticillioides* revealed that OK_17, OK_39, and OK_45, belonging to the *Pseudomonas* genus, were found to be effective against all the three fungal species (Figs. 3 and 4). In particular, we showed for the first time that strains homologous to *P. lactis* (OK_39 and OK_45) are effective in inhibiting the growth of these three pathogen fungal species although an indication on the biocontrol activity of *P. lactis* isolate was already observed against the phytopathogenic fungal species *C. pseudonaviculata*[51].

The role of *Pseudomonas* spp. in the suppression of plant pathogens is well established and some commercial products are based on different *Pseudomonas* species[52]. *Pseudomonas* spp. EM85 showed the suppression of the disease caused by *F. graminearum* and *F. verticillioides* through the production of antifungal as antibiotics, siderophore, fluorescent pigments and cyanides[53]. Multi-trait *Pseudomonas* spp. isolated from wheat and producing siderophores, cellulase and protease, were found to interfere with the growth of *F. oxysporum* and *F. menoliforme*, suppressing *Fusarium* root rot[54]. Nayaka et al.[55] observed that *P. fluorescens* successfully inhibited the growth of *F. verticillioides* and suppressed the synthesis of fumonisins. An aspect that deserves consideration

is that OK72 plants had never shown symptoms of disease such as powdery mildew and rust during the trials conducted by Bertola et al.[29] and Pogna et al.[56], even if attacks by these fungi have been observed on wheat crops adjacent to the experimental area over the different year of cultivation. The results indicate increased OK72 resistance to pathogens, to which the presence of bacterial strains capable of inhibiting the development of pathogens may contribute. Indeed, the *Pseudomonas* genus was found to be significantly correlated with the response to stripe rust[57].

The co-inoculation test significantly increased the effectiveness of bacterial strains in inhibiting *A. flavus*, *F. proliferatum*, and *F. verticillioides* mycelia (Fig. 4). Strains OK_17, OK_30, OK_39, OK_42, OK_45, and OK_54, which exhibited the highest antifungal activity, also showed the highest siderophore production (Table 1). Siderophores can inhibit pathogen growth by depriving fungi of iron, reducing conidia germination and mycelia development[58,59]. For instance, *P. syringae* BAF.1's siderophores inhibited *F. oxysporum* spore germination and damaged spore and mycelia ultrastructure[60], and *E. coli* siderophores reduced *A. nidulans* growth[61].

While iron limitation likely contributes to the high inhibitory activity of our bacterial isolates, it is not the sole mechanism. Indeed, during their growth, the isolated strains were also found to produce VOCs (Table 2; Fig. 5). In particular, the antifungal activity of these bacterial strains may result from a combination of several VOCs, which could have additive or synergistic effects. VOCs are recognized for their potential antifungal properties, often affecting fungal cell membranes by increasing lipid peroxides and causing cellular damage[62]. Among the bacterial strains tested, those with high antifungal activity (OK_17, OK_30, OK_39, OK_45, OK_54) were notably characterized by the production of 1-undecanol, a compound not produced by other strains. 1-Undecanol has demonstrated potent antifungal activity against various fungi, including *Saccharomyces cerevisiae* and *Zygosaccharomyces* spp.[63,64]. Its mechanism is likely due to its surfactant properties, disrupting fungal membrane integrity[65]. In addition, OK_30 and OK_54 produced 1-octanol; thus the inhibitory effect on fungal growth could be due to a synergism between these two antifungal compounds both altering the function of fungi cytoplasmatic membrane[65,66].

Although dimethyl disulfide was detected in low concentrations in some strains (OK_15, OK_16, OK_27, OK_32, OK_42), it did not show significant antifungal effects, likely due to its insufficient concentration relative to minimal inhibitory levels reported in previous studies[67].

1-Butanol, found in various samples (OK_9, OK_14, OK_16, OK_18, OK_22, OK_27, OK_28, OK_90), exhibited limited antifungal activity, which is consistent with previous findings that longer-chain alkanols are generally more effective[65]. 2-Nonanone, detected in strains OK_30, OK_35, and OK_54, showed inhibition against several fungal species, likely due to its impact on the structure of fungal cell membranes[68,69].

2-Hexen-1-ol, identified in strains effective against *F. verticillioides* (OK_22, OK_30, OK_39, OK_42, OK_45), is known for its antifungal properties, attributed to its hydroxyl group that disrupts enzyme activity and inhibits the growth of fungi[70]. Organic acids such as acetic and butanoic acids, though linked to antifungal activity in some studies[71], showed limited effects in our tests. Benzyl alcohol was found in several bacterial cultures, with higher concentrations in OK_35. While benzyl alcohol has antifungal properties against certain pathogens[72], it has not been extensively tested against the fungi in our study.

Conclusions

Our study found that the perennial wheat line OK72, after two years of being in the soil, hosts a rich community of cultivable rhizosphere bacteria with plant growth-promoting traits and able to inhibit the growth of phytopathogenic fungi. Notably, strains from keystone genera such as *Microbacterium*, *Pedobacter*, *Dyadobacter*, *Plantibacter*, and *Flavobacterium* are present, which could enhance nutrient availability and plant productivity. Additionally, *Pseudomonas* strains, in particular those producing high concentrations of 1-undecanol showed the highest inhibition growth effect over *Fusarium* and *Aspergillus* genera, thus suggesting the key role of this compound in the control of these plant pathogens. The results obtained in this study suggest that the selected bacteria could be used to enhance yields and health also in annual grains cultivations while reducing chemical fertilizer inputs and minimizing soil impact. Further analyses will be conducted to assess their applicability as consortia and their effective biocontrol activity in green house and in-field experiments. Additionally, from a long-term perspective, the identification of high number of keystone taxa in the rhizosphere of OK72 suggests that perennial grains could be also strategically used in crop rotation to enrich soils with beneficial microbes, thereby supporting the health and productivity of annual grains.

Materials and methods

Isolation and molecular identification of bacterial strains from the rhizosphere of the OK72 perennial hybrid line

Three plants of the OK7211542 (OK72) genotype (*T. aestivum* X *Th. ponticum*)[73] kindly provided by Richard Hayes (Graham Centre for Agricultural Innovation, NSW, Wagga Wagga, Australia) were collected from the field set up at the Montelibretti (Rome) experimental farm of CREA-IT, (Lat 42°08'N; Long 12°44'E; 20 m a.s.l.) in the Tiber valley. The samples were collected in May 2023, in the second year of cultivation, by excavating from the top 60 cm of soil, and the entire plants with the soil clod containing the root system were taken to the laboratory for subsequent analyses.

For rhizosphere bacteria isolation, all the non-adherent soil was removed from the roots by scrolling them, and the roots were separated from the plants. Roots were placed in a 50 mL tube with 0.3% (w/v) of sodium pyrophosphate under stirring conditions for 1.5 h. The isolation of culturable bacteria was carried out by diluting the suspension from 10^{-1} to 10^{-4} and 100 µL of each dilution was spread on plate count agar (PCA) medium (Sigma Aldrich, St. Louis, MO) + cycloheximide (20 mg/L) (Sigma Aldrich, St. Louis, MO). Petri dishes were incubated at 28 °C and the bacterial colonies were selected after 5 days based on their morphology. Colonies with

different morphology were purified on PCA. Selected bacterial isolates were stored in 15% glycerol at − 80 °C and routinely grown on PCA.

Genomic DNA was released from bacterial strains by diluting each colony in 40 µL of sterile water and exposing it to 3 cycles of freezing in liquid nitrogen and thawing at 90 °C as previously described[74]. For the amplification of the 16S rDNA gene fragment, the released DNA from each colony was amplified by PCR in a thermocycler Applied Biosystems (Foster City, CA, USA). The reaction was performed in 20 µL total volume with 10 µL of the "EasyTaq PCR SuperMix" (Transgenbiotech, Beijing, China), 0.4 µL of each universal 16S rDNA primers (27 F 5′- AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-ACGG CTACCTTGTTACGACT T-3′), and 2 µL of of liquid culture containing DNA. The PCR conditions were: 7 min at 94 °C; 30 cycles 1 min at 94 °C, 1 min annealing at 56 °C, 1 min and 30 s extension at 72 °C; final extension step at 72 °C for 8 min.

The 16S rDNA PCR products were purified using the "Nucleospin Gel and PCR Cleanup" kit (Macherey-Nagel) and subsequently sequenced (https://www.macrogen-europe.com).

Partial 16S rDNA sequences were matched against nucleotide sequences available in GenBank using the BLAST tool (using rRNA/ITS databases) in the NCBI website. The nucleotide sequences were deposited in the GenBank database under accession numbers PQ015127 through PQ015164. Phylogenetic analysis of the 16 S rDNA sequences of isolated strains was carried out using MEGA11.0 software[75]. The sequences were aligned using the ClustalW algorithm and the output was used to build a phylogenetic tree by calculating distance matrices for Neighbor Joining (NJ) analysis with the maximum composite likelihood and a bootstrapping analysis with 1000 replicates. Several 16S rDNA sequences of previously identified bacteria were included as references in the phylogenetic tree.

In vitro determination of bacterial IAA production and ACC-deaminase activity

Indole-3-acetic acid was assessed according to Sheng et al.[76] with some modifications[77]. Bacteria were grown in sucrose-minimal salt (SMS) medium (sucrose 1% (w/v), $(NH_4)_2SO_4$ 0.1%, K_2HPO_4 0.2%, $MgSO_4$ 0.05%, NaCl 0.01%, yeast extract 0.05%, $CaCO_3$ 0.05%, tryptophan 0.5 mg mL^{-1}) for three days. Cultures were then centrifuged and 500 µL of supernatant was added to 1 mL of Salkowski's reagent[78] (0.5 M $FeCl_3$, $HClO_4$ 35%). After 25 min incubation at RT the absorbance was read at 530 nm wavelength. IAA concentration was determined using a calibration curve of pure IAA.

ACC-deaminase activity was assessed on DF salts media[79] amended with 3mM ACC and prepared according to Penrose and Glick[80]. Bacterial strains were inoculated on DF agar plates and incubated for five days at 28 °C. Bacterial strains growing on the plates with ACC as the sole nitrogen sources were taken as positives. Negative control plates were prepared without any nitrogen source.

In vitro determination of bacterial siderophores production

To measure siderophores activity, bacteria were grown in SMS medium for three days; cultures were then centrifuged to remove the cells and 500 µL of supernatant was added to the same volume of CAS assay solution (6 mL of 10 mM hexadecyltrimethylammonium bromide (HDTMA), 1.5 mL of 1 mM $FeCl_3$, 7.5 mL of 2 mM CAS, 4.307 g of piperazine, and 6.25 mL of 12 M HCl, then diluted to 100 mL with double-distilled water)[81], and incubated for 20 min at RT. The activity of siderophores was determined reading the absorbance at 630 nm wavelength[81]. The results are expressed as percentage siderophores unit (PSU)[82].

In vitro determination of bacterial biofilm formation

Biofilm formation was evaluated following the protocol described by O'Toole[83], applying some modifications[77]. Briefly, an overnight bacterial culture in liquid PCA medium was diluted 1:100 in fresh liquid PCA medium and 100 µL was inoculated in a well of a 96-well plate in for 5 days at 28 °C. To quantify the biofilm formation, the medium was discarded submerging the plate in water two times. After water removal, 125 µL of a 0.1% solution of crystal violet was added in each well and incubated for 15 min at RT. The plate was rinsed three times with water and, after water removal, dried for 2 h. A volume of 125 µL of 30% acetic acid solution was added and after 15 min of incubation, the absorbance was quantified at 595 nm wavelength.

In vitro determination of bacterial protease activity and phosphate solubilization activity

To evaluate inorganic phosphate solubilization activity and protease activity, 5 µL of an overnight bacterial cell culture were inoculated in a Petri dish containing Pikovskaya (PVK) medium (dextrose 10 g L^{-1}, yeast extract 0.5 g L^{-1}, $Ca_3(PO_4)_2$ 5 g L^{-1}, $(NH_4)_2SO_4$ 0.5 g L^{-1}, KCl 0.2 g L^{-1}, $MgSO_4$ 0.1 g L^{-1}, $MnSO_4$ 0.0001 g L^{-1}, $FeSO_4$ 0.0001 g L^{-1}, agar 10 g L^{-1})[84] and skim milk agar plate medium (casein hydrolysate 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 4 g L^{-1}, skim milk powder 20 g L^{-1}, agar 10 g L^{-1})[85], respectively. After 5 days of growth at 28 °C, positive activity was indicated by the presence of a clear halo surrounding the bacterial colony, and the solubilization index (SI) was calculated according to Joshi et al.[86].

Evaluation of bacterial growth promoting ability in a commercial annual wheat cultivar at the germination stage

Each bacterial strain was grown for two nights in 20 mL of liquid Luria Bertani medium on shaking (130 rpm) at 28 °C. After measuring OD at 600 nm with a spectrophotometer (Varian Cary 50 UV-Visible), the suspension of bacterial cells was diluted to 10^8 cells mL^{-1} with bi-distilled sterile water. Commercial common wheat seeds (*Triticum aestivum cv* Bologna; SIS, Bologna, Italy) were surface sterilized for 1 min with 70% v/v EtOH, then for 15 min with a 40% v/v NaClO solution, and finally rinsed six times with double distilled sterile water. The sterilized seeds were imbibed in the bacterial suspension for 1 h (for the control treatment, the seeds were imbibed in sterile water) on shaking (200 rpm). The seeds were allowed to dry in a laminar airflow hood for 2 h and then transferred to sterile plastic pots (125 mm length x 65 mm wide x 80 mm height) containing a sterile

filter paper soaked with 5 ml of sterile water. All pots were incubated for 3 days in a growth chamber at 24 °C in complete darkness and then for a further 7 days in a growth chamber with photoperiod (24 °C; 16/8 h light/dark photoperiod; 120 µmol m^{-2} s^{-1} PAR, 75% RU). After 10 days of growth, the fresh weight, and the percentage of root length induction were analysed. For the root length induction percentage, the following formula was used:

$$\%\ Root\ length\ induction\ = \left[\frac{Lc - Li}{Lc}\right] \times\ 100$$

where Lc is the root length of the uninoculated seedlings and Li is the root length of the inoculated seedlings. Three biological replicates for each treatment were performed with 10 seeds per replicate. Data are expressed as mean ± standard deviation (S.D.).

Inhibition of mycelial growth in solid and liquid medium of *Aspergillus flavus*, *Fusarium proliferatum*, and*Fusarium verticillioides*

The phytopathogenic *A. flavus*, *F. proliferatum*, and *F. verticillioides* were used to evaluate the antagonism activity of the selected bacterial strains previously characterised and listed in Table 1. *A. flavus* conidia production was allowed cultivating fungi on plate count agar (PCA; tryptone 5 g L^{-1}, yeast extract 2.5 g L^{-1}, glucose 1 g L^{-1}) for 14 days at 28 °C, while *F. proliferatum* and *F. verticilliodes* conidia production was allowed by cultivating fungi on SNA medium (KH_2PO_4 1.0 g L^{-1}, KNO_3 1.0 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.5 g L^{-1}, KCl 0.5 g L^{-1}, glucose 0.2 g L^{-1}, sucrose 0.2 g L^{-1}, agar 15.0 g L^{-1}) for 14 days at 28 °C.

The antifungal activity of the bacterial isolates was evaluated using the dual culture antagonistic assay following the method described by Guevara-Avendaño et al.[87]. Five µL of a conidia suspension were spotted in the centre of a Petri dish containing PCA medium. The bacterial cells culture was diluted to 10^7 cells mL^{-1} with bi-distilled sterile water, then 5 µL of the bacterial suspension were spotted at 2 cm distance from the fungal inoculum. Three spots for each bacterial strain per plate were performed and the assay was carried out in triplicate. Five µL of bi-distilled sterile water were used as a control. After 5 and 7 days of incubation at 28 °C, the percentage of inhibition in the fungal radial growth was calculated according to Idris et al.[88]:

$$\%\ Radial\ growth\ inhibition\ = \left[\frac{R - r}{R}\right] \times 100$$

where R is the radius of fungal growth from the centre of the plate towards the control treatment and r is the radius of fungal growth towards the bacterial treatment. Values are expressed as a percentage of inhibition with respect to the control ± S.D.

A second assay was performed to evaluate the antifungal activity of bacterial strains in liquid culture. Conidia and bacterial cells of each fungal species and bacterial strains were diluted in liquid PCA medium to a final concentration of 10^5 conidia or cells mL^{-1}. Fifty µL of the conidia suspension and 50 µL of the bacterial suspensions were both inoculated in a 96-multiwell plates with 100 µL of liquid PCA to obtain a final volume of 200 µL/well. The control condition was represented by the inoculation of the same quantity of conidia in a final volume of 200 µL/well. For *A. flavus*, the biomass production was evaluated after five days of incubation, while for *F. verticilloides*, and *F. proliferatum* the biomass production was evaluated after 7 days; mycelia from single wells were recovered, slightly dried on paper, and weighted[89]. Values were expressed as percentage of inhibition with respect to the control. Six biological replicates for each bacterial strain and fungal species were performed. Values are expressed as a percentage of inhibition with respect to the control ± S.D.

Analysis of bacterial VOCs by SPME–GC–MS

The bacteria listed in Table 1 and selected based on their PGP traits were also evaluated to produce VOCs. VOCs produced by bacteria were determined by solid phase microextraction (SPME) coupled with gas chromatography and mass spectrometry (GC-MS) approach, according to Cirlini et al.[90], with some modifications. Samples (liquid cultures containing bacteria) were inserted in 20 mL SPME glass vials and VOCs were captured on a tri-phasic SPME coated with 50/30 µm of Divinylbenzene–Carboxen–Polymethylsiloxane (DVB/Carboxen/PDMS) (Supelco, Bellefonte, PA, USA), applying a sampling temperature of 40 °C for 30 min, after an equilibration time of 15 min at the same temperature. VOCs were then desorbed at 250 °C for 2 min in the GC-MS injector and separated on a ZB-WAX *plus* capillary column (Phenomenex, 30 m x 0.25 mm, f.t. 0.25 µm) placed in a Thermo Scientific Trace 1300 gas chromatograph coupled to a Thermo Scientific ISQ single quadrupole mass spectrometer (Thermo Fisher Waltham, MA, USA). Oven conditions were as follows: temperature started at 50 °C for 3 min, then it was increased of 5 °C/min until 200 °C and maintained at this value for 12 min, for a total run time of 45 min. Helium was the carrier gas used at a flow of 1 mL/min. The mass spectrometer was operated in the electron ionization impact mode, in full scan modality, monitoring an m/z range of 40–500. VOCs identification was achieved comparing the registered mass spectrum of each compound with those present in the instrumental library (NIST version 2.3). In addition, all the components were semi-quantified based on a reference compound (toluene) added in a specific concentration to the samples (5 µL of an aqueous solution, 100 mg L^{-1}); results were expressed as ng mL^{-1}. To exclude compounds already present in the medium used for the cultivation of bacteria, also this component was analysed and used as control sample.

Statistical analysis

Data obtained from bacterial growth promoting ability in common wheat and from in vitro antifungal assays were statistically analysed using One-way ANOVA test in the Past 4.06b software[91]. Tukey post-hoc test was applied. Differences were considered significant at $p < 0.05$. Data obtained from HS-SPME/GC-MS analyses

were statistically elaborated to verify differences and/or analogies among the different bacterial strains in terms of volatile compounds production. In particular, one-way ANOVA test was applied using Tukey post-hoc test ($p < 0.05$).

Data availability

The data of 16S rDNA sequences presented in the study are deposited in the NCBI database (accession numbers listed in Table 1 and Supplementary Table S1). All the data generated during the current study are presented in the main manuscript and in the supplementary information. The OK72 perennial wheat line is maintained in an on farm collection, funded, under the RGV – FAO program (Programma triennale 2023-2025 per la conservazione, caratterizzazione, uso e valorizzazione delle risorse genetiche vegetali per l'alimentazione e l'agricoltura) by MASAF (Ministerial Decree No. 5005 dated 1/02/2023). The line was initially provided in 2012 by Dr. Richard Hayes Hayes (Graham Centre for Agricultural Innovation, NSW, Wagga Wagga, Australia), as part of an international network promoted by FAO, which included among the others, The Land Institute (https://landinstitute.org/) with Dr. Shuwen Wang and CREA-IT (https://www.crea.gov.it/) with Dr. Laura Gazza.

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Author contributions

Conceptualization: G.G., G.V.; investigation, G.G., L.D.V., M.G., S.P., L.G.; Data curation: G.G., M.C., G.V.; supervision: G.V.; funding acquisition: G.G..; writing—original draft: G.G., L.D.V., M.G.; writing review and editing: G.V., M.C., G.G.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to G.V.

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