Hydrogen Peroxide Metabolism in Interkingdom Interaction Between Bacteria and Wheat Seeds and Seedlings

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In endophytes, the abundance of genes coding for enzymes processing reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), argues for a crucial role of ROS metabolism in plant-microbe interaction for plant colonization. Here, we studied H₂O₂ metabolism of bread wheat (*Triticum aestivum* L.) seeds and their microbiota during germination and early seedling growth, the most vulnerable stages in the plant life cycle. Treatment with hot steam diminished the seed microbiota, and these seeds produced less extracellular H₂O₂ than untreated seeds. Using a culture-dependent approach, *Pantoea* and *Pseudomonas* genera were the most abundant epiphytes of dry untreated seeds. Incubating intact seedlings from hot steam-treated seeds with *Pantoea* strains triggered H₂O₂ production, whereas *Pseudomonas* strains dampened H₂O₂ levels, attributable to higher catalase activities. The genus *Pantoea* was much less represented among seedling endophytes than genus *Pseudomonas*, with other endophytic genera, including *Bacillus* and *Paenibacillus*, also possessing high catalase activities. Overall, our results show that certain bacteria of the seed microbiota are able to modulate the extracellular redox environment during germination and early seedling growth, and high catalase activity is proposed as a key trait of seed endophytes.

Keywords: catalase, endophytes, epiphytes, microbiota, *Pantoea agglomerans*, *Pantoea eucalypti*, *Pseudomonas fluorescens*, *Pseudomonas oryziphobans*, reactive oxygen species

We now understand plants as holobionts that continuously interact with their associated microbiota (Bordenstein and Theis 2015; Zilber-Rosenberg and Rosenberg 2008). The microbiota consists of all microorganisms colonizing any plant niche, and the

sum of the genomes of the microbiota is defined as the microbiome (Brader et al. 2017). Seed-borne microbes represent the pioneer microbial community of the next plant generation and may play important roles in early plant growth and establishment, as well as affecting later microbial colonization from the soil microhabitat surrounding the seed, termed spermosphere (Barret et al. 2015; Nelson 2004; Rodríguez et al. 2018; Schiltz et al. 2015; Shade et al. 2017; Verma et al. 2019). Based on their colonization niche and irrespectively of their functions, seed microbiota are distinguished as epiphytes if they live on the seed surface (e.g., seed coat) and endophytes when they colonize internal seed tissues (termed endosphere), including embryo, endosperm, and eventually aleurone and perisperm (Nelson 2018).

Differently from other plant compartments such as the rhizosphere, the bacterial community of seeds is composed of a relatively limited number of phyla that are mostly grouped in the *Proteobacteria*, *Firmicutes*, and *Actinobacteria* (Hardoim et al. 2015; Nelson 2018; Santoyo et al. 2016). Seed microbiota may be intrinsically adapted to the seed habitat, thus surviving after seed maturation drying and eventually benefitting from solute leakage and storage nutrient mobilization upon germination (Barret et al. 2015; Bewley 1997; Mano et al. 2006). In the past decade, seed endophytes have received increasing attention regarding their vertical transmission routes (i.e., passing with the seed from one plant generation to the next). For instance, a culture-independent analysis reveals that 45% of the endophytes in rice seeds of the first generation are subsequently found in the second generation (Hardoim et al. 2012). Using 16S ribosomal (r)RNA gene analyses, Cope-Selby et al. (2017) showed that about 30% of bacterial endophyte families are shared between adult plants and seedlings germinated from surface-sterilized seeds, also suggesting that vertical transmission contributes to the adult plant microbiome. Furthermore, genetic selection on seed endophyte diversity was assessed using the closest maize wild relative (*Zea mays* subsp. *parviglumis*), landraces, and modern maize (*Zea mays* L.) cultivars. A core maize seed microbiome was identified with high specificity of taxa (e.g., genera *Clostridium*, *Paenibacillus*), which are tightly associated within genus *Zea* (Johnston-Monje and Raizada 2011). Similarly, barley seeds of five cultivars share a great part of their microbiome, which includes genera *Phyllobacterium*, *Paenibacillus*, and *Trabusiella* (Yang et al. 2017). Recently, the seeds of *Malus pumila* were found to be a hot spot of microbial abundance, in comparison with other apple fruit tissues (Wassermann et al. 2019). Moreover, when wheat flowers are sprayed with a suspension of the bacterial endophyte *Paraburkholderia*

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phytofirmans PsJN, this strain is transmitted to the seeds, confirming vertical inheritance of endophytes via seed production (Mitter et al. 2017).

Numerous studies report on the beneficial effects of bacterial endophytic strains on plant growth and stress tolerance, possibly mediated by endophyte-derived metabolite secretion (Bergna et al. 2018; Brader et al. 2014; Díaz Herrera et al. 2016; Khare et al. 2018; Naveed et al. 2014; Shahzad et al. 2018). Differently from seed endophytes, studies on seed epiphytes were mostly directed toward their pathogenicity, which led to the development of various seed sanitation methods (Halmer 2006; Nelson 2018).

The “oxidative burst” (Lamb and Dixon 1997; McDowell and Dangel 2000; Wojtaszek 1997) is a well-known response of plant cells to microbial challenge, involving a rapid and transient apoplastic production of reactive oxygen species (ROS), mostly superoxide anion radical and hydrogen peroxide (H_2O_2). The H_2O_2 -mediated signaling cascades activated by microbes in plants are well-described at a cellular level (Bigéard et al. 2015; Garcia-Brugger et al. 2006; Nanda et al. 2010; Pel and Pieterse 2013). Only in few cases, microbe-triggered H_2O_2 responses have been studied in seedlings (Lei et al. 2017; Morkunas et al. 2004). However, it is poorly understood if ROS production by seeds plays a role in inter-kingdom signaling with endophytes. In turn, the molecular mechanisms that enable a bacterium to adopt an endophytic lifestyle also remain unresolved. Compared with other micro-organisms, the endophytes studied hitherto appear to have an elevated number of genes coding for ROS-processing enzymes, including catalases, which dismutate H_2O_2 to water and molecular oxygen (Kandel et al. 2017; Liu et al. 2017; Mitter et al. 2013; Pauly et al. 2006; Reinhold-Hurek and Hurek 2011; Sessitsch et al. 2012; Zamocky et al. 2008). In plants, ROS (e.g., H_2O_2) are part of signaling networks influencing the activity of plant hormones, such as gibberellins and abscisic acid (Kranner et al. 2010a; Weitbrecht et al. 2011). In seeds, ROS are implicated in endosperm weakening, cell-wall remodeling to support radicle protrusion, and programmed cell death in the aleurone layer of cereals (Bailly et al. 2008; Bahin et al. 2011; Kranner et al. 2010b; Mhamdi and Van Breusegem 2018; Wojtyla et al. 2016). Furthermore, mitochondrial respiration, β -oxidation of fatty acids, alongside activities of NADPH oxidases, oxalate oxidases, amine oxidases, and extracellular peroxidases can all contribute to H_2O_2 production during seed germination (Gerna et al. 2017; Ishibashi et al. 2010; Kranner et al. 2010a; Li et al. 2017; Roach et al. 2010, 2015). Nonetheless, the influence of microbes on H_2O_2 metabolism in seeds and seedlings has not been appreciated.

Here, we used a culture-dependent approach to elucidate H_2O_2 metabolism associated with the interaction between seeds and their native epiphytes or endophytes during germination and early seedling growth, focusing on bacteria. We hypothesized that seed-borne microbiota induce H_2O_2 production (i.e., an oxidative burst) by seeds during germination. We chose to use seeds of bread wheat (*Triticum aestivum* L.), one of the most important crops globally (the FAO website; Laino et al. 2015), and the naturally occurring microbiota that they host. The production of H_2O_2 was compared between seeds treated with hot steam, a method of seed-surface sterilization obviating the use of chemicals affecting the seed redox state (e.g., H_2O_2 , sodium hypochlorite), and untreated control seeds. Epiphytes were isolated from dry control seeds and were used to inoculate intact seedlings from hot steam-treated seeds, to study and quantify subsequent oxidative H_2O_2 production. Finally, the H_2O_2 metabolism of these epiphytic bacterial strains was assessed. Altogether, our results provide physiological and biochemical evidence that breaking down H_2O_2 via catalase is implicated in the interaction of bacteria with the seedling endosphere.

RESULTS

Hot steam-treated seeds produced less H_2O_2 .

The hot steam treatment (Fig. 1A) affected H_2O_2 production in bread wheat seeds and seedlings (Fig. 1B). After the hot steam treatment, seeds and seedlings produced between 1.9-fold to 1.2-fold less H_2O_2 than untreated (control) seeds and seedlings at the same timepoint (Fig. 1B). First radicle protrusion was observed 12 h after the onset of imbibition, and, after 48 h, all viable seeds had germinated and produced seedlings (Supplementary Fig. S1). Prior to completion of germination, nitroblue tetrazolium chloride (NBT) staining indicated that ROS production was mostly localized in the coleorhiza and areas around the embryo, whereas, at the seedling stage, ROS were produced by the roots but not by the seed coat, coleoptile, or first foliage leaves (Fig. 1C and D).

Isolates of seed epiphytes comprised families *Enterobacteriaceae* and *Pseudomonadaceae*.

Quantifying the colony-forming units isolated from the surface of seeds showed that hot steam-treated seeds hosted around 10-fold fewer culturable seed epiphytes than control seeds (Fig. 2A). Eighteen bacterial isolates obtained from the surfaces of dry control seeds were identified using 16S rRNA gene sequencing, showing that they were members of class *Gammaproteobacteria*. Approximately 69 and 31% were assigned to families *Enterobacteriaceae* and *Pseudomonadaceae*, respectively (Fig. 2D; Table 1). To identify different strains, PCR amplification of the hypervariable intergenic spacer (IGS) region of the 16S-23S rDNA operon, followed by restriction fragment length polymorphism (RFLP) analyses, was combined with DNA gyrase subunit B (*gyrB*) gene sequencing. RFLP of IGS amplicons of the epiphytic *Enterobacteriaceae* spp. distinguished two clear ribotypes, suggesting that the epiphytic isolates corresponded to at least two different strains, as confirmed by *gyrB* sequencing (Table 1). Nine isolates of the same strain (approximately 82% of the *Enterobacteriaceae* spp.) were most closely related to *Pantoea agglomerans*, and two isolates of the same strain (approximately 18% of the *Enterobacteriaceae* spp.) to *Pantoea eucalypti* (Table 1).

Among the *Pseudomonadaceae* spp., three of five isolates showed identical IGS RFLP ribotypes, indicating that they were closely related. Sequence alignments of *gyrB* attributed this common ribotype to two different strains of *Pseudomonas oryzae* spp. (NT0-1 and NT0-6). Furthermore, two additional *Pseudomonadaceae* isolates exhibited slightly different ribotypes, and *gyrB* sequencing indeed assigned them to the distinct strains NT0-2 and NT0-8 of *Pseudomonas fluorescens* (Table 1). The remaining isolates belonged to two additional ribotypes, which differed from the other three and between each other. Analyses of *gyrB* sequences of these isolates revealed values of similarity with known species lower than 97%, which did not allow reliable identification.

Seedlings from hot steam-treated seeds hosted fewer culturable epiphytes and endophytes.

Compared with dry seeds, the number of epiphytic colony-forming units exponentially rose 10^4 -fold in seedlings 48 h after the onset of imbibition (Fig. 2A and B). Furthermore, seedlings from control seeds hosted about 2.3-fold more epiphytic colony-forming units than seedlings from hot steam-treated seeds (Fig. 2B), indicating that the hot steam treatment applied to dry seeds resulted in lower microbial load also at the seedling stage. Additionally, 16S rRNA gene sequencing of selected culturable epiphytic isolates of seedlings from control

seeds revealed an increase in the genus diversity of the epiphytes compared with dry seeds. The majority of the isolates were class *Gammaproteobacteria* of the genera *Pantoea* and *Pseudomonas*, but isolates of phylum *Firmicutes* genus

Paenibacillus and of phylum *Actinobacteria* genus *Rhodococcus* were also identified (Fig. 2D).

For isolating endophytes, control and hot steam-treated seeds were chemically surface sterilized. This procedure

Fig. 1. Hot steam treatment decreased hydrogen peroxide (H₂O₂) production by wheat seeds during germination and seedling growth. **A**, Conditions during the hot steam treatment prior to seed imbibition. Gray and black curves indicate intensity of the steam flow and temperature, respectively. **B**, Production of H₂O₂ by untreated control (black) and hot steam-treated (white) seeds from the onset of imbibition (0 h) until attainment of total germination (48 h). Data are means $\pm$ standard error (three replicates of 20 seeds). Bars labeled with the same letters do not differ significantly (one-way analyses of variance followed by post hoc Tukey's honest significant difference test, $\alpha = 0.05$). **C**, Visualization of nitroblue tetrazolium chloride (NBT) staining (right side, white background) in seed tissues 8 h after the onset of imbibition and **D**, in seedling tissues 48 h after the onset of imbibition. Dark purple staining indicates the production of reactive oxygen species. The corresponding samples before NBT staining are shown to the left.

thoroughly eliminated seed epiphytes from both seed types, as revealed by the absence of colony-forming units. Independent of the hot steam treatment, endophytes were only culturable from seedlings but not from dry seeds, and seedlings from hot steam-treated seeds hosted 75-fold fewer endophytes than seedlings from control seeds (Fig. 2C). Sequencing the 16S rRNA genes of culturable endophytic isolates revealed the presence of both firmicutes (approximately 41%) and gammaproteobacteria (approximately 59%). Among the first group, isolates of the genera *Bacillus* and *Paenibacillus* were identified, and within class Gammaproteobacteria, the genus *Pseudomonas* dominated over the genus *Pantoea* (Fig. 2D). Finally, the last two endophytic isolates were assigned to the genera *Kosakonia* and *Stenotrophomonas* (Fig. 2D).

In summary, compared with dry seeds, genera diversity of culturable bacteria increased in the seedling bacterial community. The only two epiphytic genera isolated from dry seeds were *Pantoea* and *Pseudomonas*. In seedlings, all genera of epiphytic isolates, except *Rhodococcus* sp., were also identified in the endophytic pool. Particularly, the genus *Pantoea* appeared to dominate the epiphytic community. However, the genus *Pseudomonas* was also found in the seedling epiphytic community and was the most represented endophyte (Fig. 2D).

Effects of seedling inoculation with *Pantoea* and *Pseudomonas* strains on H₂O₂ metabolism.

Seedlings from hot steam-treated seeds, with a significantly decreased microbial load compared with seedlings from control seeds ($P = 0.016$ for epiphytes and $P < 0.001$ for endophytes), were utilized to elucidate whether early interactions between

seedlings and seed microbiota affected H₂O₂ metabolism (Fig. 3). These seedlings were inoculated with the bacterial strains *P. agglomerans* NT0-7 and NT0-11, *P. eucalypti* NT0-3 and NT0-4, *Pseudomonas fluorescens* NT0-2 and NT0-8, and *Pseudomonas oryzae* NT0-1 and NT0-6 isolated from the surface of dry control seeds and assigned to distinct IGS RFLP groups (Table 1).

Seedlings grown from hot steam-treated seeds produced approximately 0.91 ± 0.05 (standard error) pmol H₂O₂ s⁻¹ 48 h after the onset of imbibition. The H₂O₂ levels increased by around 2.1-fold upon inoculation with *P. agglomerans* NT0-7 and NT0-11 or *P. eucalypti* NT0-3 and NT0-4, compared with seedlings incubated in sterilized tryptic soy broth (TSB) medium, included as control (Fig. 3A and B; Table 1). For the *Pantoea* strains, maximal H₂O₂ levels were detected 1 h after seedling inoculation, before returning to levels similar to those measured in control seedlings (Fig. 3A and B). In contrast, seedling inoculation with *Pseudomonas fluorescens* NT0-2 and NT0-8 was not accompanied by any H₂O₂ burst. Instead, a 14-fold decrease in H₂O₂ production occurred 1 h after inoculation (Fig. 3C). *Pseudomonas oryzae* NT0-1 and NT0-6 interacted differently with seedlings, whereby only *Pseudomonas oryzae* NT0-1 induced an H₂O₂ burst after 1 h, before both strains decreased H₂O₂ levels around 3.6-fold below those of control seedlings starting from 1.5 h after inoculation (Fig. 3D).

In summary, these results indicated that the early interaction between a seedling and seed epiphytes is characterized by a strain-dependent H₂O₂ signature.

Due to their greater mass, the seedlings were the most likely source of the majority of H₂O₂ measured during the interaction with epiphytic bacteria of the seed microbiota. Nonetheless,

Fig. 2. Effects of hot steam treatment on the microbial load of seeds and seedlings and changes in the composition of culturable bacterial microbiota. **A**, Epiphytic colony-forming units isolated from the surface of dry seeds. **B** and **C**, Epiphytic and endophytic, respectively, colony-forming units isolated from seedlings 48 h after the onset of imbibition. Black and white refer to control (i.e., untreated samples) and hot steam treatment, respectively. Whiskers indicate minima and maxima, circles indicate outliers, and asterisks denote significant differences between control and hot steam-treated samples (t tests for independent samples, $P < 0.05$, four replicates of 10 seeds or seedlings). **D**, Genera composition of the epiphytic bacterial microbiota isolated from the surface of control dry seeds (Ep 0 h) and seedlings (Ep 48 h), and of bacterial endophytes isolated from the endosphere of control seedlings (En 48 h). The genera are shown as follows: *Bacillus* = yellow, *Kosakonia* = green, *Paenibacillus* = orange, *Pantoea* = pink, *Pseudomonas* = light blue, *Rhodococcus* = dark red, and *Stenotrophomonas* = gray.

some of these bacteria produced H₂O₂ also as pure culture. Particularly, *P. agglomerans* NT0-7 and NT0-11 produced the most H₂O₂ among all strains and about 8.4-fold more than the *P. eucalypti* NT0-3 and NT0-4 and *Pseudomonas oryzihabitans* NT0-6 (Fig. 3E). Within genus *Pseudomonas*, both *Pseudomonas fluorescens* NT0-2 and NT0-8 produced H₂O₂ below the limit of detection, whereas *Pseudomonas oryzihabitans* NT0-1 generated about 3.2-fold more H₂O₂ than strains of *Pseudomonas oryzihabitans* NT0-6 (Fig. 3E). Regarding H₂O₂ breakdown, *Pseudomonas fluorescens* NT0-2 and NT0-8 had the highest levels of catalase activity among all strains, which may have contributed to the lowest H₂O₂ levels 1 h after combining with seedlings (Fig. 3). Finally, *Pseudomonas oryzihabitans* NT0-1 and NT0-6 resulted in lowered H₂O₂ levels 1.5 h after seedling inoculation

relative to control seedlings (Fig. 3D). These isolates had an intermediate catalase activity (Fig. 3E). Overall, H₂O₂ metabolism of tested seed epiphytes clearly differed between species. Additionally, decreased H₂O₂ levels in inoculated seedlings agreed with higher catalase activities of the bacterial strains used for inoculation.

High catalase activities of seedling endophytes.

All endophytic isolates of seedlings showed the ability to decompose H₂O₂ via catalase activity in the microkatal range. One third of the *Pseudomonas* isolates from the seedling endosphere displayed the highest catalase activities (Fig. 4). Differently, the lowest catalase activities (around 4.8-fold lower than those of *Pseudomonas* isolates NT48-18, NT48-12, and NT48-15) were

Table 1. Molecular identification of bacterial epiphytic and endophytic isolates from bread wheat seeds and seedlings^a

Isolate ID	Origin ^b	Stage	16S rRNA gene (RDP classifier)	gyrB (NCBI BLASTN)			
				Closest identified relative (GenBank hit)	Accession	Sequence identity (%)	RFLP group
NT0-1	s. s.	Dry seed	<i>Pseudomonas</i> sp.	<i>Pseudomonas oryzihabitans</i> BCRC 16007	AB176836.1	99	A
NT0-2	s. s.	Dry seed	<i>Pseudomonas</i> sp.	<i>Pseudomonas fluorescens</i> 89F1	KX696674.1	97	B
NT0-3	s. s.	Dry seed	<i>Pantoea</i> sp.	<i>Pantoea eucalypti</i>	EF988762.1	98	C
NT0-4	s. s.	Dry seed	<i>Pantoea</i> sp.	<i>Pantoea eucalypti</i>	EF988762.1	98	C
NT0-5	s. s.	Dry seed	<i>Pantoea</i> sp.	<i>Pantoea agglomerans</i> CFSAN047153	CP034469.1	99	D
NT0-6	s. s.	Dry seed	<i>Pseudomonas</i> sp.	<i>Pseudomonas oryzihabitans</i> MS8	CP022198.1	100	A
NT0-7	s. s.	Dry seed	<i>Pantoea</i> sp.	<i>Pantoea agglomerans</i> CFSAN047153	CP034469.1	99	D
NT0-8	s. s.	Dry seed	<i>Pseudomonas</i> sp.	<i>Pseudomonas fluorescens</i> LBUM570	MG491882.1	97	E
NT0-9	s. s.	Dry seed	<i>Pantoea</i> sp.	<i>Pantoea agglomerans</i> CFSAN047153	CP034469.1	99	D
NT0-10	s. s.	Dry seed	<i>Enterobacteriaceae</i> bacterium	<i>Pantoea agglomerans</i> CFSAN047153	CP034469.1	100	D
NT0-11	s. s.	Dry seed	<i>Pantoea</i> sp.	<i>Pantoea agglomerans</i> CFSAN047153	CP034469.1	100	D
NT0-12	s. s.	Dry seed	<i>Pantoea</i> sp.	<i>Pantoea agglomerans</i> CFSAN047153	CP034469.1	100	D
NT0-13	s. s.	Dry seed	<i>Pantoea</i> sp.	<i>Pantoea agglomerans</i> CFSAN047153	CP034469.1	99	D
NT0-14	s. s.	Dry seed	<i>Pseudomonas</i> sp.	<i>Pseudomonas oryzihabitans</i> MS8	CP022198.1	100	A
NT0-15	s. s.	Dry seed	<i>Pantoea</i> sp.	<i>Pantoea agglomerans</i> CFSAN047153	CP034469.1	99	D
NT0-16	s. s.	Dry seed	<i>Enterobacteriaceae</i> bacterium	<i>Pantoea agglomerans</i> CFSAN047153	CP034469.1	99	D
NT48-1	e.	Seedling	<i>Bacillus</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-2	e.	Seedling	<i>Bacillus</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-3	e.	Seedling	<i>Paenibacillus</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-4	e.	Seedling	<i>Bacillus</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-5	e.	Seedling	<i>Bacillus</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-6	e.	Seedling	<i>Paenibacillus</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-7	e.	Seedling	<i>Pseudomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-8	e.	Seedling	<i>Pseudomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-9	e.	Seedling	<i>Pseudomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-10	e.	Seedling	<i>Pseudomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-11	e.	Seedling	<i>Pseudomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-12	e.	Seedling	<i>Pseudomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-13	e.	Seedling	<i>Stenotrophomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-14	e.	Seedling	<i>Paenibacillus</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-15	e.	Seedling	<i>Pseudomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-16	e.	Seedling	<i>Pseudomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-17	e.	Seedling	<i>Bacillus</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-18	e.	Seedling	<i>Pseudomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-19	e.	Seedling	<i>Kosakonia</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-20	e.	Seedling	<i>Bacillus</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-21	e.	Seedling	<i>Pantoea</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-22	sl. s.	Seedling	<i>Pantoea</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-23	sl. s.	Seedling	<i>Pseudomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-24	sl. s.	Seedling	<i>Paenibacillus</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-25	sl. s.	Seedling	<i>Pseudomonas</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-26	sl. s.	Seedling	<i>Pantoea</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-27	sl. s.	Seedling	<i>Pantoea</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-28	sl. s.	Seedling	<i>Rhodococcus</i> sp.	n.d.	n.d.	n.d.	n.d.
NT48-29	sl. s.	Seedling	<i>Pantoea</i> sp.	n.d.	n.d.	n.d.	n.d.

^a Isolates were obtained from nontreated seeds (NT) in the dry state (NT0) and from seedlings 48 h after the onset of imbibition (NT48). All isolates were characterized by 16S ribosomal (r)RNA gene sequencing and were assigned to genera according to the Ribosome Database Project (RDP) classifier. Epiphytic isolates from the surface of dry control seeds were further molecularly identified based on ribotypes and *gyrase B* (*gyrB*) sequencing. Ribotypes were revealed by restriction fragment length polymorphism (RFLP) of the intergenic spacer region (IGS), and only isolates resulting in values of *gyrB* sequence similarity of at least 97% are listed. *Pantoea* sp. isolates were assigned to IGS groups based on polymorphisms revealed by IGS digestion with *AluI* and *HhaI*. For *Pseudomonas* sp. isolates the restriction enzyme *RsaI* was also included.

^b s. s. = seed surface, e. = endosphere, sl. s. = seedling surface.

measured in all isolates from the seedling surface assigned to genus *Pantoea* and in the seedling epiphytic isolate of genus *Rhodococcus* (Fig. 4). High catalase activities appeared also in other endophytic isolates assigned to genera *Pseudomonas* and *Bacillus*, which were among the most represented cultured endophytes (Fig. 2D and 4). Catalase activities of the other bacteria, including isolates of genera *Paenibacillus*, *Stenotrophomonas*, and *Kosakonia*, had intermediate catalase activities between those of *Pseudomonas* endophytes and *Pantoea* and *Rhodococcus*

epiphytes, without differing significantly (Fig. 4). Ultimately, among seedling epiphytic isolates, isolates of genera *Pantoea* and *Rhodococcus* revealed lower catalase activities in comparison with isolates of genera *Pseudomonas* and *Bacillus*. (Fig. 4).

DISCUSSION

Physical sanitation treatments based on hot steam have been optimized to eradicate seed-born pathogens from crops for the

Fig. 3. Hydrogen peroxide (H₂O₂)-mediated interactions between wheat seedlings and epiphytic bacteria isolated from dry seeds. **A**, Seedlings from hot steam-treated seeds (four replicates of 10 seedlings), 48 h after the onset of imbibition, were inoculated by incubation with pure cultures of two strains of *Pantoea agglomerans* (NT0-7, light pink squares; NT0-11, dark pink squares), **B**, two strains of *Pantoea eucalypti* (NT0-3, light purple squares; NT0-4, dark purple squares), **C**, two strains of *Pseudomonas fluorescens* (NT0-2, light green triangles; NT0-8, dark green triangles), and **D**, two strains of *Pseudomonas oryzae* (NT0-1, light blue triangles; NT0-6, dark blue triangles). Seedlings were also incubated in sterile bacterial medium to be used as a control (black circles). Asterisks denote significant differences ($P < 0.05$) between control and inoculated seedlings at individual timepoints; data shows means of $n = 9 \pm$ standard error (SE) for seedlings only and $n = 3$ to $4 \pm$ SE for seedlings inoculated with each strain. **E**, Average H₂O₂ production (open bars) and catalase activity (closed bars) of strains in pure culture. Different upper and lower case letters denote significant differences of H₂O₂ production and catalase activity, respectively ($n = 3$ to $4 \pm$ SE). All data were tested for significance by one-way analyses of variance followed by post hoc Tukey's honest significant difference test.

benefit of agricultural productivity (Baker 1969; Forsberg et al. 2002, 2005; Groot et al. 2006; Koch et al. 2010; Watts et al. 1993). Microorganisms tend to have lower tolerance to hot steam than seeds, resulting in seed sanitation without compromising seed vigor and viability (Groot et al. 2006). Previously, we found that the hot steam-treated bread wheat seeds used in this study germinate faster and also possess an advanced germination-associated metabolism (Gerna et al. 2018). Here, the hot steam treatment was shown to also entail seed-surface disinfection (Fig. 2A), which was useful to investigate the communication between seeds and their inborn bacteria during germination and early seedling growth. Notably, 48 h after the onset of imbibition, the number of colony-forming units exponentially increased (Fig. 2B and C). This result agrees with other studies indicating that bacteria of the seed microbiota, especially endophytes, proliferate during and after germination (Barret et al. 2015; Nelson 2018; Torres-Cortés et al. 2018).

Separately isolating endophytes and epiphytes from seeds and seedlings germinated under sterile conditions was useful to analyze the redistribution dynamics of surface-inhabiting and endosphere-inhabiting microbiota. Conversions from endophytic to epiphytic lifestyles and vice versa are expected to occur during germination and seedling growth, making the definition of endophyte and epiphytes quite transitory (Barret et al. 2016; Nelson 2018).

Upon completion of seed germination, new bacterial genera were detected among the epiphytes. Genus *Rhodococcus*, mostly including nonsporulating species sensitive to desiccation (Elsayed et al. 2017), was not represented among epiphytes of dry seeds but was later identified on the seedling surface. This finding suggests that the *Rhodococcus* sp. isolates presumably originated from the seed endosphere, where they could have resided in local pockets of hydration found in dry seeds (Manz et al. 2005; Rathjen et al. 2009), then becoming epiphytic during germination and early seedling growth. Eventually, bacterial epiphytes unculturable from dry seeds were apparently reactivated during seed imbibition and germination, enabling their isolation from seedlings. This could be the case for the mostly endospore-forming genus *Paenibacillus* (Grady et al. 2016). The other seedling epiphytic genera *Pantoea* and *Pseudomonas* have been recently described as copiotrophic taxa, which become particularly abundant during seedling growth (Torres-Cortés et al. 2018). At this stage, storage reserves are remobilized to support the heterotrophic seedling, also increasing nutrient availability to its microbiota (Barret et al. 2015; Gerna et al. 2017; Ho et al. 2017; Nonogaki et al. 2010).

In this study, bacterial endophytes isolated from seedlings were assigned to genera commonly described in the seed endosphere microbiota of different plant species (Compant et al. 2011; Hardoim et al. 2015; Rahman et al. 2018; Truyens

Fig. 4. Catalase activity of individual endophytic and epiphytic isolates from wheat seedlings identified by 16S ribosomal RNA gene sequencing. Endophytic (closed circles) and epiphytic (open circles) isolates were obtained from seedlings 48 h after the onset of imbibition of control untreated seeds. The genera are shown as follows: *Bacillus* = yellow, *Kosakonia* = green, *Paenibacillus* = orange, *Pantoea* = pink, *Pseudomonas* = light blue, *Rhodococcus* = dark red, and *Stenotrophomonas* = gray. Data are means $\pm$ standard error (three colonies for each isolate), and those labeled with the same letter do not differ significantly (nonparametric Kruskal-Wallis test by ranks, followed by post hoc Tukey's honest significant difference test, $\alpha = 0.05$).

et al. 2015; Walitang et al. 2017). Genus *Pantoea* is extremely versatile in terms of ecological niches, hosts, and lifestyles ranging from parasitism to mutualism, including promotion of plant growth (Walterson and Stavrinides 2015). Notably, the species *P. agglomerans* is a common epiphyte in several crops and a dominant species of the epiphytic microbiota of dry wheat seeds (Dutkiewicz et al. 2016; Walterson and Stavrinides 2015). High metabolic versatility and adaptation to multiple host niches also characterize genus *Stenotrophomonas*, comprising common endophytes and some strains applied as plant growth-promoting rhizobacteria to improve wheat stress resistance (Ryan et al. 2009; Singh and Jha 2017). Finally, seedlings from hot steam-treated seeds hosted fewer endophytes compared with untreated controls (Fig. 2A and C). Therefore, it can be inferred that the hot steam treatment presumably also contributed to decreasing endophytic microbial load of dry seeds, from which no endophytes could be cultured.

Hot steam-treated seeds produced lower amounts of H_2O_2 compared with control seeds (Fig. 1B), showing that a higher microbial load of the natural wheat seed microbiota is associated with increased H_2O_2 production and thus validating our hypothesis that seed microbiota induce H_2O_2 production during seed germination. Interestingly, seedlings from control seeds produced extracellular ROS predominantly from their roots but not from the shoot (Fig. 1D). This evidence indicates that the seedling microbiota eliciting the oxidative burst were most active in roots and agrees with data by Rahman et al. (2018), who found that seed endophytic species of surface-sterilized barley seeds colonized roots during seedling growth. Intense ROS staining was particularly visible in the root elongation zone, supporting previous findings that extracellular ROS production by the plant has a well-characterized function in cell-wall remodeling for growth (Camejo et al. 2016; Foreman et al. 2003; Kranner et al. 2010b; Müller et al. 2009; Singh and Jha 2017). Besides its key signaling role in seed germination and seedling growth, H_2O_2 also mediates plant interaction with bacteria, possibly including those between seeds and endophytes (Grant and Loake 2000; Hammond-Kosack and Jones 1996; Kranner et al. 2010a; Levine et al. 1994; Wojtyla et al. 2016). Initially, plant-microbe interaction induces an oxidative burst, which includes a rapid release of H_2O_2 by plant cells (Lamb and Dixon 1997; Wojtaszek 1997). Therefore, we quantified extracellular H_2O_2 production in untreated control and hot steam-treated seeds during and after germination. Additionally, to simulate initial interactions with epiphytes, seedlings from hot steam-treated seeds were exposed to strains isolated as epiphytes from dry control seeds. Notably, seedlings responded differently upon exposure to *Pantoea* and *Pseudomonas* strains from dry seeds (Fig. 3A to D; Table 1). An H_2O_2 burst was detected after 0.5 h in response to *P. agglomerans* NT0-7 and NT0-11 and *P. eucalypti* NT0-3 and NT0-4 (Fig. 3A and B), supporting results on wheat cell cultures in which elevated H_2O_2 production by plant cells was triggered by *P. agglomerans* exopolysaccharides released into the growing medium (Ortmann and Moerschbacher 2006; Ortmann et al. 2006). However, as cultures of *P. agglomerans* NT0-7 and NT0-11 themselves produced more H_2O_2 than *P. eucalypti* NT0-3 and NT0-4, H_2O_2 produced by the bacteria may have contributed to measurements after pure cultures were mixed with seedlings (Fig. 3D). Differently, decreased H_2O_2 levels were revealed after inoculation of seedlings with *Pseudomonas* seed epiphytic strains, pointing to a breakdown of seedling-released H_2O_2 by these epiphytes (Fig. 3C and D). This finding is consistent with high catalase activities and undetectable (*Pseudomonas fluorescens* NT0-2 and NT0-8) or low (*Pseudomonas oryzae* NT0-1 and NT0-6) H_2O_2 production in

pure cultures of both *Pseudomonas* species (Fig. 3E). Other studies report on isolates of *Pseudomonas oryzae* as seed-borne endophytes with catalase activity and beneficial effects on plant growth (Bhagat et al. 2016; Cottyn et al. 2009; Hardoim et al. 2012; Kodama et al. 1985). Furthermore, inducible catalase activity is also a distinctive trait of some *Pseudomonas fluorescens* strains (Alhasawi et al. 2015; Rodríguez-Bravo and Pionetti 1981).

In summary, strains of genus *Pantoea* triggered an early H_2O_2 burst whereas strains of genus *Pseudomonas* decomposed plant-generated H_2O_2 , thus showing that the extracellular redox environment of the seedling can be modulated by bacterial epiphytes. Since a greater native microbial load was associated with more H_2O_2 production during germination (Fig. 1B), H_2O_2 -eliciting strains, such as those of *Pantoea* spp., contributed more to the net extracellular H_2O_2 levels (Fig. 3A and B). Scavenging of H_2O_2 by, for example, *Pseudomonas* spp. (Fig. 4) would have been more influential in the endosphere, in which isolates of this genus were more abundant (Fig. 2D).

Several genomic and metagenomic studies on bacterial endophytes identified diverse gene clusters of ROS-processing enzymes, suggesting their relevance to endophytic colonization of plants (Alquéres et al. 2013; Araújo et al. 2015; Mitter et al. 2013; Sessitsch et al. 2012; Sheibani-Tezerji et al. 2015; Taghavi et al. 2010). Particularly, catalases are among the best-characterized and largest group of H_2O_2 -degrading enzymes that maintain redox poise in eubacteria (Imlay 2015; van der Heijden et al. 2016; Zamocky et al. 2008) and are recurrently found in seed endophytic microbiomes (Hardoim et al. 2015). However, to date, very few studies have actually quantified catalase activity of endophytes. Much more commonly, isolates are simply reported as “catalase positive” or “catalase negative” (Naveed et al. 2014; Okunishi et al. 2005; Sheibani-Tezerji et al. 2015; Walitang et al. 2017). In this study, we provide physiological support to the importance of ROS-processing systems of seed endophytic bacteria through spectrophotometric quantification of catalase activity. Notably, many isolates assigned to genera *Pseudomonas* and *Bacillus* were identified as most abundant seedling endophytes and displayed higher catalase activity compared with other culturable isolates of less abundant genera (Fig. 4). Some epiphytic isolates of genera *Pseudomonas* and *Bacillus* had relatively high catalase activities, which may provide versatility to also live as an endophyte.

In conclusion, seed germination and seedling growth are highly dynamic processes entailing morphological, physiological, and redox changes in both endosphere and surface tissues. This requires physiological plasticity of the seed microbiota, including a capability to tolerate and modulate H_2O_2 production by the seed and seedling. Therefore, we conclude that rapid H_2O_2 metabolism by catalases is a key attribute of seed endophytes.

MATERIALS AND METHODS

Seed treatment, germination, and staining of ROS.

Bread wheat seeds (*Triticum aestivum* L. ‘Rebelde’) produced in the 2013 to 2014 growing season were provided by the Council for Agricultural Research and Economics (CREA) of Sant’Angelo Lodigiano (Italy). Seeds were harvested at maturity (growth stage 90 [Zadoks et al. 1974]) and were pooled in a 2-kg lot, of which 1 kg was left untreated (control) and 1 kg was exposed to hot steam treatment, using an industrial method (BioFlash, Hoopman Equipment & Engineering, Aalten, The Netherlands). This treatment was developed as a physical seed sanitation procedure against seed-borne pathogens, using a

controlled flux of hot steam in a chamber containing the seeds. Within 18 s, air temperature was increased to $65.0 \pm 0.3^\circ\text{C}$ (standard deviation [SD]) and was maintained for 1 s (flash duration), after which the steam flux was interrupted, allowing cooling to $18.5 \pm 0.2^\circ\text{C}$ (SD) within 85 s. This cycle was repeated twice, for an overall duration of 4 min (Fig. 1A). After the treatment, seeds were re-dried at ambient conditions, were then stored at 4°C in a sealed box over silica gel, and were analyzed within 32 months. In this report, seeds treated with BioFlash and untreated seeds are termed hot steam-treated and control, respectively.

For germination under sterile conditions, seeds were sown in 90-mm Petri dishes on two layers of filter paper (Whatman 1; GE Healthcare, Little Chalfont, U.K.) imbibed with 3 ml of autoclaved distilled H_2O (dH_2O) at 20°C in the dark. Total germination was scored at regular intervals over 48 h, which was the time required by all viable seeds to complete germination (Supplementary Fig. S1). A seed was scored as germinated once the radicle protruded through the coleorhiza by at least 1 mm.

To stain ROS produced during germination and early seedling growth, control and hot steam-treated seeds (four replicates of 10 seeds each for both treatments) were germinated for 8 and 48 h prior to incubation in a 5 mM solution of NBT in 20 mM Tris-HCl buffer (pH 7.40) at room temperature for 30 min. After thoroughly rinsing with dH_2O , seeds were analyzed with an Olympus KL 1500 LC (Olympus Corporation, Tokyo) stereomicroscope and snapshots captured with the software Intervideo WinDVR v3.0. Insoluble purple formazan precipitates were indicative of ROS production.

Isolation of epiphytes and endophytes from seeds and seedlings.

Seed epiphytes were isolated by washing the surface of dry seeds (four replicates of 10 seeds) with 700 μl of sterilized 0.9% (wt/vol) NaCl (780 rpm, 15 min, 4°C), using a compact digital microplate shaker (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Unless specified differently, all chemicals were purchased from Sigma Aldrich (St. Louis).

To quantify epiphytic colony-forming units of control and hot steam-treated seeds, 10- μl aliquots of the washing suspensions were 10-fold serially diluted with sterilized 0.9% (wt/vol) NaCl in sterile 96-well Brand plates-pureGrade S (Brand GmbH & Co KG, Wertheim, Germany) and were plated on 10% (wt/wt) tryptic soy agar (TSA) (VWR International, Radnor, PA, U.S.A.) prior to incubation (28°C , 24 to 48 h). Sterilized 0.9% (wt/vol) NaCl was included as a blank and nine technical replicates for each biological replicate ($n = 4$), and the two treatments were measured. The optical density of the seed-washing suspensions were spectrophotometrically quantified at a wavelength (λ) of 600 nm (OD_{600}), using a microplate reader (Synergy-HTX multimode reader; BioTek Instruments, Inc., Winooski, VT, U.S.A.). Following a culture-dependent approach, 15 μl of the seed-washing suspensions ($n = 4$, 10 control seeds each) were streaked on 10% (wt/wt) TSA and were incubated at 28°C for 24 to 48 h. Based on phenotypic differences, single colonies ($n = 18$) were selected from all four Petri dishes, were individually loop-inoculated in 100-ml Erlenmeyer flasks containing 10 ml of 10% (wt/vol) TSB (Corning Inc., Corning, NY, U.S.A.) and were grown overnight (28°C , 180 rpm) prior to storage at -80°C as glycerol stocks. Similarly, seedling epiphytes with different morphologies were isolated from the surface of seedlings 48 h after the onset of imbibition of control and hot steam-treated seeds.

Endophytes were isolated from control and hot steam-treated seeds and corresponding seedlings (four replicates of 10 seeds for both treatments) after chemical surface sterilization. This

consisted of sequential exposure of seeds to 70% (vol/vol) ethanol for 3 min and 2% (vol/vol) sodium hypochlorite (NaOCl) for 5 min, followed by three rinsing steps of 1 min each with autoclaved ultrapure H_2O (aUPW), as described by Naveed et al. (2014). One blank without seeds was processed to exclude any sources of contamination from solutions and equipment. To check eradication of seed epiphytes, seeds were resuspended in 700 μl of sterilized 0.9% (wt/vol) NaCl; 15 μl of all suspensions were plated on 10% (wt/wt) TSA and were incubated at 28°C for 1 week. Immediately after chemical surface sterilization, control and hot steam-treated seeds were germinated for 48 h under sterile conditions. Thereupon, samples were transferred to autoclaved 5-ml Teflon capsules (Sartorius AG, Göttingen, Germany) and were ground with 1-cm diameter agate beads, using a Mikro-Dismembrator S (B. Braun Biotech International, Melsungen, Germany) at 3,000 rpm for 30 s. Obtained seedling slurries were collected into 2-ml Eppendorf tubes containing 3-mm diameter autoclaved glass beads (Carl Roth GmbH & Co, Karlsruhe, Germany), prior to resuspending (30 Hz, 1 min) in 1.5 ml of sterilized 0.9% (wt/vol) NaCl with a Tissue-Lyser (Qiagen, Hilden, Germany). After resuspension, ground homogenized seedlings were decanted for 90 min and were centrifuged ($450 \times g$, 2 min) to obtain clear supernatants. Finally, 900- μl aliquots of supernatants were used to quantify endophytic colony-forming units by 10-fold serial dilutions, as described for seed epiphytes. The approach adopted to isolate individual colonies of seedling endophytes ($n = 21$) was then equivalent to that used for epiphytes. To normalize the colony-forming units to a dry weight (DW) basis, the weights of intact (for epiphytes) and ground (for endophytes) seeds and seedlings were recorded with an analytical balance (XS105, Mettler-Toledo GmbH, Columbus, OH, U.S.A.) after drying at 103°C for 17 h.

Quantification of H_2O_2 production in seeds, seedlings, and isolated seed epiphytes.

To quantify the extracellular production of H_2O_2 by control and hot steam-treated seeds during germination and early seedling growth, a horseradish peroxidase (HPOX)-coupled fluorimetric assay was developed. Briefly, seeds were incubated for 30 min in the dark with 950 μl of 50 mM Sørensen phosphate buffer (pH 7.40, hereafter called assay buffer), 40 μl of 20 U of HPOX per milliliter, and 10 μl of 2 mM AmpliFlu Red (10-acetyl-3,7-dihydroxyphenoxazine) in dimethyl sulphoxide. Reaction mixtures were twofold diluted prior to measuring fluorescence ($\lambda_{\text{excitation}} = 540 \pm 35$ nm, $\lambda_{\text{emission}} = 590 \pm 20$ nm) of resorufin (7-hydroxy-3H-phenoxazin-3-one), the H_2O_2 -HPOX product generated from AmpliFlu Red, using a microplate reader (Synergy-HTX multimode reader, BioTek Instruments, Inc.). Data were corrected for background fluorescence occurring in the presence of pure assay buffer. Three technical replicates were measured for each biological replicate ($n = 3$, each containing 20 seeds). Seed and seedling H_2O_2 contents were calculated from a standard curve of H_2O_2 (0 to 10 μM range), and data were normalized on a time and DW basis, after drying samples at 103°C for 17 h. Selected epiphytic bacterial isolates of *P. agglomerans* (strains NT0-7 and NT0-11), *P. eucalypti* (strains NT0-3 and NT0-4), *Pseudomonas fluorescens* (strains NT0-2 and NT0-8), and *Pseudomonas oryzae* (strains NT0-1 and NT0-6), obtained from the surfaces of dry control seeds, were grown by loop-inoculating single colonies (three to four colonies for each isolate) in 10% (wt/vol) TSB. Following overnight incubation (28°C , 180 rpm), the OD_{600} values of pure cultures were measured spectrophotometrically and were adjusted to 0.4 to obtain uniform populations of bacteria and to normalize the H_2O_2 production. This was determined as for seeds, using a reaction mixture of 200 μl

combined with 30 µl of each bacterial cultures. After brief vortexing and spinning, the reaction mixtures were incubated for 30 min in the dark and were then twofold diluted with assay buffer, and the resorufin fluorescence of three technical replicates for each biological replicate ($n = 3$ to 4) was measured. As negative controls, individual reactions for each isolate were prepared by mixing 170 µl of assay buffer with 30 µl of bacterial cells at an OD_{600} of 0.4. Finally, blanks containing assay buffer and TSB were included to correct for background fluorescence. Data were expressed as H_2O_2 contents per flask of pure bacterial culture and were normalized over time.

Quantification of H_2O_2 production by hot steam-treated seedlings exposed to seed epiphytes.

Hot steam-treated seeds were germinated under sterility for 48 h, prior to inoculation with pure cultures of *P. agglomerans* NT0-7 and NT0-11, *P. eucalypti* NT0-3 and NT0-4, *Pseudomonas fluorescens* NT0-2 and NT0-8, and *Pseudomonas oryzae* NT0-1 and NT0-6, isolated as epiphytes from the surfaces of control seeds. Nongerminated seeds were disregarded, and pools of 10 intact seedlings ($n = 4$, for each bacterial strain) were inoculated by incubating in Erlenmeyer flasks containing 20 ml of pure cultures of each bacterial strain at an OD_{600} of 0.4, at 20°C and 80 rpm. As a control, 10 intact seedlings were incubated in sterilized 10% (wt/vol) TSB without bacteria. At 30 min intervals, 160-µl aliquots were collected from each flask and were mixed with 2 µl of 2 mM AmpliFlu Red, 8 µl of 20 U of HPOX per milliliter, and 30 µl of assay buffer. Additionally, at each 30-min interval, OD_{600} values of suspensions from each flask were monitored, thus excluding the potential that the H_2O_2 contents were biased by bacterial growth. All seedlings remained visually undamaged throughout the analyses, which were stopped as soon as the suspensions exceeded an average OD_{600} of 0.45. Finally, data were expressed as H_2O_2 contents per flask of pure bacterial culture.

Determination of catalase activity of isolated seed epiphytes and endophytes.

Individual isolates from seedlings were loop-inoculated in sterilized 10% (wt/vol) TSB and were grown overnight under shaking (28°C, 180 rpm). Pure cultures ($n = 3$ to 4, for each isolate) were adjusted to an OD_{600} value of 0.4, to obtain uniform populations of bacteria. Catalase (EC 1.11.1.6) activity was measured in 150 µl of 50 mM Sørensen phosphate buffer (pH 7.00), 20 µl of 150 mM H_2O_2 , and 30-µl aliquots of pure bacterial cultures. As positive control, 0.2 U of catalase from bovine liver were included in each set of measurements. Catalase activity was quantified by following H_2O_2 consumption at substrate saturation, indicated by the linear decrease in absorbance at 240 nm (Bailly and Kranner 2011), and were calculated with a pathlength-corrected molar extinction coefficient ($\epsilon = 25.30 \text{ mM}^{-1} \text{ cm}^{-1}$), using a microplate reader (Synergy-HTX multimode reader, BioTek Instruments, Inc.) and 96-well UV plates (Corning Inc.). Reaction rates were calculated for biological replicates (i.e., cultures from individual colonies of the same isolate, $n = 3$ to 4), and four technical replicates were averaged.

Molecular identification of seed and seedling epiphytes and endophytes.

DNA extraction. Individual colonies of epiphytes isolated from control dry seeds ($n = 18$) and of epiphytes ($n = 8$) and endophytes ($n = 21$) from seedlings 48 h after the onset of imbibition were grown by loop-inoculating in 10% (wt/vol) TSB. Cultures were incubated for 24 h at 20°C and 180 rpm. DNA was extracted using an UltraClean Microbial DNA isolation kit (Mo Bio Laboratories, Inc., Qiagen, Carlsbad, CA,

U.S.A.) and was eluted in 60 µl of sterile aUPW, according to manufacturer instructions. Aliquots of 5 µl of DNA were visualized by electrophoresis (100 V, 40 min) on 1% (wt/vol) agarose gel in TBE (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.00) buffer (Sambrook and Russel 2001) and were stained with 0.005% (vol/vol) Midori Green (Bulldog Bio, Inc., Portsmouth, England). DNA concentrations and purities were quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

16S rRNA gene PCR and sequencing. To group epiphytic and endophytic isolates according to their family and genus, the 16S rRNA gene was PCR-amplified using primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3') (White et al. 1990) and 1520r (5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards et al. 1989). In a total volume of 50 µl of reaction buffer, the PCR mixture contained 1 µl of extracted DNA, 2.5 mM $MgCl_2$, 0.15 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate, and 2.5 U of FIREPol DNA polymerase (Solis Bio-Dyne, Tartu, Estonia). PCR cycling conditions included 5 min of denaturation at 96°C, followed by 30 cycles consisting of denaturation for 30 s at 90°C, primer annealing for 1 min at 52°C, and polymerization for 2 min at 72°C, with a final extension of 10 min at 72°C. The quality of the PCR products was assessed by gel electrophoresis, using the same conditions reported for genomic DNA. Isolates were characterized by partially sequencing their 16S rRNA genes with the Sanger method (LCG Genomics GmbH, Berlin) and primers 8f and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') (Lane 1991). Retrieved sequences were visualized with the sequence alignment editor package BioEdit (Ibis Biosciences, Carlsbad, CA, U.S.A.), were quality filtered, and were trimmed. Finally, Ribosome Database Project (RDP) Classifier and RDP Sequence Match were used to identify epiphytic and endophytic isolates (Wang et al. 2007).

Analysis of the 16S-23S IGS region and gyrB gene PCR and sequencing of seed epiphytes. To distinguish closely related epiphytic isolates of dry seeds, the IGS region of the 16S-23S rDNA operon was amplified by PCR using primers pHr (5'-TGCGGCTGGATCACCTCCTT-3') and P23SR01 (5'-GGCTGCTTCTAAGCCAAC-3') (Massol-Deya et al. 1995). Thermal cycler conditions included 5 min at 95°C, followed by 35 cycles consisting of denaturation for 30 s at 95°C, primer annealing for 1 min at 55°C, and polymerization for 2 min at 72°C, with a final extension of 10 min at 72°C. After checking DNA quality by gel electrophoresis, 5 µl of PCR products were digested with 5 U of the restriction enzymes *AluI*, *HhaI*, and *RsaI*, used in individual reactions and incubated for at least 2 h at 37°C. The resulting DNA fragment patterns were visualized on 2% (wt/vol) agarose in TBE after gel electrophoresis (100 V, 200 min). To further characterize seed epiphytes, *gyrB* genes were amplified using a mixture of universal degenerate primers: UP1 (5'-GAAGTCATCATGACCGTTCTGCAYGCNNGGNAARTTYGA-3'), UP1G (5'-GAAGTCATCATGACCGTTCTGCAYGCNNGGNAARTTYGG-3'), UP2r (5'-AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNCGTCAT-3') and UP2Ar (5'-AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNCGTCNGYCAT-3') (Yamamoto and Harayama 1995). Primers were added to reaction mixtures prepared as reported for the other PCR assays. Cycler conditions included 5 min at 95°C and 35 cycles consisting of 1 min at 95°C, 1 min at 58°C, and 2 min at 72°C, followed by a final elongation step of 10 min at 72°C. The approximately 1,200-bp-long PCR products were visualized by electrophoresis on 1% (wt/vol) agarose gel and were sequenced with the Sanger method (GATC Biotech AG, Konstanz, Germany), using primers UP1S (5'-GAAGTCATCATGACCGTTCTGCAY-3') and UP2rS (5'-AGCAGGGTACGGATGTGCGAGCC-3'). The quality of sequence chromatograms was evaluated using the sequence alignment editor software BioEdit.

Sequences that showed reliable quality were further processed, aligned, and trimmed with BioEdit. Finally, to identify the closest related species of seed epiphytes, BLASTN algorithm on the National Center for Biotechnology Information sequence repository was run for nucleotide search in 2017, excluding sequences from uncultured/environmental samples.

Statistical analyses. Data were assessed for significance ($\alpha = 0.05$) by Student's *t* tests and one-way analysis of variance combined with Tukey's honest significant difference (HSD) tests for post hoc comparisons of means, using the SPSS Statistics 24 software package (IBM, Armonk, NY, U.S.A.). The assumption of normal distribution was assessed by the Shapiro-Wilk test and analysis of QQ-plots. The assumption of homoscedasticity of variance across groups was ascertained through Levene's test and analysis of the residuals plotted against fitted values. To correct for heteroscedasticity, arithmetical transformations (e.g., $\log_{10}$, square root) were applied to the data before analysis. Whenever this was not possible, bias-corrected accelerated bootstrap analyses were run with a sample size of 10^5 and two different seeds (i.e., 2000 and 200), using the Mersenne Twister random number generator. The generated 95% confidence intervals showed sensitivity to both seeds at the first decimal digit. Alternatively, whenever data were not normally distributed, results were tested for significance by the nonparametric Kruskal-Wallis rank variance analysis, followed by Tukey's HSD tests for post hoc comparisons of ranks. The *P* values for Kruskal-Wallis analyses were adjusted by the Bonferroni correction for multiple tests.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

FAO website: <http://www.fao.org/faostat/en/#home>

Ribosome Database Project (RDP) Classifier: <https://rdp.cme.msu.edu>

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