



The seeds of invasion: a comparison of endophytic seed bacteria of *Acacia longifolia* between its native and invasive ranges

Mónica Condessa¹ · Joana G. Jesus^{1,2} · Cristina Máguas^{1,2} · Johannes J. Le Roux³ · Helena Trindade^{1,2}

Received: 8 September 2023 / Accepted: 5 March 2024
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Abstract

Acacia longifolia is an aggressive invader in Mediterranean-type ecosystems severely impacting biodiversity and ecosystem functions. The species' invasiveness has been linked to its ability to thrive in nutrient poor soils, high seed production, and quick establishment after fire. In this study, we identify and compare the bacterial endophytes of *A. longifolia* seeds collected from populations in the species' native (Australia) and invasive (Portugal) ranges. For this, we characterised the morphology (length, width, and weight) of seeds from two sites in each range and isolated and cultivated bacteria from seeds. DNA fingerprinting and cluster analyses revealed slightly higher, and distinct, bacterial diversity associated with seeds collected from native range populations in comparison to those collected from invasive populations. Sequencing of the 16S rDNA gene identified 119 bacterial isolates from 15 genera, with *Curtobacterium* strains being common in both ranges. Several differences in bacterial genera were found among ranges and sites: *Dermacoccus*, *Frigoribacterium*, *Kocuria*, *Pantoea* and *Phyllobacterium* taxa were each unique to seeds from the native populations, while *Brevundimonas*, *Microbacterium*, *Rhizobium* and *Sphingomonas* taxa were only found in the invasive seeds. The genus *Paraburkholderia* occurred in all invasive-range seeds but was not isolated from the native-range. *Bacillus* and *Paenibacillus* co-occurred in seeds collected from all invaded sites, but the simultaneous presence of both taxa was not found in native-range seeds. We propose that the bacterial endophytes present in invasive-range seeds may be important players for the invasiveness of *A. longifolia*, due to their role as plant growth promoters, providing extra capabilities helping acacia expansion.

Keywords Australian acacia · Invasive species · Nitrogen fixation · Nodulation · Seed endophytes · Symbiosis

1 Introduction

The global movement of humans and goods has led to the introduction of many alien species beyond their native ranges, where some have become invasive (Jeschke et al. 2014). Invasive species are one of the main drivers of global change and impact the composition and functioning of ecosystems worldwide (Van der Putten et al. 2007; Essl et al.

2020). The ecology of invasive species has been the subject of many studies, yet invasiveness – the inherent ability of an alien species to colonise and spread in certain environments – are still not fully understood (but see Gioria et al. 2023). Knowing these factors is important to better design and implement management strategies that can be applied on a global scale (Pyšek et al. 2020).

Some of the most problematic woody plant invaders are from the genus *Acacia* (family Fabaceae), commonly known as wattles. The genus contains 1082 species, most of which are found in Australia (Binggeli 1996; Richardson et al. 2023) and many of which have been introduced outside Australia. Numerous *Acacia* species are considered as some of the world's worst invasive species, especially in Mediterranean and temperate climate regions (Lorenzo et al. 2010; Richardson et al. 2011). For example, in Portugal approximately 20% of the total area covered by invasive species is invaded by wattles (Marchante et al. 2003). One of the most widespread wattles in the country is *A. longifolia* (Andrews)

✉ Helena Trindade
htrindade@fc.ul.pt

¹ Faculdade de Ciências, Departamento de Biologia Vegetal, Universidade de Lisboa, 1749-016 Lisbon, Portugal

² cE3c – Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências da Universidade de Lisboa, Lisboa & CHANGE - Global Change and Sustainability Institute, 1749-016 Campo Grande, Portugal

³ School of Natural Sciences, Macquarie University, North Ryde, NSW, Australia

Willd., which was introduced at the end of the nineteenth century for coastal dune stabilisation, forestry, ornamental use, and to prevent sand erosion (Marchante 2011). This species threatens native plant communities and reduces native species cover and diversity (Marchante et al. 2003). *Acacia longifolia* is also a major invader in other parts of the world, notably in South Africa, and it is considered a “transformer” species (Richardson et al. 2000) that negatively impacts ecosystem services (Le Maitre et al. 2011), soil physicochemical properties and functions (Marchante et al. 2008), and that has high water and resource consumption (Morris et al. 2011). These changes can be partially evaluated considering carbon and nitrogen cycles and isotopic analyses can provide insights into several ecological processes ascribed to plant growth and physiology within seed formation, especially in relation to climatic conditions (Kelly et al. 2005; Gonzalez et al. 2009; McCue et al. 2020).

The success of invasive wattles has partly been attributed to their ability to establish mutualisms with nitrogen-fixing bacteria, known as rhizobia (Novoa et al. 2023). Rhizobia are free-living soil bacteria capable of forming specialised structures, called root nodules, on most legumes. These bacteria fix atmospheric nitrogen in root nodules into forms that legumes can utilise, in turn receiving carbon-rich photosynthates from their host plants (Spaink 2000). Forming symbioses with compatible rhizobia can be an important factor underlying the invasiveness of legumes (Parker 2001; Parker et al. 2006). *Acacia* species are generally able to associate with a wide range of rhizobia, primarily from the genus *Bradyrhizobium* (Le Roux and Wandrag 2023). Despite this promiscuity, many invasive wattles have been co-introduced with their *Bradyrhizobium* strains from Australia into new environments (Rodríguez-Echeverría 2010; Crisóstomo et al. 2013; Ndlovu et al. 2013; Warrington et al. 2019; Le Roux and Wandrag 2023). Strains in other rhizobia genera, such as *Rhizobium*, have also been isolated from invasive wattles (Rodríguez-Echeverría et al. 2011; Birnbaum et al. 2016; Keet et al. 2017; Jesus et al. 2020).

Multiple and independent acacia-rhizobium co-introductions, coupled with the known symbiotic promiscuity of acacias, raises the question about the role of other seed microbiome taxa in the invasiveness of these plants. Seed microbiota is diverse, mostly comprising epiphytic or endophytic bacteria (Malfanova et al. 2013; Truyens et al. 2015) and fungi (Rodríguez et al. 2009). Epiphytes are microorganisms found on the seed’s surface and can be internalised within seed tissues and transferred vertically or horizontally to seedlings (Nelson 2018). Seed endophytes are microorganisms that are present in the inner seed tissues and are transferred vertically (Truyens et al. 2015; Nelson 2018). Vertical transmission allows the retention of certain microbiota over successive generations, including mutualists (Ewald 1987; Truyens et al. 2015). The direct and indirect benefits of seed endophytes for plant growth

are well known. For example, direct effects are related to nutrient acquisition, including nitrogen, phosphorus, and iron, and phytohormone production (e.g. Khan et al. 2014; Goswami et al. 2016; Brígido et al. 2019). Indirect effects may include protection from pathogens through the production of antibiotics and lytic enzymes by seed endophytes (Ma et al. 2016; Santoyo et al. 2016; Liu et al. 2017).

Among the studies considering bacterial seed endophytes in a wide range of plant taxa, four different phyla seem to always be represented, with Pseudomonadota (formerly Proteobacteria) being dominant, followed by Actinomycetota (formerly Actinobacteria), Bacillota (formerly Firmicutes), and Bacteroidota (formerly Bacteroidetes). Among these, some genera are commonly represented, including *Bacillus*, *Methylobacterium*, *Pseudomonas*, *Paenibacillus*, *Pantoea*, *Rhizobium*, *Sphingomonas*, while others are less common, e.g., *Acinetobacter*, *Micrococcus* and *Staphylococcus* (Truyens et al. 2015; Nelson 2018; Simonin et al. 2022). Plant associated bacterial endophytic communities are thought to be more deterministic while fungal endophytes appear more randomly associated with plants (Powell et al. 2015). This may be due to their response to different edaphoclimatic conditions, since fungal endophytes are more often described to be associated with plant resistance to biotic and abiotic stresses compared to bacterial endophytes (Yan et al. 2019). The most common phylum of plant fungal endophytes is Ascomycota, including the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium* and *Penicillium*, but also *Mucor* from the phylum Mucoromycota (Shahzad et al. 2018).

Considering that seeds are the natural propagules responsible for dissemination and invasion, in the present study, we wanted to compare *A. longifolia* seeds from the native and invasive ranges. Specifically, we compared seed bacterial endophyte communities given their key physiological roles allowing a more successful early plant establishment and survival. We hypothesised that (1) invasive range seeds would harbour unique endophytic bacterial communities, characterised by taxa involved in nitrogen fixation and plant growth promotion and (2) bacterial endophytes would be more diverse in the native than in the invasive range, due to loss of endophyte diversity associated with the introduction events of the species into Portugal. Furthermore, seeds from the species’ native and invasive ranges were compared morphologically and isotopically, to check for differences between ranges.

2 Materials and Methods

2.1 Seeds collection and characterisation

Seeds of *Acacia longifolia* were collected at each of two sites from the species’ native (Australia) and invaded

(Portugal) ranges. Australian seeds were acquired from the Indigo Nursery in Sydney, New South Wales, and were collected from Long Reef (LR) in November 2007 and Belrose (BE) in November 2010 (GPS coordinates not available). Portuguese seeds were collected in July 2020 from Vagos (VA; 40.52451, -8.67253) and Vila Nova de Milfontes (VN; 37.682060, -8.762254). Collected seeds were stored in paper bags at room temperature until further use. Fifty seeds from each site were randomly selected and morphologically characterised (length, width, and weight). Seed volumes were calculated using an oblate spheroid as a proxy for seed shape, using the following equation:

$$V = \frac{3}{4} \pi \cdot a \cdot b^2,$$

where a is the semi-major axis (i.e., seed length) and b is the semi-minor (i.e., seed width), being the spheroid made by the semi rotation of the shortest axis (i.e., seed width is equal to seed height).

2.2 Isotopic analysis

Isotopic analysis was conducted for Carbon (C) and Nitrogen (N) fractionation for *A. longifolia* seeds from the species' native and invasive (Portugal) ranges. For these analyses, seeds were dried for 48 h at 60 °C, combined into composite samples (5 composites per site with 10 seeds each), and ground using a ball mill (Retsch, Haan, Germany). Stable isotope ratio analysis was performed at the Stable Isotopes Analysis Facility (SIAF) at the Faculdade de Ciências from the University of Lisbon (Portugal). Carbon and nitrogen isotopic fractionation signatures were determined by continuous flow isotope mass spectrometry (CF-IRMS) (Preston and Owens 1983) on a Sercon Hydra 20–22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas-combustion. The δN and δC values were determined as $\delta N/C = \left[\frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \right] \cdot 1000$, where R is the ratio between the heavier and lighter isotope of N or C. $\delta^{15}\text{N}_{\text{Air}}$ values are referred to Air and $\delta^{13}\text{C}_{\text{VPDB}}$ values are referred to PDB (Pee Dee Belemnite). The reference materials used were IAEA N1, IAEA N2, IAEA-C3 and IAEA-CH7 (Coleman and Meier-Augenstein 2014) and the laboratory standard used was Rice Flour. Uncertainty of the isotope ratio analysis, calculated using values from 6 to 9 replicates of laboratory standard interspersed among samples in every batch of analysis, was $\leq 0.1\%$. The major mass signals of C and N were used to calculate total C and N abundances, using Wheat Flour Standard OAS (Elemental Microanalysis, UK, with 1.47%N, 39.53%C) as elemental composition reference materials.

2.3 Seed surface-disinfection and pre-germination

To isolate endophytic bacteria, the seeds were surface-sterilised using a modified protocol from Vincent (1970). The seeds were immersed in 70% ethanol for 1 min, followed by immersion in commercial bleach for 6 min, followed by five thorough rinses with sterile distilled water at room temperature. After this, the seeds were soaked for 5 min in sterile distilled water at approximately 100 °C to induce germination. The efficiency of the protocol was tested by surface printing the seeds on Tryptic Soy Agar (TSA) plates, incubated at 28 °C for four days. Seeds that did not exhibit visible microbial growth (i.e., contamination) were germinated with a photoperiod of 16 h at 23 ± 2 °C until root emergence.

2.4 Isolation of seed endophytic bacteria

We tested for seed viability and only used germinated seeds for endophyte isolations, leading to the inclusion of 40 seeds (10 per collection site). In each pool, two seeds were macerated in 500 μL 0.85% sodium chloride. Serial dilutions (10^0 – 10^{-5}) were used to inoculate Yeast Mannitol Agar (YMA) and TSA plates supplemented with 0.01% cycloheximide to avoid fungal growth. The plates were incubated for 5–10 days at 28 °C and checked daily for visible bacterial growth. Colonies with different morphologies were selected and sub-cultured until purification, which was determined by morphological and microscopic observations. Five isolation pools (of two seeds/collection site) were plated out as described above until no new morphologically distinct bacterial colonies were observed (assessed through macro- and microscopic observation).

2.5 Phenotypic and biochemical characterisation

To evaluate bacterial growth patterns we characterised colony size, pigmentation, form, margin, and elevation as described by Cappuccino and Sherman (1998). Gram-staining allowed us to distinguish between Gram-positive and Gram-negative rods and cocci. Other routine tests were performed such as potassium hydroxide (3% KOH, or string test, where Gram-negative bacteria cell walls are broken down releasing stringy or viscous material), catalase (bubble formation when a colony loop is mixed with 3% hydrogen peroxide), and oxidase tests (checks for cytochrome oxidase; in its presence the reduced colourless reagent becomes an oxidized coloured product). All tests were performed after 24–48 h of visible colony growth following streaking onto new growth media. Phenotypical and biochemically different

isolates were used for further clustering analyses, together with the molecular fingerprinting.

2.6 DNA extraction and fingerprinting

Bacterial DNA extraction was performed using a guanidinium thiocyanate, ethylenediamine tetraacetic acid (EDTA) and Sarkosyl (GES) protocol with some modifications (Pitcher et al. 1989). A loopful of one pure colony from each isolate was used and DNA was resuspended in 50 μL of 1 \times Tris–EDTA (TE) buffer.

Genetic discrimination between the different isolates was performed using repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprinting. DNA fragments were amplified in two separate PCR reactions using the primers csM13 (5' – GAGGGTGGCGGTTCT – 3') (Meyer et al. 1993) and (GTG)₅ (5' - GTGGTGGTGGTGGTG -3') (Versalovic et al. 1994). Each PCR reaction was performed in a final reaction volume of 12.5 μL , containing 1 \times NZYTaQ II Green Master Mix (NZYTech, Portugal), 0.5 μM of each primer and 1 μL (10 ng) of DNA template. All PCR reactions were performed in a BioRad T100 thermal cycler (BioRad, USA) with the following thermal cycle: initial denaturation at 95 $^{\circ}\text{C}$, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 1 min, 50 $^{\circ}\text{C}$ for 2 min, 72 $^{\circ}\text{C}$ for 2 min, and a final extension at 72 $^{\circ}\text{C}$ for 5 min. PCR products were separated by electrophoresis on a 1% (w/v) agarose gel using 0.5 \times TBE Buffer, ran at 85 V for 5 h. The gel was stained with 0.5 ng. μL^{-1} ethidium bromide solution for 10 min and washed in water to remove excess staining. Gel images were obtained using an Alliance 4.7 UV Transilluminator (Uvitec, Cambridge) and processed with the associated software, Alliance version 15.15 (Uvitec, Cambridge).

2.7 Taxonomic identification of bacterial endophytes via 16S rRNA sequencing

For 16S rRNA gene sequencing, isolates representative of all clusters obtained by DNA fingerprinting were included. For these samples, the 16S rRNA gene was amplified using the primers PA(8f) (5'–AGAGTTTGATCCTGG CTCAG–3') (Massol-Deya et al. 1995) and 907r (5-CCG TCAATTCMTTTRAGTTT-3) (Lane et al. 1985). The final PCR volume for each reaction was 25 μL , containing 2 μL of template DNA, 1U of NZYTaQ II DNA polymerase (NZYTech, Portugal), 1 μM of each primer, 3 mM MgCl₂ (NZYTech, Portugal), 0.2 mM dNTPs and 1 \times PCR buffer (NZYTech, Portugal). The thermocycle for amplification was as follows: initial denaturation at 95 $^{\circ}\text{C}$, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 1 min, 55 $^{\circ}\text{C}$ for 2 min, 72 $^{\circ}\text{C}$ for 1 min, and a final extension at 72 $^{\circ}\text{C}$ for 5 min. Aliquots of 5 μL of each PCR product were ran on a 1% (w/v) agarose gel

to confirm amplification success. All PCR products were purified and sequenced using the reverse primer (STAB VIDA, Portugal).

DNA sequences were edited and analysed using the software Geneious (v5.3 2010). All the sequences were compared with data available in GenBank using BLAST (16S ribosomal RNA database). Analysis of the dendrogram obtained with the fingerprinting clusters allowed us to assign identification based on the sequences isolates that fell in same the clustering. All DNA sequences were submitted to GenBank (accession numbers OR039116 to OR039161; Supplementary Table S1).

2.8 Statistical analyses

Seed morphological and ecophysiological data (i.e., seed length, width, weight, N and C isotopic signatures), were analysed using Kruskal–Wallis tests ($\alpha=0.05$). The link between seed volume and seed weight was evaluated through a Spearman correlation for each site separately (Fig. S1). Data analyses were performed using the package *stats* and *rstatix* in R (v.4.2.2) (R Core Team 2022).

The software BioNumerics (Applied Maths, Belgium) was used to construct the dendrograms, which compares and clusters DNA fingerprints. For better resolution and discrimination between isolates, fingerprinting profiles obtained from the amplification with both csM13 and (GTG)₅ primers were used in the analysis. For clustering we used the unweighted pair-group method with arithmetic mean (UPGMA) with Pearson correlation coefficients. A cut-off level for each dendrogram was established based on the analysis of the reproducibility, in which a random sample of 10% of the total isolates was used. Diversity and evenness between the isolates at each location were assessed and compared as the Shannon-Weiner (H'), Simpson (D), and Pielou (J') indices (Krebs 1989).

3 Results

3.1 Seed characterisation

Acacia longifolia seed origin significantly affected the width of the seed, considering the two sites in each range ($p < 0.05$) (Table 1). Native seeds displayed the largest differences in width, from 2.46 to 3.04 mm, while the seeds from the invaded range were more uniform (2.7–2.81 mm). For seed shape, seeds from Belrose (BE) were more elongated and narrower, than Long Reef (LR) seeds, which were shorter and wider. Seeds weight also revealed differences, with BE presenting the lighter seeds (16.8 mg) followed by VN (20.2 mg) ($p < 0.05$). LR and Vagos (VA) seeds were heavier (25.3–23.7 mg) however

Table 1 Mean and standard error (SE) of *A. longifolia* seed morphological traits (length, width, and weight) from each location (n=50). Means followed by different letters are significantly different according to Kruskal–Wallis test ($\alpha=0.05$)

Range	Location ¹	Seed length (mm)	Seed width (mm)	Seed volume (mm ³)	Seed weight (mg)
Native	BE	4.91 ± 0.06 ^a	2.46 ± 0.04 ^a	124 ± 5.05 ^a	16.80 ± 0.48 ^a
	LR	4.55 ± 0.07 ^b	3.04 ± 0.04 ^b	172 ± 4.68 ^b	25.30 ± 0.83 ^b
Invaded	VA	4.46 ± 0.08 ^b	2.81 ± 0.03 ^c	146 ± 4.74 ^c	23.70 ± 0.93 ^b
	VN	4.80 ± 0.08 ^a	2.70 ± 0.03 ^d	145 ± 5.03 ^c	20.20 ± 0.70 ^c

¹Seed origin: BE – Belrose, AUS; LR – Long Reef, AUS; VA – Vagos, PT; VN – Vila Nova de Milfontes, PT

differences between these two sites were not statistically significant. Similar to seed width, the highest differences in seed weight were in the native range (16.8–25.3 mg vs. 20.2–23.7 for invasive range). For seed length, significant differences ($p < 0.05$) were found in seeds between sites in each range (native BE vs LR and invaded VA vs Vila Nova de Mil Fontes (VN)). Seed volume was similar between sites in the invaded region (146 and 145 mm³ for VA and VN, respectively). LR seeds had the highest volume (172 mm³) while BE had the lowest volume (124 mm³). Seed volume significantly correlated with seed weight ($p < 0.001$) in all sites (Fig. S1), with higher values in the invaded region ($\rho = 0.88$, $p < 2.2 \times 10^{-16}$ and $\rho = 0.82$, $p = 2 \times 10^{-13}$) for VA and VN, respectively). Once again, the lowest correlation was found for BE followed by LR ($\rho = 0.35$, $p = 0.013$ and $\rho = 0.45$, $p = 0.00093$, respectively).

Seed viability was similar among seeds from different origins (native and invasive) and sites (results not shown).

3.2 Isotopic analysis

The analysis performed on *A. longifolia* seeds showed significant differences when comparing sites for most parameters of isotopic composition ($p < 0.05$), but these were unrelated to range (Table 2). Regarding $\delta^{13}\text{C}$, differences were statistically significant between all the sites. The seeds from native populations, LR and BE showed average values of -25.9 and -28.9 ‰, respectively, while in invaded seeds means were more uniform (-27.1 in VN

to -28.2 ‰ in VA). Values of $\delta^{15}\text{N}$ were close to 0 ‰ in all locations, but seeds from the invaded range had the highest variation (-0.21 and + 1.42 ‰ for VA and VN respectively, ($p < 0.05$). Australian seeds had mean values of -0.44 and + 0.80 ‰ for BE and LR, respectively ($p < 0.05$). Nitrogen values (‰N) were different ($p < 0.05$) between sites within Australia (BE vs LR) and sites within Portugal (VA vs VN). BE seeds had the highest nitrogen content (‰N; 4.64), followed by similar values for LR (4.35) and VA (4.31). Seeds from VN had the lowest ‰N (3.95). The carbon content (‰C) did not differ between native and invaded ranges, with a low interval of values (40.5–41.6%), but seeds from invaded range had statistically significant differences. C/N ratios did not show high variability, ranging from 8.78 to 10.5. Differences were significant between sites ($p < 0.05$), except for the comparison between LR and VA.

3.3 Bacterial isolation and fingerprinting

We observed no microbial growth after imprinting seed coats of sterilised seeds onto growth media. In total, 119 bacterial isolates were obtained from our culture-dependent method. Sixty-five isolates were obtained from native *A. longifolia* seeds: 30 from BE and 35 from LR and 54 isolates were obtained from the invaded range: 28 from VA and 26 from VN (Table 3). Our UPGMA clustering analysis (Fig. 1) separated BE13AUS (later identified as *Kocuria*) from all the other accessions (cluster I). A second

Table 2 Mean and standard error (SE) of nitrogen and carbon isotopic signatures of *A. longifolia* seeds in the studied locations [BE (n=5); LR (n=5); VA(n=5); VN(n=5)]. Nitrogen and carbon isotopic signatures ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$), as well as their percentage in the

Range	Location ¹	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$	‰N	‰C	C/N
Native	BE	-0.44 ± 0.06 ^a	-28.90 ± 0.08 ^a	4.64 ± 0.04 ^a	40.70 ± 0.21 ^{ab}	8.78 ± 0.10 ^a
	LR	0.80 ± 0.17 ^b	-25.90 ± 0.15 ^b	4.35 ± 0.09 ^b	41.20 ± 0.10 ^{ac}	9.48 ± 0.18 ^b
Invaded	VA	-0.21 ± 0.14 ^a	-28.20 ± 0.10 ^c	4.31 ± 0.04 ^b	40.50 ± 0.07 ^b	9.40 ± 0.08 ^b
	VN	1.42 ± 0.26 ^b	-27.10 ± 0.27 ^d	3.95 ± 0.10 ^c	41.60 ± 0.26 ^c	10.50 ± 0.23 ^c

¹Seed origin: BE – Belrose, AUS; LR – Long Reef, AUS; VA – Vagos, PT; VN – Vila Nova de Milfontes, PT

Table 3 Number of isolates identified within the seeds of *A. longifolia* in each location from native and invasive ranges. Colours follow those attributed to the different genera as in Fig. 2. Unclassified: sequences which could not be classified at the genus level

Range	Country	Location ¹	Phylum ²	Genus	Isolates	Total				
Native	Australia	BE	Bacillota	<i>Bacillus</i>	12	30				
				<i>Curtobacterium</i>	2					
				<i>Frigoribacterium</i>	1					
				<i>Kocuria</i>	4					
				<i>Phyllobacterium</i>	1					
			Pseudomonadota	Unclassified	10					
				LR	Bacillota		<i>Niallia</i>	4	35	
			Actinomycetota	<i>Paenibacillus</i>			1			
				<i>Staphylococcus</i>			1			
				<i>Curtobacterium</i>			14			
<i>Dermacoccus</i>	2									
<i>Pantoea</i>	9									
Pseudomonadota	Unclassified	4								
	Invaded	Portugal	VA	Bacillota		<i>Bacillus</i>	4	28		
<i>Paenibacillus</i>						6				
Actinomycetota						<i>Curtobacterium</i>	7			
					<i>Microbacterium</i>	1				
Pseudomonadota					<i>Paraburkholderia</i>	1				
				<i>Rhizobium</i>	8					
				<i>Sphingomonas</i>	1					
VN						Bacillota	<i>Bacillus</i>		4	26
							<i>Paenibacillus</i>		13	
							<i>Staphylococcus</i>		1	
	<i>Niallia</i>	1								
	Actinomycetota	<i>Curtobacterium</i>	3							
		Pseudomonadota	<i>Paraburkholderia</i>			1				
	<i>Brevundimonas</i>		3							

¹Seed origin: BE – Belrose, AUS; LR – Long Reef, AUS; VA – Vagos, PT; VN – Vila Nova de Milfontes, PT. ²Recent alterations were made in the nomenclature of the prokaryote phyla showed, in accordance with Oren and Garrity (2021)

cluster contained eight accessions, all from the native range (LR34AUS—LR14AUS, mostly *Curtobacterium* and *Dermacoccus*), followed by another group consisting of the native range isolates BE15AUS (*Kocuria*) and BE16AUS (*Frigobacterium*) (cluster III). In another hierarchical level, two main clusters, IV-A and IV-B, are evident, one including most of the bacteria isolated from the invasive range and the other containing most of the isolates from the native range, respectively. However, both these clusters contained

bacteria isolated from both ranges (Fig. 1). This is evident for the subclusters containing *Curtobacterium* and *Bacillus* (including *Paenibacillus*) isolates from both the native and invasive ranges. Some bacteria show identical profiles for both primers.

Cluster analysis was also performed for accessions obtained from each site separately (Fig. S2). In Belrose (BE) a major cluster (IV-B) contained the *Bacillus* group with some *Kocuria* accessions; in Long Reef (LR) two

different clusters of *Curtobacterium* were present; in Vagos (VA) *Rhizobium*, *Curtobacterium*, *Bacillus* and *Paenibacillus* fell into four clusters, while in Vila Nova de Milfontes (VN) *Curtobacterium*, *Bacillus* and *Paenibacillus* clustered separately.

3.4 Bacterial identification based on 16S rRNA gene sequence analysis

BLAST results on partially sequenced 16S rRNA genes identified 41 isolates to genus level with up to 98.9% pairwise identity (Table S1). For 14 isolates, identification was only possible to family level. These isolates (referred as Unclassified) corresponded to Australian seeds, 10 from Belrose (with a combined relative abundance of 33.33%) and four isolates from Long Reef (with a combined relative abundance of 11.43%) (Table 3, Table S1). Based on our BLAST results, it was possible to group bacterial genera as ‘core taxa’ (i.e., present at all seed collection sites) or ‘flexible taxa’ (only present in some seed collection sites; Simonin et al. 2022). *Curtobacterium* was the only core taxon while all other genera, including *Bacillus*, *Brevundimonas*, *Dermacoccus*, *Frigoribacterium*, *Kocuria*, *Microbacterium*, *Niallia*, *Paenibacillus*, *Pantoea*, *Paraburkholderia*, *Phyllobacterium*, *Rhizobium*, *Sphingomonas* and *Staphylococcus*, were considered flexible (Fig. 2). Native and invasive seeds harboured the same richness of endophytic bacterial genera (10 in each) that differed compositionally (Table 3). Native and invasive range seeds had five genera in common, *Bacillus*, *Curtobacterium*, *Niallia*, *Paenibacillus*, and *Staphylococcus*. The genus *Bacillus* was dominant in native range seeds and *Paenibacillus* in invasive range seeds. The genera *Dermacoccus*, *Frigoribacterium*, *Kocuria*, *Pantoea*, and *Phyllobacterium* were only present in native range seeds, while *Brevundimonas*, *Microbacterium*, *Paraburkholderia*, *Rhizobium* and *Sphingomonas* were only present in invasive range seeds.

In native *A. longifolia* seeds, *Bacillus* was considered the most represented genus in Belrose with 12 isolates (corresponding to 40% of all sequenced isolates), while *Curtobacterium* was the most represented genus in Long Reef with 14 isolates (corresponding to 40% of all sequenced isolates). In Portuguese seeds, *Rhizobium* was the most represented genus in Vagos (corresponding to 28.57% of all sequenced isolates) and *Paenibacillus* was the most represented genus in Vila Nova de Milfontes (corresponding to 50% of all sequenced isolates) (Table 3, Fig. 3). An interesting result was the presence of *Rhizobium* only in seeds from Vagos (Portugal) and the absence of *Bradyrhizobium* strains in all locations. Isolates from Australia and Portugal were from three different phyla: Bacillota (formerly Firmicutes), Actinomycetota (formerly Actinobacteria) and Pseudomonadota (formerly Proteobacteria), with different representation at

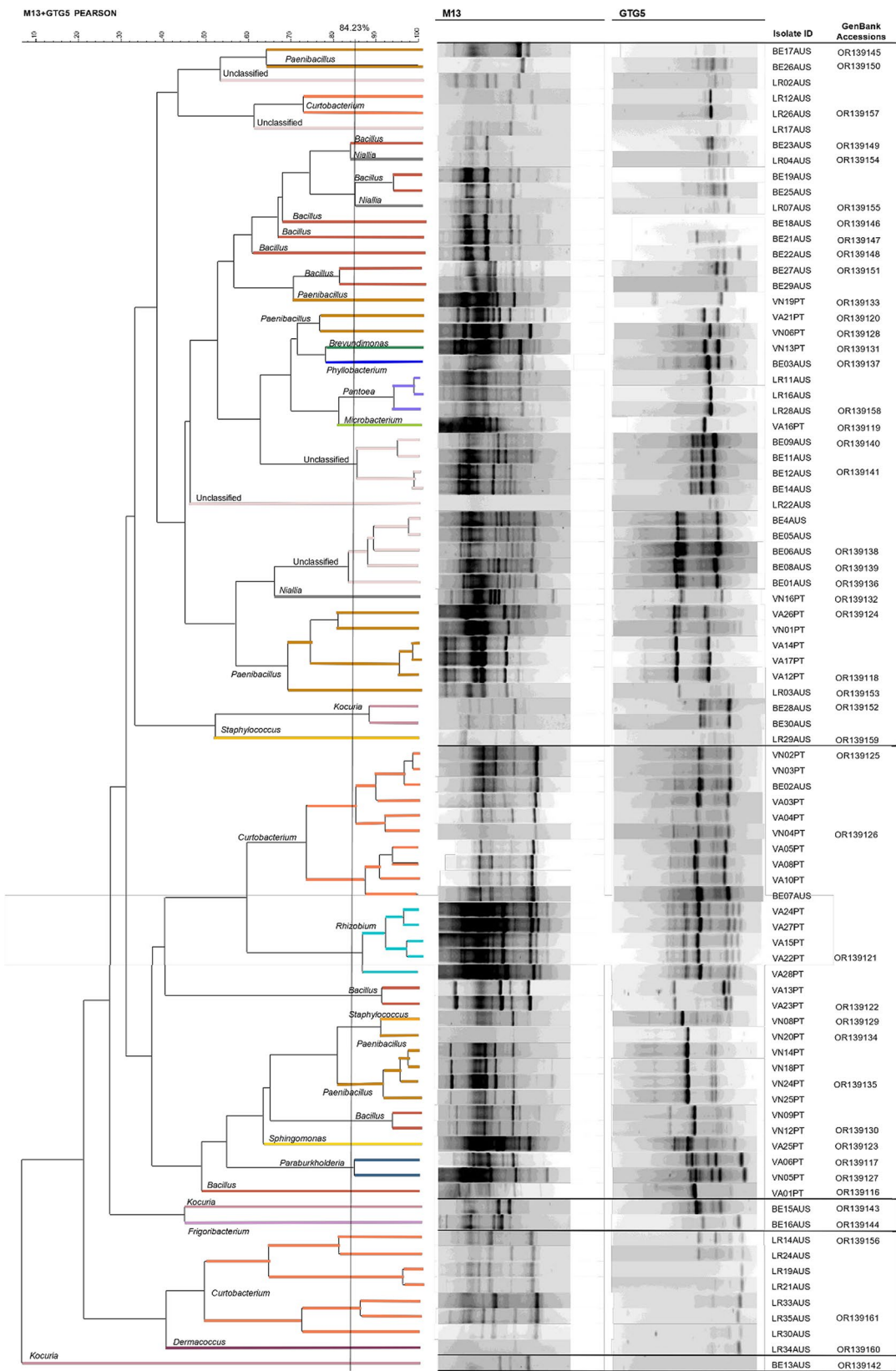
each site (Table 3). While the limited number of sites we sampled in each region precluded us from doing statistical comparisons of diversity between regions we do note a higher bacterial diversity in seeds from the native range and no dominance by a specific genus in both seed origins (Australia vs. Portugal; Table 4). The Shannon-Weiner diversity of native seeds was higher in BE ($H' = 1.21$) in comparison to LR ($H' = 1.13$), while non-native seeds from VA had higher diversity than VN (respectively $H' = 0.96$ and $H' = 0.93$). The Simpson diversity index was highest in the native-range seeds from LR ($D' = 0.98$), while in invasive seeds, VN had the highest diversity ($D' = 0.92$). Simpson diversity indexes were very similar both within native and invasive ranges. The Pielou evenness index was similar ($J' = 0.92$ – 0.94) in all locations, from native and invasive range, meaning that the isolates found were equally distributed and that no dominant isolate was found.

4 Discussion

Understanding plant invasiveness is an area of immense research and for several invasive species differences in traits between native and invaded ranges have been identified. Yet, few studies to date have compared specific seed traits between ranges and how these may facilitate invasion, either considering morphological characteristics or the endophytic communities they harbour. Here, we compared seeds from native and invasive sites of the globally important invader, *Acacia longifolia*, based on morphological, isotopic, and bacterial endophyte community data.

The link between seed size and range (invasive vs. native) has been receiving a lot of research attention, however, no clear picture has emerged. For example, contradictory results have been found in a study on *Cytisus scoparius* (Scotch broom) and *Ulex europaeus* (European gorse), with *C. scoparius* (Scotch broom) producing significantly heavier seeds in its invasive range compared to in the native range, while *U. europaeus* produced seeds of similar size in both ranges (Buckley et al. 2003). Our study illustrates that, although seeds of *A. longifolia* do not differ morphologically between native and invasive populations, significant differences were found among sites within the same range (e.g. seed length within the native and invasive ranges) and between different ranges and sites (e.g. for seed width).

Isotopic analyses can provide information on the physiological responses of plants to local environmental conditions such as their water use efficiency (WUE) (Farquhar et al. 1982, 1989; Brugnoli and Farquhar 2000). For example, higher $\delta^{13}\text{C}$ values (less negative) are generally linked to drier habitats (Cernusak et al. 2013). In the present study, one can infer that native LR seeds ($\delta^{13}\text{C} = -25.9$) collected from



IV - B

IV - A

III

II

I

Fig. 1 Dendrogram based on cluster analysis of fingerprinting PCR profiles using universal primers M13 and (GTG)₅, using UPGMA clustering algorithm with Pearson correlation coefficient, of all the bacterial isolates obtained from *A. longifolia* seeds in native (Australia) and invasive (Portugal) range. Native: BE- Belrose; LR- Long Reef. Invaded: VA- Vagos, VN-Vila Nova de Milfontes are the sites from where bacteria were isolated from the seeds. The cut-off level determined was 84.23%, whereby isolates below this level were considered different. On the left side the labels with genus identification of several isolates are included. The main clusters are represented, from I to IV (A, B)

plants close to the sea, are more influenced by salt spray than plants from BE ($\delta^{13}\text{C} = -28.9$), from more inland areas. Similarly, in the invaded range VN seeds ($\delta^{13}\text{C} = -27.1$) originated from trees closer to the Atlantic Ocean than VA seeds ($\delta^{13}\text{C} = -28.2$).

The isotopic analysis of the $\delta^{15}\text{N}$ revealed values close to 0 ‰ in seeds from all sites, suggesting that most of the nitrogen present in the seeds was derived from the atmosphere and not soil (i.e. soil nitrate or ammonium). While $\delta^{15}\text{N}$ of seeds collected from the native and invasive range of *A. longifolia* were generally similar, we note differences among sites that suggest different soil conditions between BE ($\delta^{15}\text{N} = -0.44$) and LR ($\delta^{15}\text{N} = 0.8$), as well as between VA ($\delta^{15}\text{N} = -0.21$) and VN ($\delta^{15}\text{N} = 1.42$). Our results also suggest that for VN and LR, some NO_3^- and NH_4^+ were utilised by plants and translocated to seeds. The total % N obtained in our study are within the range of values reported previously (2.8–5.1) for other *Acacia* seedlings (Konate et al. 2016). Overall, in accordance with other seed studies (e.g. Alegria et al. 2020), our isotope results suggest a site-dependent response due to local climatic and geological characteristics. Therefore, isotope analyses have been used to trace the geographical origins of seeds from diverse species such as coffee (Rodrigues et al. 2011), *Quercus* species (Alegria et al. 2020) and argan (Elgadi et al. 2021). In conclusion, the seeds isotopic composition of *A. longifolia* reflects site characteristics and no distinctive pattern was found that could differentiate native from invasive seeds.

Our study also provided clear evidence that *A. longifolia* seeds harbour a large variety of endophytic bacteria. The 15 cultivable genera that we identified in these communities are likely a gross underestimate of the true endosymbiont diversity associated with the seeds of this species. Our finding that most cultivable endophytic bacteria belong to the phyla Pseudomonadota (formerly Proteobacteria) and Actinomycetota (formerly Actinobacteria), and some to Bacillota (formerly Firmicutes), is in accordance with a recent meta-analysis on the diversity of seed microbiomes (Simonin et al. 2022). This meta-analysis also identified the phylum Bacteroidota (formerly Bacteroidetes) as being commonly associated with seed microbiomes, which we did not find, either

because in the present study we only characterised cultivable bacteria or because they are not present in *A. longifolia* seeds. Different studies have shown that seed vigour can be influenced by the microorganisms living inside and on the surface of seeds (e.g. Nelson 2018), by protecting seedlings from pathogens or harsh environmental conditions (e.g., Sahu et al. 2019; Xia et al. 2022). As outlined below, we propose that the seed endophytic bacteria identified in our study may be associated with the establishment and growth of young plants (see below), facilitating the invasiveness of *A. longifolia*.

Seed endophytes have been found to colonise the ovules of plants (Sahu et al. 2019), to facilitate vertical transfer from the mother plant through developmental stages (embryogenesis). Another possible pathway of transmission is horizontal transfer from various sources such as soil, water, air, and insects, which may explain why the diversity of bacterial seed endophytes in our study was site-specific (Afzal et al. 2019). These different pathways for transmission can explain the core (transmitted across multiple generations) and flexible (depending on habitat) bacteriomes (Shahzad et al. 2018; Simonin et al. 2022) that we found in our study. The dominance of *Curtobacterium* strains in all seeds (Figs. 2 and 3) suggests the existence of a possible vertical transmission pathway for this taxon and warrants further research. *Curtobacterium* is an Actinomycetota (formerly Actinobacteria) with a cosmopolitan distribution, predominantly found in soils. Although traditionally described as pathogens, the genus plays various ecological roles such as the decomposition of organic matter (Chase et al. 2016), attributed to its genomic potential for carbohydrates degradation, specifically structural polysaccharides (Chase et al. 2016). In the case of *A. longifolia*, we can hypothesise that the presence of *Curtobacterium* as a core endophytic phytopathogen may benefit its establishment, since it may negatively affect the native flora.

Considering the Bacillota phylum, *Bacillus* was a prominent genus isolated from *A. longifolia* seeds collected from most sites (except LR), while *Paenibacillus* was present in seeds from most sites (except BE). These two genera co-occurred in the seeds collected from invasive populations. Previous research has described these genera as including plant growth promoting (PGP) bacteria, capable of phytohormones production, but also involved in atmospheric nitrogen fixation and phosphate solubilisation (e.g., Goswami et al. 2016; Grady et al. 2016). These functions may contribute to the successful establishment of invasive plants, in terms of growth, by granting them a competitive advantage over native plants (Rout et al. 2013). Some *Bacillus* and *Paenibacillus* species also produce several metabolites that have antifungal activities and play an important role in pathogen suppression (Hu et al. 2017). These genera are also

Fig. 2 Core and flexible bacterial genera found within each location from *Acacia longifolia* seeds. Overlaps in the diagram indicate shared genera in those locations. (BE – Belrose, AUS; LR – Long Reef, AUS; VA – Vagos, PT; VN – Vila Nova de Milfontes, PT)

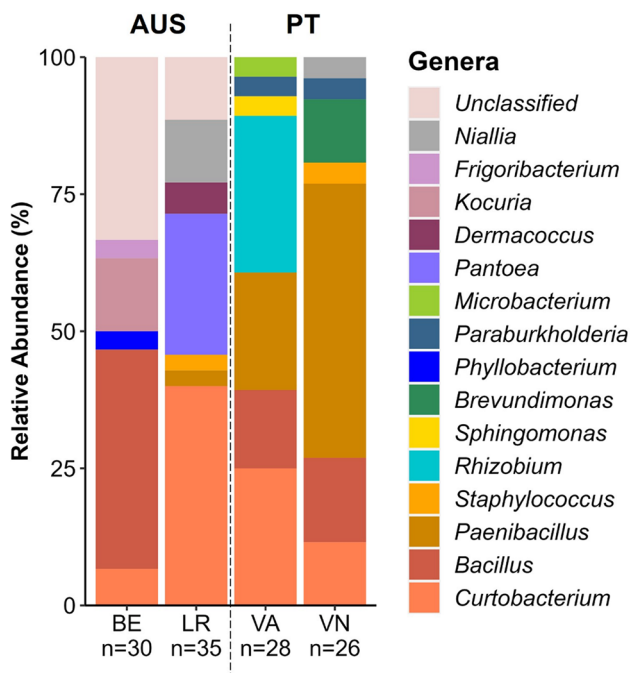
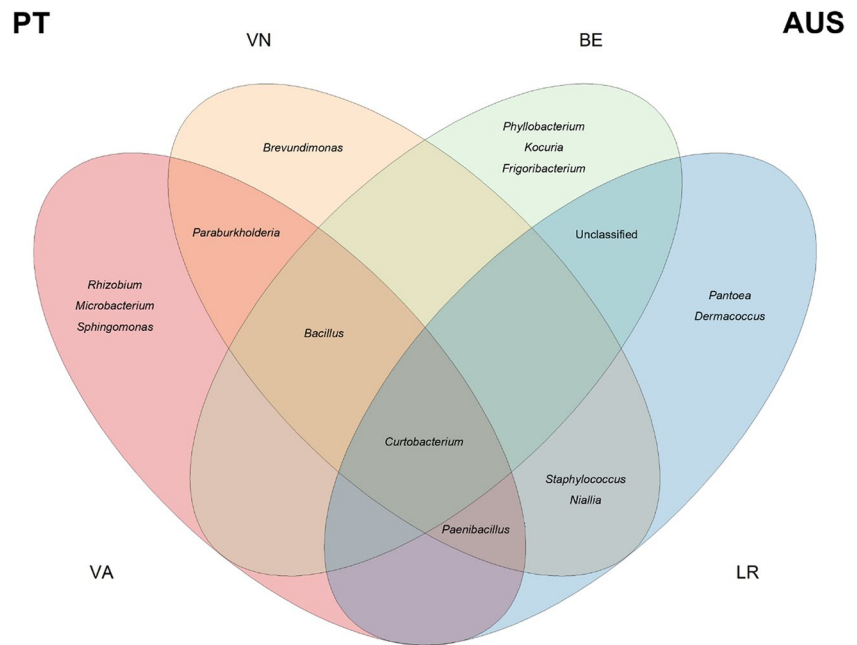


Fig. 3 Relative abundance (%) of the different bacterial genera found in the different seed origins [BE (n=30);LR (n=35);VA (n=28);VN (n=26)]. Unclassified: sequences which could not be classified to the genus level. (BE – Belrose, AUS; LR – Long Reef, AUS; VA – Vagos, PT; VN – Vila Nova de Milfontes, PT) AUS, Australia; PT, Portugal

considered as drought resistant (Köberl et al 2013), which recently have been associated with the drought tolerance capacity of plants. Drought affects plant growth, and some studies have correlated this abiotic stress with the presence of

Table 4 Bacterial seed endophyte diversity of *Acacia longifolia* calculated as Shannon-Weiner and Simpson diversity indices and the Pielou evenness index

Range	Country	Location ¹	Shannon–Wiener (H')	Simpson (D')	Pielou (J')
Native	Australia	BE	1.21	0.96	0.94
		LR	1.13	0.98	0.94
Invaded	Portugal	VA	0.96	0.91	0.92
		VN	0.93	0.92	0.93

¹Seed origin: BE – Belrose, AUS; LR – Long Reef, AUS; VA – Vagos, PT; VN – Vila Nova de Milfontes, PT

drought-resistant bacteria in endophyte communities that are capable of producing PGP substances, as well as compounds that cope oxidative stress (Ullah et al. 2019; Verma et al. 2021; Ashry et al. 2022). In the context of ongoing climate change, these bacterial endophytes may be important to their host plants and may increase the performance strength of invasive species. Furthermore, these bacteria may also be involved in auxin and cytokinin production (de Melo Pereira et al. 2012; Xia et al. 2022). Apart from all the roles played by these phytohormones in plant development, higher auxin levels have also been directly linked to higher nodulation in legumes (Pii et al. 2007). The presence of *Bacillus* and *Paenibacillus* in *A. longifolia* seeds in both VA and VN sites suggests that plants in the invaded range benefit from these endosymbionts, the extent of which remains to be determined.

Kocuria and *Rhizobium* species have also been identified as capable of auxin production (Haidar et al. 2018). *Pantoea* is one of the most common bacterial genera associated

with plant seeds (Nelson 2018) and has been associated with biocontrol activity against a wide range of phytopathogens (Walterson and Stavrinos 2015). The presence of *Pantoea* species in the seeds of *A. longifolia* suggest similar functions may be present and deserves further research. The role played by these bacteria in the seed is difficult to prove unequivocally, and the ecological characterisation of microorganisms is difficult, since they display dynamic alterations of life cycles depending on the environmental conditions they live in (Chase et al. 2016).

In this study, we also wanted to find out whether rhizobia are common endophytes of the seeds of *A. longifolia*, as has been shown for other legume seeds (Chimwamurombe et al. 2016; Aguilar et al. 2018; Laranjeira et al. 2022). *Rhizobium* was the only representative genus found in the seeds of *A. longifolia*, from one site in the species' invasive range. *Rhizobium* strains fix atmospheric nitrogen and solubilise phosphorus, which are important attributes to legume performance. *Bradyrhizobium*, commonly associated with Australian acacias (Lafay and Burdon 2001; Rodríguez-Echeverría et al. 2007; Birnbaum et al. 2016), was not found in our study in either native or invasive seeds. It has been identified in *A. longifolia* nodules as the predominant bacterium from both invasive and native plants (Rodríguez-Echeverría et al. 2012; Crisóstomo et al. 2013; Birnbaum et al. 2016; Jesus et al. 2020). Previous phylogenetic studies have shown that *Bradyrhizobium* species have been co-introduced from Australia (native range) with various *Acacia* species into Brazil, California, New Zealand, Portugal and South Africa (Crisóstomo et al. 2013; Warrington et al. 2019; Le Roux and Wandrag 2023). Assessing whether *Bradyrhizobium* strains can act as *Acacia* seed endosymbionts provides exiting future research opportunities.

5 Conclusion

Although several studies have reported on the seed endophytic communities of plants, as far as we know, our study is the first to report on the bacterial endophyte communities of *Acacia longifolia* seeds. We show that seed endophytic communities from the species' native and invasive ranges share common taxa, such as *Curtobacterium* while some ranges harboured unique associations (e.g., the presence of *Paraburkholderia* or the co-occurrence of *Bacillus* and *Paenibacillus* in seeds from the invasive range). We propose that the seed endophytic bacteria of *A. longifolia* might be playing an important role underlying its invasiveness. The bacterial taxa we identified are known to aid plant establishment and early development, especially in stressful environments. Future studies should include an evaluation of the ecological roles of seed endophytic bacteria and their contributions to plant performance.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13199-024-00987-3>.

Acknowledgements This research was funded by Fundação para a Ciência e a Tecnologia (FCT, Portugal), FCT/MCTES through financial support to cE3c, Research Unit grant number UIDB/00329/2020 (<https://doi.org/10.54499/UIDB/00329/2020>). We thank Rodrigo Maia from SIIAF for the technical support on isotopic analysis.

Funding Open access funding provided by FCTIFCCN (b-on).

Data availability Further data and details are provided in the Supplementary Information file. The full dataset used in this study is available upon request to the corresponding Author.

Declarations

Conflicts of interest On behalf of all the authors, the corresponding author states that there are no conflicts of interest.

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