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RESEARCH PAPER

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Cointroductions of Australian acacias and their rhizobial mutualists in the Southern Hemisphere

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Abstract

Aim: Mutualisms are often disrupted for plants introduced to new ranges, yet many of these plants have managed to obtain effective mutualistic associations in their new ranges. There are two potential pathways for non-native plants to reassemble mutualisms: cointroduction (i.e. familiar associations with cointroduced mutualists) or ecological fitting (i.e. forming or adapting novel associations with resident native mutualists). We assessed the importance of each pathway for mutualist reassembly in four Australian *Acacia* species (*A. baileyana*, *A. dealbata*, *A. decurrens* and *A. melanoxylon*) and their associated nitrogen-fixing rhizobial symbionts in two non-native locations.

Location: Native ranges of acacias in south-eastern Australia and two non-native ranges in New Zealand and South Africa.

Methods: Rhizobia associated with each acacia species in each country were isolated and identified based on DNA sequencing of the housekeeping *recA* gene and the symbiotic *nodA* gene. Separate phylogenies were reconstructed for each gene region to infer biogeographic histories of acacia-associated rhizobia. Selected rhizobial strains for each acacia species by country combination were used as inocula in a glasshouse experiment and early growth kinetics and nitrogen fixation efficiency of acacia seedlings were compared between inoculum treatments to determine symbiotic effectiveness.

Results: All isolated rhizobial strains belonged to the genus *Bradyrhizobium*. Phylogenetic analyses revealed almost no country- or species-specific clusters of these strains for either gene region and indicated that most acacia-associated bradyrhizobia in New Zealand and South Africa were cointroduced from Australia. These results were supported by little variation in the growth performances of acacia seedlings, irrespective of inoculum treatment.

Main conclusions: This study revealed that cointroduction of Australian acacias and their rhizobia may be more prevalent than previously thought. Additionally, a single rhizobium cointroduction event may be sufficient to facilitate the establishment of effective mutualisms in numerous *Acacia* species, potentially leading to an invasion meltdown.

KEYWORDS
Australian acacias, biological invasions, *Bradyrhizobium*, host-switching, invasional meltdown, mutualist cointroduction

1 | INTRODUCTION

Mutualistic interactions, such as those associated with pollination, seed dispersal, and nutrient acquisition by soil mutualists, are critical for many plants to complete their life cycles. For some introduced non-native plants, disruption of these interactions can pose a significant hurdle to successful establishment (Traveset & Richardson, 2014). Such plants have two avenues to re-establish mutualistic interactions; they can either be cointroduced with familiar mutualists from their native range (so-called cointroduction pathway) or they can associate with, or adapt to, novel mutualists in their new ranges (so-called ecological-fitting pathway; Le Roux, Hui, Keet, & Ellis, 2017). The establishment success of introduced plants with highly specialized mutualist associations may be more reliant on cointroductions relative to plants with more generalist associations, because generalist species may more readily establish novel interactions (La Pierre, Simms, Tariq, Zafar, & Porter, 2017; Le Roux et al., 2017; Richardson, Allsopp, D'Antonio, Milton, & Rejmánek, 2000; Richardson & Rejmánek, 2011; Rodríguez-Echeverría, Le Roux, Crisóstomo, & Ndlovu, 2011; van der Putten, Klironomos, & Wardle, 2007).

It has been suggested that when introduced species are cointroduced with their mutualists they may become more invasive and have greater ecological impact than those that establish mutualisms by ecological fitting (Le Roux et al., 2017). Novel associations may not be optimal and thus limit the performance of non-native plants. This may be due to direct competition for available mutualists with native host plants that may be superior in attracting them, and/or because such associations have lower effectiveness (e.g. Rodríguez-Echeverría, Fajardo, Ruiz-Díez, & Fernández-Pascual, 2012). Thus, non-native plants that form novel mutualist associations may experience substantially longer lag phases between the time from first introduction to becoming widespread. The severity of ecological impacts caused by non-native plants is expected to be higher when they are cointroduced with their mutualists, because these familiar associations typically involve positive feedbacks between co-invading partners (Le Roux et al., 2017).

Evidence from non-native legumes and their associated mutualistic bacteria, known as rhizobia, suggests both novel as well as familiar associations via cointroduction are commonplace during invasions. Rhizobia are capable of forming nodules on the roots and, less frequently, on the stems of most legumes. Within nodules, rhizobia fix atmospheric nitrogen into ammonium that legumes can utilize. In return, legumes provide rhizobia with various sources of carbon. In support of novel associations, recent molecular research has demonstrated unique rhizobial communities in association with some legumes in their native versus non-native ranges (e.g. Birnbaum, Barrett, Thrall, & Leishman, 2012; Callaway, Bedmar, Reinhart, Silvan, & Klironomos, 2011; Shelby et al., 2016). On the other hand, many legumes associate with identical rhizobia in both their native and their non-native ranges (e.g. Birnbaum, Bissett, Thrall, & Leishman, 2016; Horn, Parker, Malek, Rodríguez-Echeverría, & Parker, 2014; McGinn et al., 2016; Ndlovu, Richardson, Wilson, & Le Roux, 2013), supporting cointroduction.

The interaction between legumes and rhizobia involves complex and intricate molecular signalling (van der Putten et al., 2007). Plant signalling chemicals, such as (iso)flavonoids, are released into the rhizosphere by legumes to attract rhizobia. The rhizobia subsequently colonize the root hairs of legumes through the activation of so-called nodulation (*nod*) genes (Perret, Staehelin, & Broughton, 2000), which are thought to be important determinants of legume–rhizobia symbiotic specificity (Rogel, Ormeno-Orrillo, & Martinez-Romero, 2011). Nodulation genes are located on mobile genetic elements, such as symbiotic islands within the core genome or on plasmids (Perret et al., 2000). These mobile elements can be exchanged between different rhizobium species, and even genera, through horizontal gene transfer (HGT) facilitated by conjugation (Lemaire, Dlodlo, et al., 2015). Such exchanges mean that rhizobial strains receiving mobile elements, while retaining their core genetic identity (based on non-mobile housekeeping genes), may obtain new genetic capabilities for nodulation. This can have consequences under cointroduction where nodulation genes can be transferred between introduced rhizobial strains and resident native rhizobial strains (Le Roux et al., 2017). Therefore, while the identities of rhizobia are important, the true determinants of effective nodulation lie in the nodulation genes carried by them. Additionally, given their apparent role in modulating interaction specificity, nodulation genes are important biogeographic markers of legume–rhizobia compatibility (Martinez-Romero, 2009; McGinn et al., 2018). Consequently, in order to determine the efficacy of the association, as well as the occurrence of cointroduction and HGT (of nodulation genes) versus novel associations, it is necessary to utilize both housekeeping and symbiotic genes when identifying rhizobia.

Australian acacias (genus *Acacia* Mill.) are a group of legumes which are particularly invasive globally (Richardson et al., 2011). Many acacias have been intentionally introduced for various purposes such as forestry, coastal sand dune stabilization and ornamental purposes (Le Roux et al., 2011). Successful nodulation of Australian acacias has been recorded in several regions across the globe, including Europe (Rodríguez-Echeverría, Crisóstomo, Nabais, & Freitas, 2009), Asia (Le Roux et al., 2009), southern Africa (Le Roux et al., 2018; Le Roux, Mavengere, & Ellis, 2016; Ndlovu et al., 2013), the Americas (Aronson, Ovalle, & Avendaño, 1992), New Zealand (Weir, Turner, Silvester, Park, & Young, 2004), as well as regions

outside their native range within Australia (Birnbaum et al., 2012). A few examples of cointroduction of acacias and their associated rhizobia also exist (e.g. *Acacia longifolia* in Portugal, Rodríguez-Echeverría, 2010; *Acacia pycnantha* in South Africa, Ndlovu et al., 2013; *Acacia saligna* in Portugal, Crisóstomo, Rodríguez-Echeverría, & Freitas, 2013). While cointroduction of effective rhizobial strains may be a key factor in the successful establishment of Australian acacias in novel ranges, the vast number of successful acacia introductions globally suggests high levels of generalism in their symbiotic requirements and thus the potential to also establish effective novel rhizobial associations (Rodriguez-Echeverria et al., 2011).

Here, we aim to determine how frequently cointroduction versus ecological fitting occurs for four different *Acacia* species in two regions where they are non-native (South Africa and New Zealand), and what the subsequent consequences are for plant performance. These two regions represent unique opportunities to investigate the role of cointroduction vs ecological fitting during invasion. This is because bradyrhizobia (the preferred symbionts of Australian acacias) are not naturally found in association with any of New Zealand's native legumes and only associate with few South African legumes (Lemaire, Van Cauwenberghe, et al., 2015). To address our aims, we first reconstructed phylogenies, based on both housekeeping (identity) and nodulation (symbiotic) genes, for rhizobia isolated from all four acacias in their native Australian and their non-native South African and New Zealand ranges to determine how often rhizobia have been cointroduced with acacias to these regions. Next, we grew these four acacias under glasshouse conditions and compared their performance in association with the rhizobia isolated from host plants from all regions to determine the efficacy of each association. Given that acacias have been predominantly introduced for forestry and agroforestry purposes, where purposeful inoculation may often be prevalent and/or plants may have been introduced as saplings, we hypothesized (1) that cointroduction would be more commonplace than novel associations in both non-native regions, that is that rhizobia isolated from South Africa and New Zealand will cluster phylogenetically with rhizobia from Australia and (2) that familiar associations should lead to higher plant performance compared to novel associations, such that we expected acacias to fix less nitrogen and grow slower when associated with novel bacteria isolated from non-native ranges compared to growth in association with Australian rhizobia.

2 | MATERIALS AND METHODS

2.1 | Study species, root-nodule collection and inocula preparation

We studied four Australian acacias: *Acacia baileyana* F. Muell., *Acacia dealbata* Link., *Acacia decurrens* Willd. and *Acacia melanoxylon* R. Br., and their associated rhizobia collected from Australia (AUS), New Zealand (NZ) and South Africa (SA). All four species are native to south-eastern Australia and have become naturalized and/or invasive in South Africa and New Zealand (Rejmánek & Richardson, 2013).

For each *Acacia* species, root nodules were collected from five different and well-separated individuals within a single population in all countries (see Appendix S1). Root nodules were preferentially excavated from younger trees due to their shallower root systems. Collected nodules were placed into tubes containing silica gel to dehydrate them until later use. In the laboratory, nodules were placed in 1 ml of distilled water overnight to rehydrate. Rhizobia were axenically isolated from single nodules following Somasegaran and Hoben (1994) with minor modifications: submersion in 3.5% sodium hypochlorite for 60 s instead of acid sterilization. Rhizobia were grown at 28°C on yeast mannitol agar supplemented with Congo Red dye and restreaked until purity was achieved. Colony purity was confirmed through Gram-staining (Cornell University, Animal Health Diagnostic Centre).

To prepare country- and species-specific inocula, three strains were randomly selected from the isolated rhizobia for each country by species combination, because there appeared to be a low overall diversity of strain identities associated with each *Acacia* species (see Section 3.1). We inoculated 15 ml of sterilized yeast mannitol broth with a single pure colony from the three selected strains, followed by shake incubation at 28°C for 1 week. Following growth, that is turbid growth media, the three individual strains were mixed together and made up to a 1-L bottle with sterilized distilled water for each country-species combination.

2.2 | Glasshouse experiment

All four *Acacia* species were grown from seeds obtained from the Agricultural Research Council's Plant Protection Research Institute (ARC-PPRI) in Stellenbosch, South Africa. Seeds were surface-sterilized and scarified (Rincón-Rosales, Culebro-Espinosa, Gutierrez-Miceli, & Dendooven, 2003) and subsequently placed at a depth of 1 cm in 2-L pots filled with sterile silica soil and saturated with distilled water. Two seeds were planted per pot and, in the event that both seeds germinated, one of the seedlings was haphazardly removed from each pot. All pots were placed in a glasshouse located at Stellenbosch University, South Africa. The glasshouse was exposed to ambient temperature and light conditions, and acacias were grown for a period of 6 months between August 2016 and March 2017. Each *Acacia* species was exposed to a factorial design of six different treatments of country-specific inoculum (AUS, NZ or SA) by nutrient addition (including/excluding nitrogen) combinations and a control treatment, which received no inoculum and only nitrogen-containing nutrient solution. Each treatment was replicated nine times for each species. For rhizobial inoculation, 50 ml of country- and species-specific inoculum was added to each treatment the day following planting. This was followed by two additional inoculations, at the end of the first and third month following planting. 7.5 ml nitrogen solution (2 mM NH_4NO_3) was diluted in 15 L of Long Ashton nutrient solution, of which 100 ml was added to each of the seedlings once every 2 weeks. Those seedlings exposed to the no-nitrogen nutrient treatments had no NH_4NO_3 added to the nutrient solution. Plants were watered with 150 ml of distilled water twice a

week for the duration of the experiment. The position of pots was randomized weekly. In order to avoid cross-contamination during watering and nutrient applications, each pot was placed in a water collecting saucer and all randomization was done prior to watering when saucers were completely or partially dry.

2.3 | Rhizobial phylogenies

Following the 6-month growth period, seedlings were harvested and nodules from three randomly selected replicates per treatment per species were removed from the roots and stored in tubes containing silica gel. Rhizobia were extracted, purified and grown in liquid medium as stated above. These, together with rhizobia initially isolated from field-collected nodules, were used to extract genomic DNA using the Sigma Gen-Elute Bacterial Genomic DNA kit (Sigma-Aldrich Co. LLC) according to the manufacturer's specifications. The housekeeping (identity) gene, *recA*, and the nodulation (symbiotic) gene, *nodA*, were amplified using the primers and polymerase chain reaction (PCR) conditions described in Gaunt, Turner, Rigottier-Gois, Lloyd-Macgilp, and Young (2001) and Haukka, Lindström, and Young (1998), respectively. Amplified PCR products were purified using the Qiaquick PCR purification kit (Qiagen GmbH, Germany) and purified PCR products sequenced in one direction using the ABIPRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and an automated ABI PRISM 377XL DNA sequencer (PE Applied Biosystems, Foster City, CA) and the same (forward) primer used for PCR amplification.

Separate phylogenies were reconstructed for the *recA* and *nodA* DNA regions in order to (a) verify Koch's postulates (Rivers, 1937), that is that nodulation in the glasshouse was by bacterial strains present in the original inocula and not as a result of cross-contamination and (b) to determine the genetic relatedness between rhizobia isolated from the native (AUS) and non-native ranges (NZ and SA) of acacias. DNA sequences were edited in BioEdit 7.0.5.3 and aligned using CLUSTAL W (Ndlovu et al., 2013). DNA sequences were blasted against reference data available on the online GenBank repository (https://blast.ncbi.nlm.nih.gov). All our isolated rhizobia belonged to the genus *Bradyrhizobium* (see Section 3.1), and therefore, those sequences with the highest similarity to ours, as well as bradyrhizobia previously isolated from native legumes in South Africa and Australia, were included in the phylogenetic analyses (Appendices S2–S4). *Bradyrhizobium* is not naturally found in New Zealand (Weir et al., 2004); thus, no data were included for this country. We used *Rhizobium* spp. as outgroup taxa for all phylogenetic reconstructions. Both phylogenies were reconstructed using Bayesian search criteria as implemented in the MrBayes program (Ronquist & Huelsenbeck, 2003) and best fit models based on Akaike information criterion using JModel Test (Posada, 2008). The best fit models for the *recA* and *nodA* gene regions were GTR+G and TPM3uf+I+G, respectively. The latter was substituted with a GTR+I+G model (Lecocq et al., 2013), due to the identified model's incompatibility with the MrBayes program. Topological support was inferred as posterior probabilities, and trees were visualized using FigTree 1.1.2. (Rodríguez-Echeverría, 2010). Lastly, we determined the proportion of different homologous sites (as *P*-distances, Nei & Kumar, 2000) to evaluate the likely number of unique bacterial strains (at 2% DNA similarity cut-off) represented by our *recA* and *nodA* data. It has been previously shown that different bacterial species often show high DNA sequence similarity (e.g. up to 98.65% for the frequently used *16S rRNA* gene; Kim, Oh, Park, & Chun, 2014).

2.4 | Plant growth performances and stable isotope analysis

To determine the efficacy of the various rhizobial inocula, three measures of plant growth performance were taken (shoot length, root:shoot biomass and total dry biomass). As additional measures of nitrogen fixation efficiency, we also counted the number of nodules per seedling and conducted stable isotope analyses. *Acacia* seedlings were carefully removed from soil following 6 months of growth, avoiding damage to the root systems. All remaining silica sand was removed by submersion of root systems in distilled water. The shoot length of each plant was measured before removal from pots. Following the removal of sand from roots, all visible nodules were detached and counted. Following root-nodule removal, harvested plants were divided into above- and belowground parts, placed into individual paper bags and oven-dried for 1 week at 55°C. Once dried, samples were weighed to determine total dry biomass and to calculate root:shoot biomass ratios. Under ineffective rhizobial associations, acacias tend to invest more in belowground growth in order to increase organic N absorption. Acacias with effective associations, however, tend to invest more in aboveground growth and thus have lower root:shoot ratios (Rodríguez-Echeverría et al., 2009).

Three leaflets (taken from the leaf originating directly below the shoot apical meristem) per seedling were oven-dried and crushed into a fine powder for carbon and nitrogen stable isotope analyses. Isotope analyses were conducted using a Flash HT Plus Elemental Analyser integrated via a ConFlo IV system with a Delta V Plus Isotope Ratio Mass Spectrometer (Thermo Scientific, Bremen, Germany). Samples were combusted at 1,020°C and the nitrogen isotope values corrected against an in-house standard (Merck Gel $\delta^{15}N$ = +6.80‰). Isotope values were expressed in parts per thousand (‰) following Lötter, van Garderen, Tadross, and Valentine (2014) and Rodríguez-Echeverría et al. (2009). The same procedure was followed for the $\delta^{13}C$ values. The $\delta^{15}N$ data were used as a measure of biological nitrogen fixation (BNF), with lower and more negative values indicating greater contribution of atmospheric nitrogen via BNF (Rodríguez-Echeverría et al., 2009; Unkovich, 2013). Carbon:nitrogen ratios as well as carbon construction costs of shoots were also calculated following Mortimer, Pérez-Fernández, and Valentine (2008). Carbon construction costs (Cw) are proxies for the amount of carbon required by plants to construct new tissues. When N is acquired via BNF, the costs associated with nodule development and maintenance would increase Cw (Mortimer et al., 2008) and could reduce biomass accumulation. In contrast, when N is not acquired from BNF but from soil sources, it is expected

that the Cw would be lower (Magadlela, Pérez-Fernández, Kleinert, Dreyer, & Valentine, 2016; Mortimer et al., 2008). Although root growth to acquire soil N can also impose an increase in Cw, this is expected to be less than the symbiotic costs, as was found for associations between legumes and rhizobia and arbuscular mycorrhizas (Mortimer et al., 2008). Additionally, in situations where plants are associated with effective, as opposed to ineffective rhizobia, costs should also be lower because the exchange of nutrients would be more efficient in more effective associations. Therefore, carbon:nitrogen ratios and carbon construction costs are proxies for nitrogen fixation where higher values represent inefficient nitrogen supply from BNF (Magadlela et al., 2016).

2.5 | Statistical analyses

Because each *Acacia* species was inoculated with country- and species-specific rhizobia, comparisons of performance parameters were undertaken between treatments for each species separately. All plants that had died (n = 21) before the termination of the experiment were excluded from all subsequent analyses because there were no clear relationship between treatment and number of deaths. All analyses were performed in the R statistical environment (version 3.5.0; R Development Core Team, 2018).

To test for differences in overall symbiotic effectiveness (i.e. growth performance benefits of rhizobial associations compared to no associations) between treatments, one-way analyses of variance (ANOVA) were conducted to compare the performance measurements (shoot length, root:shoot ratio, total dry biomass, nodule numbers, δ^{15}N, carbon:nitrogen ratio and carbon construction costs) between the control (nitrogen-containing nutrient solution but no inoculum added) and the treatments that received inoculum as well as nitrogen-containing nutrient solution, followed by Tukey HSD post hoc tests. To determine the relative symbiotic effectiveness (i.e. influence of rhizobial origin [country] and the influence of nitrogen addition on plant performance), factorial ANOVAs and Tukey HSD post hoc tests were conducted using those treatments that received inoculum (i.e. excluding the control treatment), with rhizobial origin and nutrient treatment as main effects.

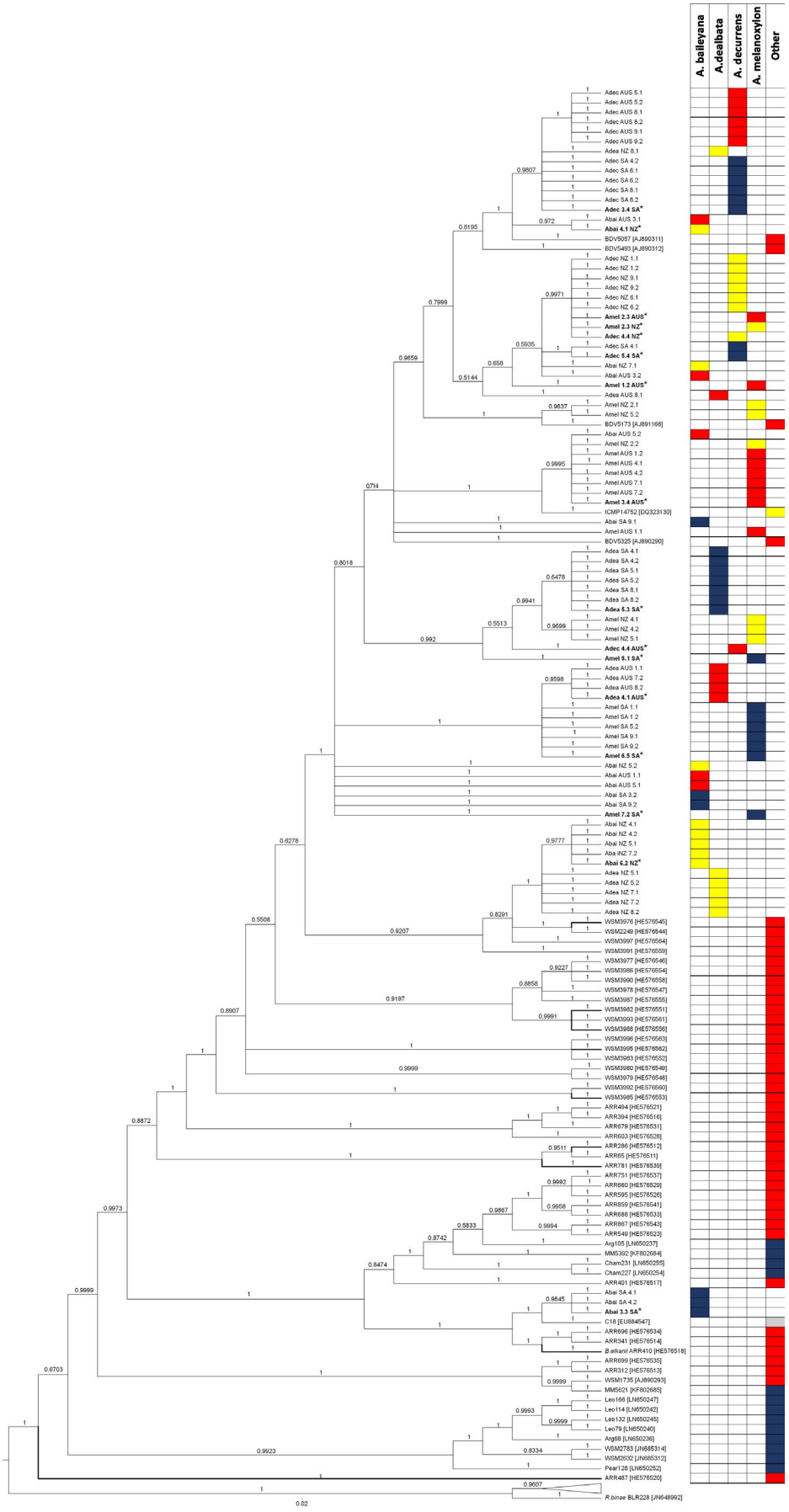
3 | RESULTS

3.1 | Rhizobial phylogenies

Based on Blast results for the housekeeping *recA* gene sequences, all rhizobia isolated from acacias belong to the genus *Bradyrhizobium* (Appendix S2). While *nodA* genes may have non-bradyrhizobial origins due to HGT, our Blast results indicated that all *nodA* gene sequences also had highest similarity to those previously described from *Bradyrhizobium* (Appendix S2). Based on *nodA* DNA sequence data, 87% of acacia-isolated strains from all three focal countries showed highest DNA sequence similarity to *Bradyrhizobium* reference strains previously isolated from native legumes in Australia. On the other hand, for the *recA* housekeeping gene, only 47% of our strains showed highest similarity to *Bradyrhizobium* reference strains of Australian origin. Koch's postulates were verified for 50% of the originally isolated strains sequenced (i.e. 18 out of 36 strains – Appendix S2). Yeast contamination of inoculum stocks prevented verification for all strains. Twelve strains were verified for both gene regions, but due to sequencing failure a further two strains could only be verified for *recA* and four only for *nodA* (i.e. a total of 14 strains verified for *recA* and 16 for *nodA*). All DNA sequences generated in this study have been submitted to the GenBank online repository (https://www.ncbi.nlm.nih.gov/genbank/, accession numbers MK759676–MK759831).

In the *nodA* cladogram (Figure 1), reference data obtained from GenBank for bradyrhizobia isolated from native Australian and South African legumes (Appendix S3) formed well-supported and country-specific phylogenetic clades, with a few exceptions. All acacia-associated rhizobia isolated in this study fell into an exclusively Australian clade, with the exception of one strain isolated from *A. baileyana* in South Africa. Genetic distances (i.e. *P*-distances) indicated that all our isolates represented two bacterial *nodA* strains (i.e. showing more than 2% DNA sequence differences): one strain representing most rhizobia isolated from all acacias and regions and a second strain representing rhizobia isolated from *A. baileyana* in South Africa (Figure S1). Reference data obtained from GenBank for bradyrhizobia isolated from native Australian and South African legumes in the *recA* cladogram retrieved poorly supported clades and also poor spatial structuring. Acacia rhizobial isolates from this study were closely related to reference strains isolated from legumes in both Australia and South Africa (Appendix S2) as well as from other origins around the world (Figure 2). For *recA*, *P*-distances indicated that acacia isolates comprised seven strains (i.e. >2% DNA sequence differences, Figure S2), most of which were shared between species and countries. However, unique *recA* strains were found in association with *A. baileyana* and *A. dealbata* in South Africa and *A. baileyana* and *A. melanoxylon* in New Zealand (Figure S2). Overall both tree topologies supported the prevalence of cointroduction and the near absence of novel associations between naturalized/invasive acacias and their bradyrhizobia. The single *A. baileyana* strain isolated from South Africa formed distinct and monophyletic clades in both phylogenies. For both gene regions, and based on Blast results, this strain was most closely related to reference strains with origins outside of Australia (Appendices S2 and S3). Therefore, it is likely that this strain is not of Australian origin and represent a novel association.

While the occurrence of HGT among rhizobium species is common, the low topological support in our *recA* phylogeny renders any inferences of HGT (see tanglegram, Figure S3) speculative at best. Despite this, we found support for three HGT events between strains that were placed in incongruent and well-supported clades in both phylogenies (Figure S3). These include strains isolated from *A. decurrens* and from *A. baileyana* in South Africa. For the *A. baileyana* strains in particular, a single *recA* strain identity which fell into a distinct clade from the rest of the acacia isolates was retrieved, while multiple, separate *nodA* identities were found, representing


A. balleyana
A.dealbata
A. decurrens
A. melanoxylon
Other
Adec AUS 5.1
Adec AUS 5.2
Adec AUS 8.1
Adec AUS 8.2
Adec AUS 9.1
Adec AUS 9.2
Adea NZ 8.1
Adec SA 4.2
Adec SA 6.1
Adec SA 6.2
Adec SA 8.1
Adec SA 8.2
Adec 3.4 SA*
Abai AUS 3.1
Abai 4.1 NZ*
BDV5057 [AJ890311]
BDV5493 [AJ890312]
Adec NZ 1.1
Adec NZ 1.2
Adec NZ 9.1
Adec NZ 9.2
Adec NZ 6.1
Adec NZ 6.2
Amel 2.3 AUS*
Amel 2.3 NZ*
Adec 4.4 NZ*
Adec SA 4.1
Adec 5.4 SA*
Abai NZ 7.1
Abai AUS 3.2
Amel 1.2 AUS*
Adea AUS 8.1
Amel NZ 2.1
Amel NZ 5.2
BDV5173 [AJ891168]
Abai AUS 5.2
Amel NZ 2.2
Amel AUS 1.2
Amel AUS 4.1
Amel AUS 4.2
Amel AUS 7.1
Amel AUS 7.2
Amel 3.4 AUS*
ICMP14752 [DQ323130]
Abai SA 9.1
Amel AUS 1.1
BDV5325 [AJ890290]
Adea SA 4.1
Adea SA 4.2
Adea SA 5.1
Adea SA 5.2
Adea SA 8.1
Adea SA 8.2
Adea 5.3 SA*
Amel NZ 4.1
Amel NZ 4.2
Amel NZ 5.1
Adec 4.4 AUS*
Amel 5.1 SA*
Adea AUS 1.1
Adea AUS 7.2
Adea AUS 8.2
Adea 4.1 AUS*
Amel SA 1.1
Amel SA 1.2
Amel SA 5.2
Amel SA 9.1
Amel SA 9.2
Amel 6.5 SA*
Abai NZ 5.2
Abai AUS 1.1
Abai AUS 5.1
Abai SA 3.2
Abai SA 9.2
Amel 7.2 SA*
Abai NZ 4.1
Abai NZ 4.2
Abai NZ 5.1
Aba iNZ 7.2
Abai 6.2 NZ*
Adea NZ 5.1
Adea NZ 5.2
Adea NZ 7.1
Adea NZ 7.2
Adea NZ 8.2
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ARR699 [HE576535]
ARR312 [HE576513]
WSM1735 [AJ890293]
MM5621 [KF802685]
Leo166 [LN650247]
Leo114 [LN650242]
Leo132 [LN650245]
Leo79 [LN650240]
Arg68 [LN650236]
WSM2783 [JN685314]
WSM2632 [JN685312]
Pear128 [LN650252]
ARR487 [HE576520]
R.binae BLR228 [JN648992]
0.02

FIGURE 1 Phylogenetic tree based on the *nodA* gene region for *Bradyrhizobium* strains isolated during this study as well as reference strains from GenBank. Outgroups (*Rhizobium* strains) are shown as collapsed clades. The inserted table shows the respective host species from which strains were isolated (*Acacia baileyana*, *Acacia dealbata*, *Acacia decurrens*, *Acacia melanoxylon* and other). Fill colour represents the country from where rhizobia were isolated, that is rhizobial origin (red = Australia; yellow = New Zealand; blue = South Africa; light grey = other). Bolded strain identities followed by an "*" represent rhizobial strains originally isolated and used in inocula in this study. Bold branches indicate strains previously isolated from *Acacia* species and downloaded from GenBank. Nodal support is given as posterior probabilities

both strains we identified based on DNA similarity. Finally, isolated rhizobial strains did not fall into distinct host species-specific clades for either gene tree, nor did they fall into distinct country-specific clades.

3.2 | Plant growth performance

With the exception of carbon construction costs, rhizobial inoculation increased plant performance of all other early growth measures for most acacia by inoculum treatments. As expected, all inoculated plants and no uninoculated plants had root nodules.

3.3 | Overall symbiotic effectiveness

Several lines of evidence suggest that symbioses with all bacterial strains were effective: (1) all inoculated plants produced nodules and (2) inoculation in all but two cases (*A. dealbata* inoculated with South African and New Zealand strains) resulted in significantly taller plants compared to uninoculated controls (*A. baileyana* – $F_{(3,12)} = 14.71$; $p < 0.001$; *A. decurrens* – $F_{(3,19)} = 17.95$; $p < 0.001$; *A. melanoxylon* – $F_{(3,26)} = 5.572$; $p = 0.04$). Insights into overall symbiotic effectiveness gained from other performance measures were more varied between the three inoculum treatments and between the four species (Figures 3 and 4, Appendix S4).

3.4 | Relative symbiotic effectiveness

For relative symbiotic effectiveness (i.e. differences in plant growth performances between inoculum and nutrient treatments), several findings supported equally effective symbioses across all three (AUS, NZ and SA) inoculum treatments. There were no significant differences between the three inoculum treatments without nutrient addition for any performance measurement, with the exception of shoot length (i.e. *A. baileyana* in association with New Zealand rhizobia and *A. dealbata* in association with South African rhizobia had shorter shoots than the other inoculum treatments) and total dry biomass (i.e. *A. dealbata* in association with South African rhizobia and *A. melanoxylon* in association with New Zealand rhizobia had lower total biomasses than the other inoculum treatments). Also, nutrient addition did not appear to have any influence on the growth of the four species, except in the case of $\delta^{15}N$ values which were lower for treatments without nutrients, which relied only on BNF for nitrogen supply, particularly for *A. melanoxylon* (see Appendix S5).

4 | DISCUSSION

Our results show that establishment of rhizobial interactions outside the native range of Australian *Acacia* species has overwhelmingly involved cointroduction of their symbionts. Both housekeeping and symbiotic gene phylogenies suggest close evolutionary relatedness between non-native (South African and New Zealand) and Australian rhizobium strains, a pattern expected for cointroduction. In line with these findings, we found rhizobia isolated from all these regions to be, overall, equally effective in terms of BNF efficiency and host plant growth performance.

Our finding that acacias exclusively associate with bradyrhizobia is not surprising as this is now well known from various studies and regions around the world (e.g. Keet, Ellis, Hui, & Le Roux, 2017; Le Roux et al., 2016; Le Roux et al., 2018; Ndlovu et al., 2013; Rodríguez-Echeverría, 2010; Rodríguez-Echeverría et al., 2011; Weir et al., 2004). However, it is surprising that we found no evidence of country-specific bradyrhizobia in association with invasive acacias, at least in Australia and South Africa, based on our *nodA* analyses. Considering that we collected multiple nodules from each acacia species in each country, the fact that these largely housed a single *nodA* strain is surprising. It is difficult to ascertain whether the predominance of a single strain across these regions reflects a single cointroduction event of one or a few *Bradyrhizobium* strains (e.g. from soil or wild seed collections), or whether multiple introductions of rhizobia that are widespread in Australia and harbour identical *nodA* genes occurred. The latter is certainly possible given that we found all acacias to share rhizobial strains of a single *nodA* identity (i.e. <2% DNA sequence divergence) in Australia (Figure S1).

Phylogenetic incongruence between *recA* and *nodA* phylogenetic clades found here also provides strong evidence for the occurrence of HGT (Lemaire, Van Cauwenberghe, et al., 2015). For example, the single *recA Bradyrhizobium* strain isolated from *A. baileyana* in South Africa harboured two distinct *nodA* strain identities. Bradyrhizobial strains associated with *A. decurrens* in South Africa showed similar incongruency. Our data indicate that these likely represent historical HGT that took place prior to cointroduction. However, due to the low resolution of the *recA* cladogram and the lack of a well-supported South African clade, we cannot rule out the possibility of post-introduction HGT. Such HGT between non-native Australian and native resident bradyrhizobia has previously been reported in Portugal (Rodríguez-Echeverría, 2010). Horizontal gene transfer may translate into more severe acacia impacts if, for example, it leads to reduced chemoattraction

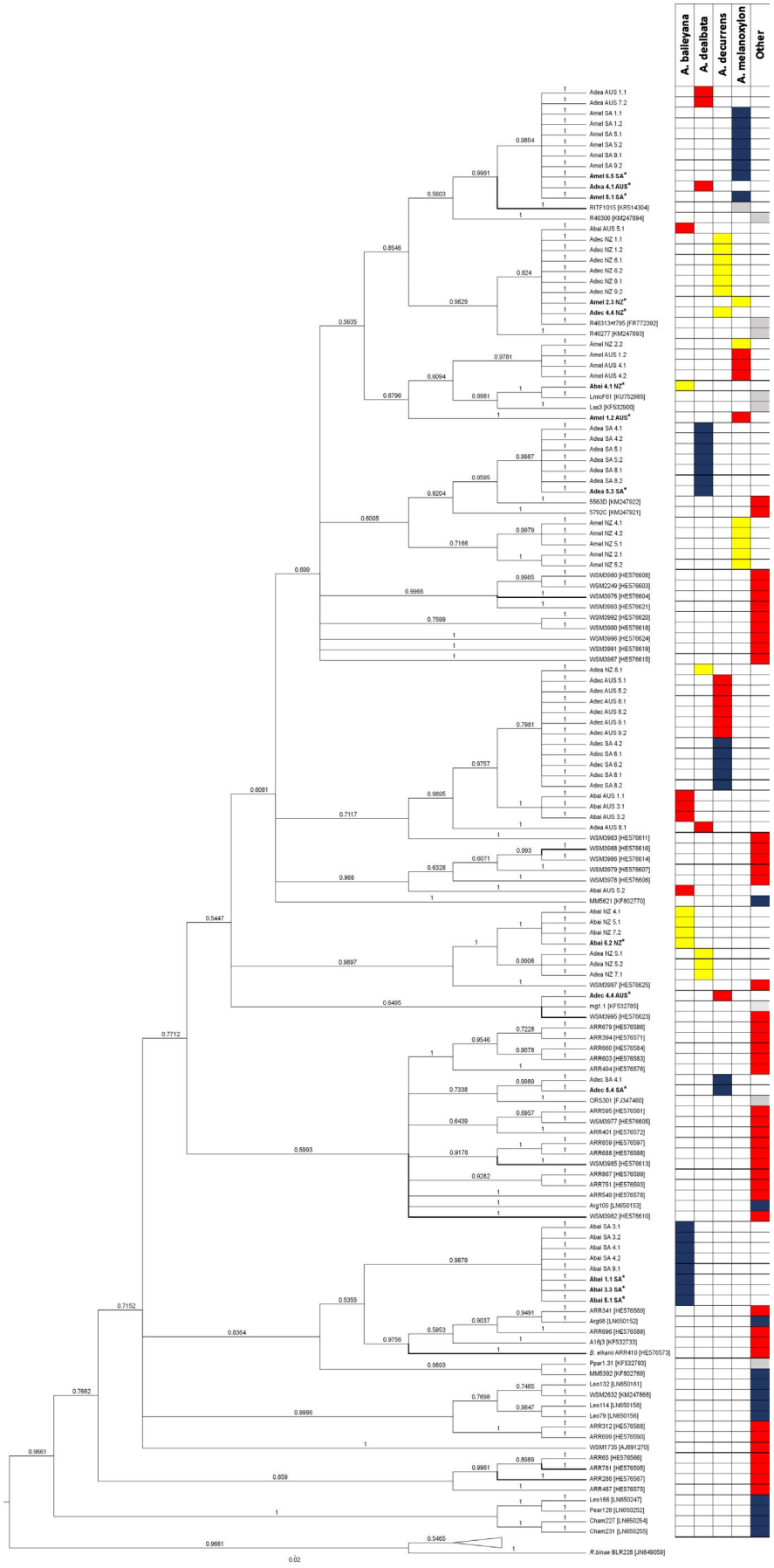

FIGURE 2 Phylogenetic tree based on the *recA* gene region for *Bradyrhizobium* strains isolated during this study as well as reference strains from GenBank. Outgroups (*Rhizobium* strains) are shown as collapsed clades. The inserted table shows the respective host species from which strains were isolated (*Acacia baileyana*, *Acacia dealbata*, *Acacia decurrens*, *Acacia melanoxylon* and other). Fill colour represents the country from which rhizobia were isolated, that is rhizobial origin (red = Australia; yellow = New Zealand; blue = South Africa; light grey = other). Bolded strain identities followed by an "*" represent rhizobial strains originally isolated and used in inocula in this study. Bold branches indicate strains previously isolated from *Acacia* species and downloaded from GenBank. Nodal support is given as posterior probabilities

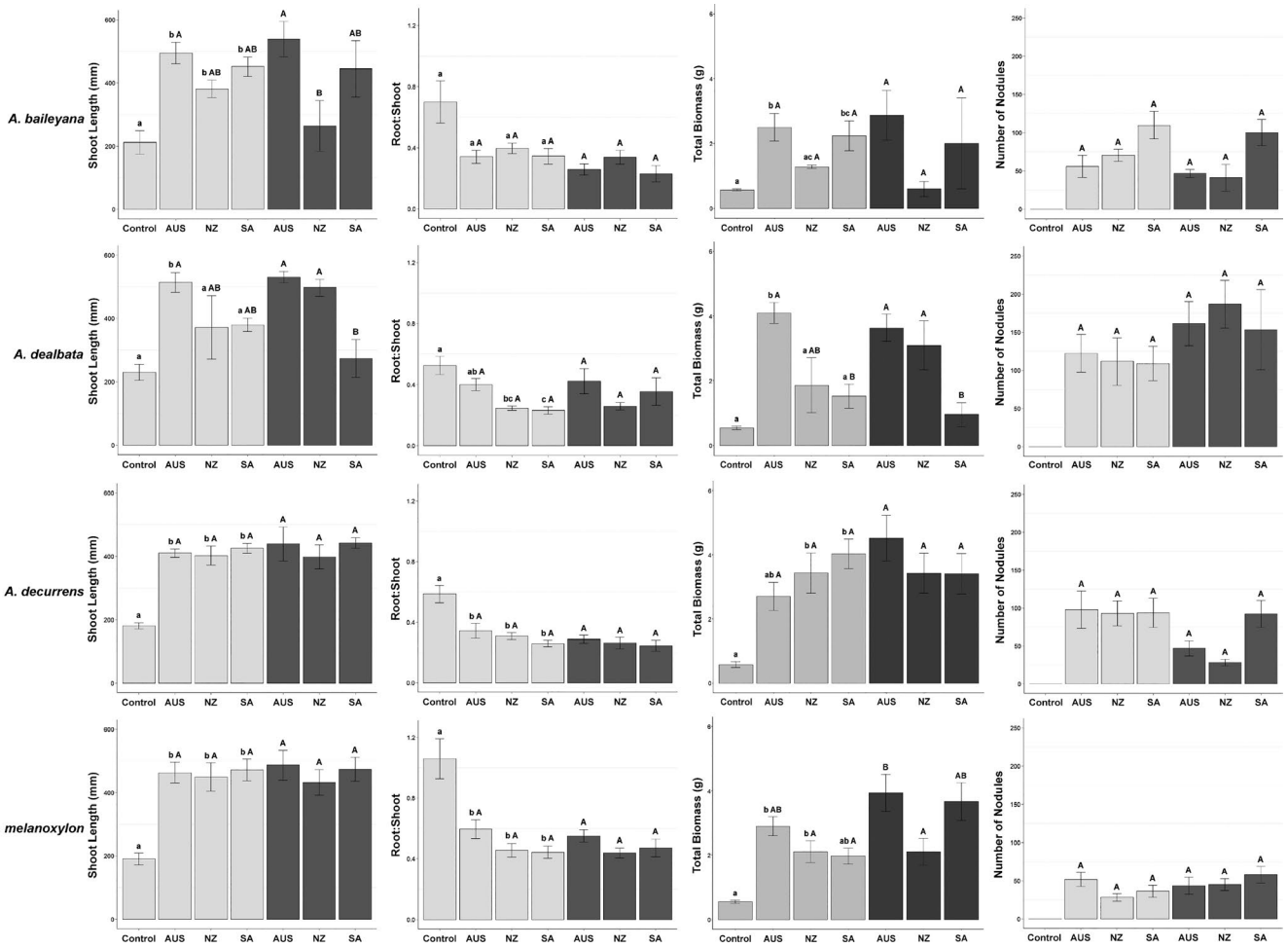

FIGURE 3 Differences in plant performance metrics between the different rhizobial and nutrient treatments for the four *Acacia* species included in this study. The various rhizobial treatments are indicated on the *x*-axis, and differences in nitrogen addition are indicated by the fill colour (i.e. light grey = addition of nitrogen; dark grey = no addition of nitrogen). Two sets of results are displayed in the figure: results of the one-way ANOVA between the control and the +Nitrogen treatments that received inoculum (i.e. overall symbiotic effectiveness) as indicated by lowercase lettering, and results for the factorial ANOVA between the three rhizobial treatments (AUS: Australia, NZ: New Zealand, SA: South Africa, that is relative symbiotic effectiveness) and the two nutrient treatments (+Nitrogen and −Nitrogen) as indicated by uppercase lettering. Bars denote *SE*s

of rhizobia by native legumes and thus lowered symbiotic effectiveness (Le Roux et al., 2017).

While some have argued that *Bradyrhizobium* has a cosmopolitan distribution, it is now accepted that the genus does show strong biogeographic structuring based on phylogenetic data from nodulation genes, with a well-supported Australian clade (Moulin, Béna, Boivin-Masson, & Stępkowski, 2004; Stępkowski et al., 2005). Based on our Blast results for the *nodA* gene region, the vast majority of acacia-isolated bradyrhizobia (87% of total) showed the highest similarities to, and clustered with, *Bradyrhizobium* strains previously isolated from legumes in New South Wales in Australia (the historical native range of the Australian acacias studied here). On the other hand, only 47% of strains showed highest similarity to Australian bradyrhizobia based on identity alone (i.e. *recA* DNA sequences). Cointroductions of acacias and their rhizobia are further supported by the fact that bradyrhizobia are probably rare in natural soils in South Africa and absent in New Zealand (Lemaire, Dlodlo, et al., 2015; Weir et al., 2004). The precise mechanism(s) underlying acacia–rhizobium cointroductions identified here remain unknown. Many Australian acacias have been imported to both countries for ornamental, forestry and agroforestry purposes (Richardson et al., 2011), and rhizobia may, therefore, have been accidently introduced along with imported seeds/seedlings, or purposefully introduced to promote the growth of the seedlings (Marques, Pagano, & Scotti, 2001). Early introductions of forestry species often involved the introduction of saplings (Poynton, 2009), and therefore, there is a high chance of cointroduction of their associated mutualistic microbes. However, the precise mechanisms governing rhizobial cointroduction with legume imports deserve further attention.

Our assessments of overall symbiotic effectiveness related to shoot length, total dry biomass accumulation and root:shoot ratio show that successful acacia–rhizobium associations almost always benefit plant performance. Relative symbiotic performances, that is plant performance comparisons among seedlings

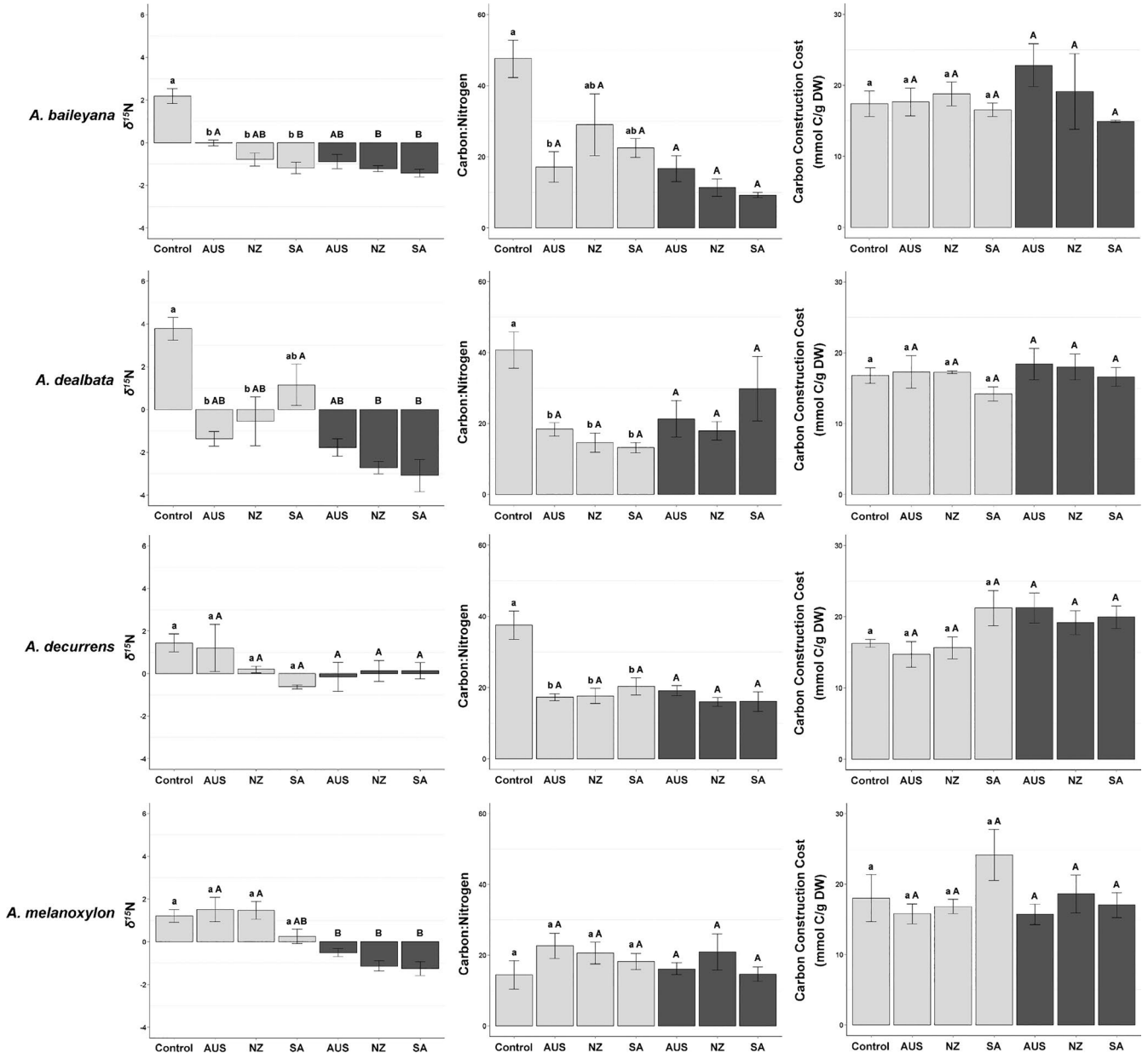

FIGURE 4 Differences in biological nitrogen fixation efficiency between the different rhizobial and nutrient treatments for the four *Acacia* species included in this study. The various rhizobial treatments are indicated on the *x*-axis, and differences in nitrogen addition are indicated by the fill colour (i.e. light grey = addition of nitrogen; dark grey = no addition of nitrogen). Two sets of results are displayed in the figure: results of the one-way ANOVA between the control and the +Nitrogen treatments that received inoculum as indicated by lowercase lettering, and results for the factorial ANOVA between the three rhizobial treatments (AUS: Australia, NZ: New Zealand, SA: South Africa) and the two nutrient treatments (+Nitrogen and −Nitrogen) as indicated by uppercase lettering. Bars denote *SE*

inoculated with rhizobia isolated from non-native and native range acacia populations, largely corroborated our inferences of cointroduction as being the principal pathway underlying acacia–rhizobium associations in both South Africa and New Zealand, and the expectation that cointroduction should be more beneficial to plant performance than ecological fitting. That is, for the eight non-native acacia species by country-specific rhizobia comparisons, most early growth and symbiotic efficiency measures were similar to those of plants inoculated with rhizobia from their native Australian range. It is also important to mention that we did not verify Koch's postulates for all glasshouse inoculum treatments (see Section 3.1), and so cannot unambiguously eliminate the possibility of cross-contamination in the glasshouse. However, the complete lack of nodulation in all uninoculated control treatments makes cross-contamination, or the accidental introduction of novel strains not used in inocula preparations, unlikely. It could be argued that the addition of nutrient solution may have inhibited nodulation by contaminant rhizobia in uninoculated controls, preventing us from detecting such cross-contamination. However, all plants that received both additional nitrogen and inoculum formed

nodules. Taken together, this suggests that successful nodulation in our glasshouse experiment was solely due to the inocula we applied. Surprisingly, we did not find nitrogen addition to have a significant impact on the growth performance of acacias. In most instances, inoculated plants, whether grown with or without additional nitrogen, had similar performance and almost always higher compared to uninoculated control plants. The latter received the same amount of nitrogen than inoculated plants. It is therefore conceivable that the amount of nitrogen we added was simply too low to overcome the reliance of acacias on rhizobia for their nitrogen needs.

Evidence from elsewhere suggests that cointroductions of acacias and their rhizobia enhance plant performance (e.g. Rodríguez-Echeverría, 2010; Rodríguez-Echeverría et al., 2012). Others have found the immediate availability of compatible rhizobia to impact the performance of some non-native acacias (Klock, Barrett, Thrall, & Harms, 2016; Wandrag, Sheppard, Duncan, & Hulme, 2013), while other acacias are capable of forming novel associations (Ndlovu et al., 2013). The lack of host species-specific clades in our phylogenies suggests that acacias are also capable of sharing the same rhizobial strains. For example, *A. dealbata* in Australia and *A. melanoxylon* from South Africa appear to utilize the same rhizobia. Similarly, *A. melanoxylon* and *A. baileyana* appear to share bradyrhizobia of Australian origin in South Africa. The most parsimonious explanation is that cointroduced rhizobia of one *Acacia* species can be utilized by others. These observations also support the notion that, while acacias mainly associate with bradyrhizobia, they show some level of interaction promiscuity to strains within this genus (Birnbaum et al., 2016; 20111). Introduced acacias are known to share rhizobial strains in places like South Africa (Keet et al., 2017) and in their non-native distributions in Australia (Birbaum et al., 2016). These findings imply that a single cointroduction can facilitate symbiont associations and invasion of other acacias, at least for the four species we investigated.

The prevalence of cointroduction in this study, as well as evidence from previous studies (Crisóstomo et al., 2013; Ndlovu et al., 2013; Rodríguez-Echeverría, 2010), may have important implications for invasion success and the rate and accrual of invasion impacts by acacias (Le Roux et al., 2017). That is, multiple and potentially strong positive feedbacks may result between cointroduced partners. These may include mutualist efficiency, whereby resident mutualists are out-competed by cointroduced mutualists, or positive feedbacks between non-native plants (e.g. leaf litter input, also see Dickie et al., 2017; Keller & Lau, 2018) and cointroduced mutualists, leading to higher non-native plant performance and competitiveness. For example, Le Roux et al. (2018) recently found acacia invasions in South Africa to affect both the diversity and structure of soil rhizobial communities by lowering rhizobial diversity and homogenizing rhizobial communities in invaded compared to uninvaded soils. They also found that overall acacia-induced soil changes further benefitted the performance of acacias. These changes may facilitate other acacias whereby host-switching between cointroduced rhizobia and acacias may allow those acacias that are introduced without their Australian bradyrhizobia to overcome the perceived negative effects linked to forming novel associations, potentially resulting in a form of invasion meltdown (Le Roux et al., 2017).

The evidence for plant–mutualist cointroductions in two geographically distinct regions identified here may indicate that cointroduction is more commonplace than previously thought. This may be particularly true for soil microbial mutualists. Our data suggest that other plant–microbial interactions (both beneficial and antagonistic) may show similar patterns of co-invasion and open the door to much needed and exciting future research opportunities.

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BIOSKETCH

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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