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Importance of soil legacy effects and successful mutualistic interactions during Australian acacia invasions in nutrient-poor environments

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Abstract

1. Non-native plants often alter environments they invade, favouring their own performance through positive feedbacks. Plant–soil interactions represent one such mechanism, but their complexity (e.g. invader-induced changes in soil nutrients, microbial communities, etc.) makes inferences of the precise mechanisms that benefit invaders difficult. Here we aimed to determine: (1) whether invasion by Australian acacias (genus *Acacia* Mill.) changes nitrogen-fixing soil rhizobial community diversity and structure, and (2) the importance of available rhizobia and overall invader-induced soil changes as significant facilitators of acacia performance.
2. We sampled soils from various invaded and nearby uninvaded areas in South Africa's Core Cape Subregion and, using next generation sequencing, compared rhizobial communities between invaded and univaded soils. We then determined the relative importance of soil status (invaded vs. uninvaded), in conjunction with rhizobial addition, to the performance of invasive acacias under common garden conditions.
3. Next generation sequencing data revealed that invaded soils generally harboured lower rhizobial diversity and were compositionally more homogenous compared to uninvaded soils. *Bradyrhizobium* strains, the most common known rhizobia associated with acacias, were more abundant in invaded than uninvaded sites. Our greenhouse experiment found significantly reduced growth performances of acacias in uninvaded relative to invaded soils for most species by site comparisons, and almost no influence of additional rhizobial inoculum. However, the overall relationship between nodulation and growth kinetics was much steeper for plants grown in uninvaded compared to invaded soils.
4. Despite invasive acacias homogenizing nitrogen-fixing rhizobial community composition and reducing diversity, it appears that mutualist availability poses no significant barrier to acacia establishment. Although acacia-induced changes to soil conditions enhance plant performance, successful nodulation seems important to early growth performance when encountering novel soil conditions.

5. *Synthesis*. We provide evidence that invasions by Australian acacias affect the diversity and structure of soil rhizobial communities. Although overall soil changes benefit their performance independent of rhizobia addition, forming successful mutualistic interactions is critical during the establishment phase under novel environmental conditions. Taken together, our results indicate that interactions between soil abiotic and biotic conditions work in concert to enhance invader performance through positive feedbacks.

KEYWORDS
Australian acacias, invasive legumes, legume–rhizobium interactions, plant–soil feedbacks, rhizobia, soil microbial community

1 | INTRODUCTION

What makes some plants successful at invading new habitats and others not, remains a central discussion in invasion ecology (Funk, Standish, Stock, & Valladares, 2016; Pyšek et al., 2012; Rejmánek & Richardson, 1996). The high context-dependency of alien species introductions (taxonomic and environmental) makes general inferences underlying their invasion success difficult. However, most successful plant invasions must cross certain functional and structural ecosystem thresholds, with these often leading to positive feedbacks (Bever et al., 2012; Brooks et al., 2004; Klironomos, 2002). For example, invasive cheatgrass (*Bromus tectorum*) in the Great Basin in the western United States is now a dominant structural component of degraded rangelands, resulting in increased fire fuel loads. As a consequence, more frequent and intense fires benefit its dominance through competitive release from fire-intolerant native species (Chambers, Roundy, Blank, Meyer, & Whittaker, 2007). Functionally, cheatgrass has also led to drastic increases in plant-available soil nitrogen, further widening its competitive advantage over resident native species (Morris, Stark, Bugbee, & Norton, 2016). Despite examples like this, the link between crossed thresholds and how they influence plant invasiveness remains largely unexplored (but see Gaertner et al., 2014).

Invasive legumes are known to dramatically alter soil nutrient cycling in their new ranges (Le Maitre et al., 2011; Yelenik, Stock, & Richardson, 2004), which can partly be explained by their ability to form symbioses with nitrogen-fixing bacteria known as rhizobia (Richardson, Allsopp, D'Antonio, Milton, & RejmÁNek, 2000; Rodríguez-Echeverría, 2010). In this symbiosis, legumes provide carbon resources and a protective environment to rhizobia, often within specialized root structures known as nodules. In exchange rhizobia fix atmospheric nitrogen into organic forms that the legumes can utilize (Franche, Lindström, & Elmerich, 2009). Often, this endosymbiosis frequently involves some degree of specialization (Porter, Stanton, & Rice, 2011), whereby complex signalling pathways between bacteria and plants initiate the formation of root nodules (Le Roux, Hui, Keet, & Ellis, 2017). Finding "compatible" rhizobia represents a significant threshold that needs to be crossed for the successful establishment and persistence of introduced legumes under new environmental conditions (Wandrag, Sheppard, Duncan, & Hulme, 2013). When not co-introduced with their co-evolved rhizobia, promiscuous (generalist) legumes may still be able to nodulate efficiently in their new ranges. On the other hand, specialized legumes might be constrained due to an overall or partial absence of abundant, high quality and compatible rhizobia (Le Roux et al., 2017). Irrespective of whether legumes are co-introduced with their rhizobia or form novel associations once introduced, symbiotic efficiency would still be dependent on abiotic conditions, such as soil nutrients and pH (Graham, 1992). Such abiotic conditions may represent an additional (functional) threshold that introduced legumes need to overcome.

Changes to abiotic soil conditions created by invasive legumes are also expected to lead to changes in the abundance and diversity of soil microbial communities (e.g. Kamutando et al., 2017). That is, as legumes increase in density in their new ranges, they functionally change soil chemistry and nutrient loads, indirectly leading to altered soil microbial communities, including mutualistic microbes (Le Roux et al., 2017; Stanton-Geddes & Anderson, 2011). Thus, functional thresholds posed by soil conditions may be crossed through positive feedbacks whereby soil alterations by legumes favour their own rhizobia and consequently their performance and densities.

Invasive Australian acacias (genus *Acacia* Mill.) provide a good system to investigate how non-native species cross numerous thresholds and how these relate to invader-induced positive feedbacks. Acacias are considered some of the most damaging invasive alien species to South Africa's Core Cape Subregion (CCR) (Le Maitre et al., 2011; Witkowski, 1991). A global biodiversity hotspot, the CCR is a Mediterranean-type fire-prone shrubland, characterized by nutrient-poor soils and exceptionally high levels of plant diversity and endemism (Goldblatt & Manning, 2000). Invasive acacias are known to impact on soil organic matter and nutrient levels (Stock, Wienand, & Baker, 1995; Yelenik et al., 2004) as well as on soil microbial communities, including rhizobial composition, diversity and abundance (Kamutando et al., 2017; Le Roux, Mavengere, & Ellis, 2016). However, the complex interactions between invasive legume densities, abiotic soil conditions, the availability of compatible rhizobia in the new environment, etc., make disentangling the relative importance of each driver to invader performance difficult.

Here we not only aim to assess the impact that invasive Australian acacias have on soil rhizobial communities in the CCR, but also to tease apart the relative roles of soil conditions (heavily invaded vs. uninvaded) and abundance (and possibly presence) of preferred rhizobial symbionts on their own performance. First, we used next generation sequencing data to determine how different acacia species impact soil rhizobial communities by comparing the diversity, structure and composition of these mutualist communities between acacia-invaded and uninvaded soils. Second, to investigate how invader-induced changes in soil conditions and the availability of compatible rhizobial mutualists impact on the performance of acacia species, we grew acacias under common garden conditions in invaded and uninvaded soils, with or without the addition of their preferred rhizobial strains. We hypothesized that invasive acacias will increase the abundance of their preferred nitrogen-fixing rhizobia in their surrounding soils under field conditions, either directly through increased population growth of favoured rhizobia in nodules, or indirectly through acacia-mediated soil changes that favour their preferred rhizobia. Under this hypothesis we expect (1) increased dominance of acacia-associated rhizobia in invaded compared to neighbouring uninvaded soil communities, (2) reduced rhizobial community diversity in invaded soil vs. uninvaded soils and (3) homogenization of rhizobial community composition in invaded vs. uninvaded soils. We further expect that acacia performance should be enhanced in invaded over uninvaded soils because of these changes in rhizobial communities. Alternatively acacia performance could largely be determined by altered soils under invasion, independent of rhizobial abundance. If this is the case, we expect that additional inoculation with preferred rhizobia to not affect acacia performance across soil types.

2 | MATERIALS AND METHODS

2.1 | Sample collection, DNA extraction, and Illumina MiSeq sequencing

To compare the structure and composition of soil rhizobial communities between areas invaded by acacias to neighbouring uninvaded areas, we collected soils from three nearby sites (*c.* 16 km apart) during June 2015 (Table S1). Sites had different combinations of dominant invasive acacias present. Vergelegen was invaded by *Acacia longifolia* (Andrews) Willd., *A. mearnsii* De Wild., and *A. saligna* (Labill.) H.L.Wendl.; Bilton by *A. cyclops* A.Cunn. ex Don, *A. longifolia* and *A. saligna*; and Rustenberg by *A. pycnantha* Benth., *A. mearnsii* and *A. melanoxylon* R.Br.. At each site we collected three soil samples 30 m apart directly underneath dense canopy of acacias and in adjacent uninvaded areas along transects (total $n = 18$).

For next generation sequencing analyses we extracted total genomic DNA from 0.25 g of soil from each sample using the PowerSoil® DNA extraction kit (MO BIO laboratories Inc., Carlsbad, CA, USA), following the manufacturer's protocol. DNA quality was checked using a NanoDrop ND-1,000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The use of next generation sequencing approaches for studying rhizobial diversity in soils has the advantage of identifying "hidden" soil diversity, since conventional methods of bacterial culturing may not detect all of these free-living soil bacteria (Birnbaum, Bissett, Thrall, & Leishman, 2016). Here, the nodulation gene, *nod*C, was amplified using the primers nodCF12F (5′-CCG GAT AGG MTG GKB CCR TA-3′) and nodCRI2R (5′-GTG CAC AAS GCR TAD RCC TTC AH-3′), with sample-specific barcodes in the forward primer. Amplification was done using a 30-cycle polymerase chain reaction (PCR) and the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) under the following PCR conditions: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min followed by a final elongation at 72°C for 5 min. After amplification, PCR products were checked on a 2% agarose gel to determine amplification success and the relative intensity of bands. Multiple PCR samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, Beverly, MA, USA) and used to prepare DNA libraries, following the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at the Molecular Research LP next generation sequencing service (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) following the manufacturer's guidelines.

2.2 | Bioinformatics

All raw MiSeq DNA sequence data were processed following standard procedures as described in Schloss, Gevers, and Westcott (2011), using mothur version 1.37.1 (Schloss et al., 2009). Briefly, after removal of low quality and ambiguous sequences, and optimizing sequence lengths (between 313 and 500 basepairs), all chimeric sequences were removed independent of a reference database using the uchime algorithm (Edgar, Haas, Clemente, Quince, & Knight, 2011) and the template as self, i.e. de novo removal. We computed pairwise sequence similarities using the Needleman–Wunsch algorithm for alignment (Subbiah & Harrison, 1989) and clustered the sequences into rhizobial operational taxonomic units (OTUs) at 97% similarity using the nearest neighbour algorithm. To determine the taxonomic affinity of OTUs we blasted representative sequences against the NCBI's Genbank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

2.3 | Soil rhizobial community diversity

From the OTU matrix we calculated species richness (S; total number of OTUs giving equal weight to both abundant and rare OTUs), the exponent of Shannon diversity (H; diversity measure that takes into account the abundance differences between dominant and rare OTUs), Inverse Simpson diversity (Si; diversity measure that weights the abundance of dominant OTUs more than rare ones) and evenness (J; which measures how equally the abundances of OTUs are spread in the sample). We calculated evenness as H/log(S) (Hill, 1973). All diversity metrics were calculated with the function renyi in the VEGAN R package (version 2.3–3; Oksanen et al., 2016). In order

to investigate the influence of soil status (invaded vs. uninvaded) on the various diversity metrics, we performed a mixed model two-way ANOVA with site locality (i.e. geographical origin) as random factor and soil status as fixed factor.

2.4 | Soil rhizobial community composition

To visualize soil bacterial community composition we performed non-metric multidimensional scaling (NMDS) based on a Bray–Curtis dissimilarity matrix for the OTU table, calculated with the functions vegdist and metaMDS in the VEGAN R package (Oksanen et al., 2016). Permutation Multivariate Analysis of Variance (PERMANOVA; Anderson, 2001) with 9,999 permutations was then used to test for significant compositional differences using the function adonis, with soil status (invaded vs. uninvaded) as fixed factor and site locality (geographical origin) as random factor, using the argument "strata". We also tested whether rhizobial communities in invaded soils were more homogenized than in uninvaded soils for each site separately. For this we used the betadisper function in the VEGAN R package (Oksanen et al., 2016) and 9,999 permutations to test for differences in multivariate homogeneity of group variances between soil types.

Finally, in order to visualize OTU abundances, we created a heat map with the following abundance categories: 1–500, 501–1,000, 1,001–5,000, >5,000. Since the 1,375 obtained OTUs made visualization impossible, we only used the first 197 OTUs, which accounted for 80% of all sequence reads. The heat map was constructed using the gplots R package and the function heatmap.2 (Warnes et al., 2015). All statistical analyses were performed in the R programming language (version 3.4.0; R Development Core Team, 2016) with functions from the base package. NMDS and diversity plots were drawn with the ggplot2 R package (Wickham, 2009).

2.5 | Greenhouse experiment

We set up a common garden experiment using soils collected from five sites spanning different soil types throughout the CCR (Table S1). These sites were chosen based on the presence and high density of invasive acacias (*Acacia longifolia*, *A. mearnsii and A. saligna*) being adjacent (<4.5 km) to uninvaded areas. At each site, bulk soils were collected during November 2013, separately from underneath the canopies of monotypic stands of acacias (invaded soils) and from neighbouring areas with no acacias present (uninvaded soils). Different soils were then stored in 110 L sealable plastic containers and transported back to the laboratory where they were kept at room temperature until further use.

From each selected site and *Acacia* species combination, six pots (18-cm diameter and 15-cm deep) were filled with invaded soil and six pots with uninvaded soil. *Acacia* seeds were obtained from the Agricultural Research Council's Plant Protection Research Institute (ARC-PPRI), Stellenbosch, South Africa. Seeds were surface-sterilized in 6% sodium hypochlorite solution for 5 min and mechanically scarified using dissection scissors. Subsequently, some species' seeds were directly planted in soils, while others were first germinated in polystyrene trays containing sterile filter sand for 2 weeks before transplanting. All plants for each species by site combination were planted out using only one of these two approaches. All germination and subsequent growth was conducted at ambient temperatures in a growth tunnel at Stellenbosch University, South Africa (33°55′52.3″S 18°51′47.9″E).

All plants were mist watered to mimic well-watered conditions throughout the experiment. For each species/site and soil type combination three pots were supplemented with species-specific rhizobial inoculum and three pots were left uninoculated. For inocula preparation, root nodules were collected from at least five individuals of all acacias and kept on silica gel (Table S2). Root nodules were rehydrated overnight in 1 mL sterile deionized water, surface-sterilized and washed (see Le Roux et al., 2016 for details). Washed root nodules were crushed in sterile water and plated onto yeast mannitol agar (YMA) supplemented with Congo red. Bacterial growth was allowed at 28°C until rhizobial colonies were observed (5–7 days) and re-streaked until pure colonies were obtained. Colony purity was confirmed by a standard Gram-staining. Identification of the selected rhizobial strains was done through colony PCR and sequencing of the 16S rRNA gene region. DNA was extracted by heating single bacterial colonies (picked up using a sterile toothpick) to 96°C in 50 μl distilled water for 10 min. Each 50 μl PCR reaction contained 5 μl 10 X Buffer, 1 μl dNTPs (10 mM), 1 μl each of forward 16S-PB36 and reverse 16S-1509R primers (Weir, 2006; both 10 mM), 5 μl of $MgCl_2$ (25 mM), 0.6 μl Super-Therm Taq polymerase (Supplied by Whitehead Scientific, Cape Town, South Africa), 30.4 μl distilled water and 6 μl DNA. PCR conditions were: initial denaturation at 95°C for 5 min; followed by 20 cycles of denaturation at 95°C for 45 s, annealing at 65°C for 45 s, elongation at 72°C for 90 s; followed by 15 cycles of denaturing at 95°C for 45 s, annealing at 60°C for 45 s, elongation at 72°C for 90 s; and a final elongation at 72°C for 7 min. PCR products were purified with the Qiagen PCR purification kit (supplied by Whitehead Scientific, Cape Town, South Africa) and sequenced in both directions using the same primers used for amplification. Aligned and edited sequences were blasted against reference data from GENBANK to identify the rhizobial species (http://www.ncbi.nlm.nih.gov/genbank).

For rhizobial inocula we seeded yeast mannitol (YM) broth with a "cocktail" of all uniquely identified rhizobial strains isolated from each acacia separately, i.e. each *Acacia* species received its own inoculum treatment containing only strains isolated from that particular species (Table S2). Inocula were shake-incubated at 28°C for 7 days until turbid. Eleven weeks after planting, all inoculum treatment pots received 5 mL of species-specific inoculum, while uninoculated pots received 5 mL sterile YM broth. These treatments were repeated 7 weeks later. Following each inoculation treatment, pots were divided into four blocks corresponding to different treatments. Separate tables were used in the growth tunnel for invaded and uninvaded soil treatments, while inoculated and uninoculated pots were separated from each other on the same table (*c.* 30 cm) to reduce chances of rhizobial cross-contamination. For randomization, pots were weekly rotated in a conveyer belt fashion. Non-acacia seedlings were also manually weeded from all pots regularly.

After 26 weeks of growth, entire plants were extracted from pots. Excess soil was removed from roots by rinsing. For each plant we counted the number of nodules as a proxy for symbiotic success and then separated below and above-ground biomass. Plant material was dried at 40°C for 7 days and weighed. Growth rate (GR) was determined as the accumulation of dried above-ground biomass over the growth period, and root:shoot ratios (R:S) were calculated from the dried above and below-ground biomass. We used generalized linear models with a Poisson error distribution to evaluate the effects of soil status (invaded and uninvaded), inoculum treatment and their interaction on the number of nodules found on each acacia species at each collection site separately. Models were checked to comply with the assumption of equidispersion (Cameron & Trivedi, 1990). To determine the response of growth kinetics (GR, R:S) to the treatments (soil status and inoculum) or their interaction, we log-transformed all response variables and performed ANOVA for each site by species combination separately. R:S was not analysed for *A. mearnsii* in Vergelegen since the roots of one of the treatment combinations were lost during harvesting, leaving us with inadequate replication. Lastly, we explored how the number of nodules generally affected early growth (GR and R:S) of acacias in each soil type using linear models. All data were analysed in the R programming language (R Development Core Team, 2016).

3 | RESULTS

3.1 | Impact of *Acacia* invasions on soil rhizobial communities

After removal of singleton and doubleton OTUs our matrix consisted of 99,661 sequences comprising 1,375 OTUs. Mixed-model ANOVAs, accounting for variability between sites, indicated that some diversity metrics (H and Si) of rhizobial communities were significantly lower in invaded soils compared to uninvaded soils (S: $F = 1.08$, $p = .32$; H: $F = 5.96$, $p < .05$, Si: $F = 4.91$, $p < .05$, J: $F = 2.12$, $p = .168$, Table S3; Figure 1).

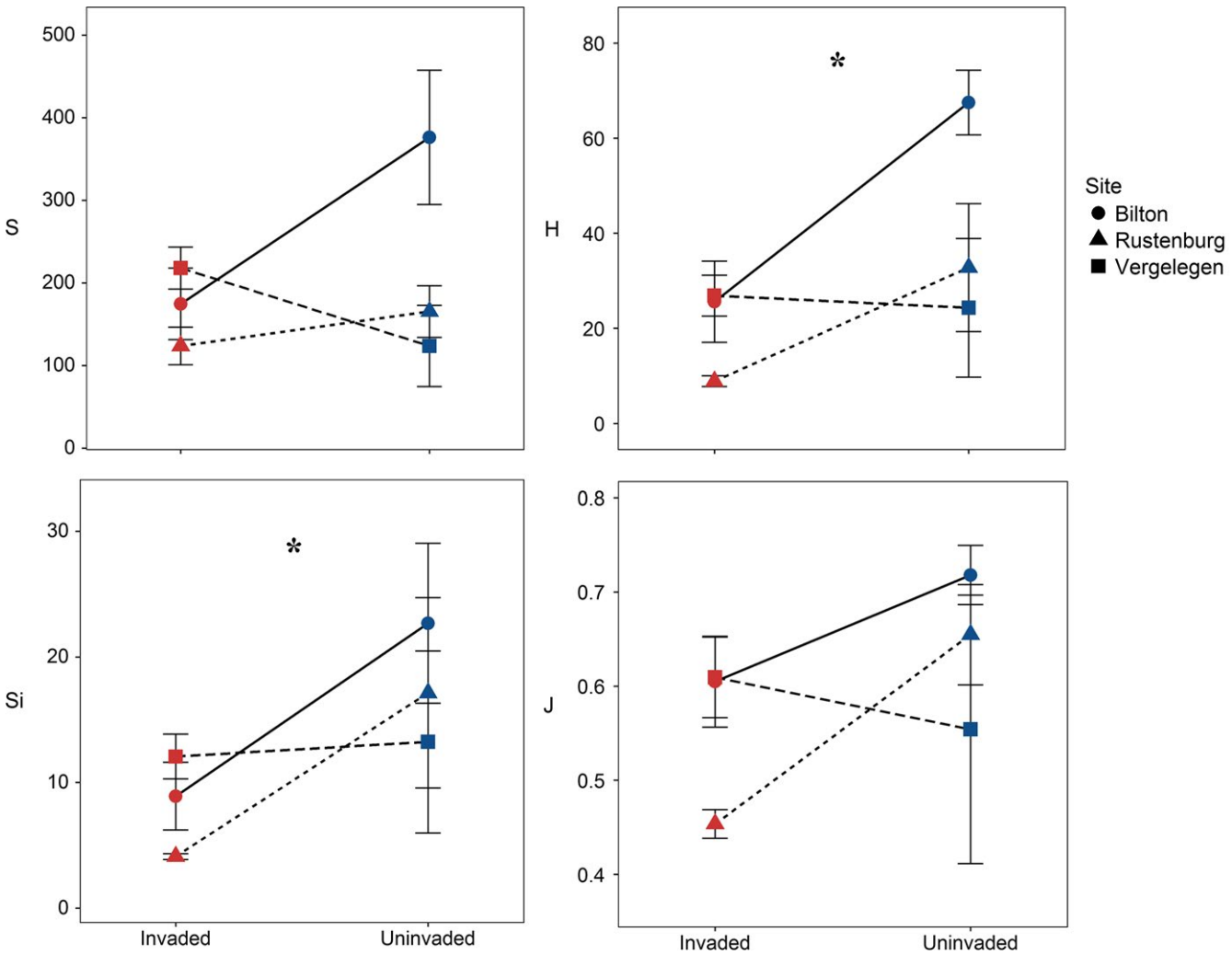

FIGURE 1 Soil rhizobial community diversity metrics (S, richness; H, Shannon diversity; Si, inverse Simpson diversity; J, evenness) for neighbouring acacia-invaded and -uninvaded soils (lines) at different sites (symbols). (*) Indicates significant differences in diversity metrics between invaded and uninvaded soils from ANOVAs accounting for variability between sites. Error bars indicate ± *SE* of the mean [Colour figure can be viewed at wileyonlinelibrary.com]

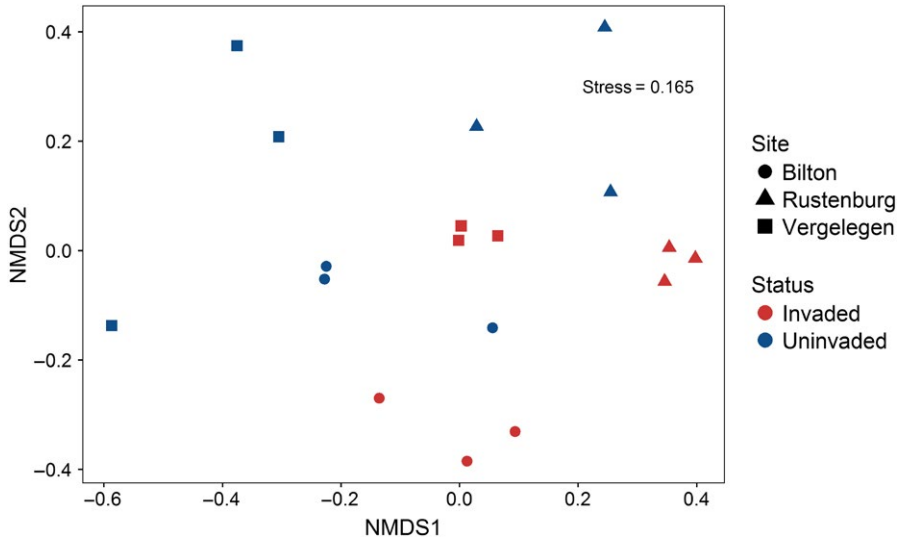

FIGURE 2 Non-metric multidimensional scaling plot (NMDS) showing differences in the composition of the *nodC*-derived rhizobial communities among sites and according to soil status (invaded vs. uninvaded) [Colour figure can be viewed at wileyonlinelibrary.com]

The NMDS analysis of bacterial community structure had a moderate stress coefficient (0.165), indicating that the plot is an acceptable representation (Clarke, 1993). The PERMANOVA model, accounting for variability between sites, indicated that invasion status significantly altered the composition of soil rhizobial communities ($F = 1.61$, $R^2 = .091$, $p < .001$, also see heatmap in Figure S1). In addition, the NMDS plot showed denser clustering of samples from invaded sites relative to uninvaded sites, indicating that the invasive acacias have an overall homogenizing effect (Figure 2). This was confirmed by significant differences in multivariate homogeneity of group variances between invaded and uninvaded soils ($p < .05$) for Rustenburg and Vergelegen, with average distances to group centroids being lower in invaded soils than uninvaded soils.

A total of 267 OTUs were unique to invaded sites (7,698 seqs, *c.* 7.7% of total seqs) with almost double that number (442 OTUs) being unique to uninvaded sites (13,494 seqs; *c.* 13.5% of total seqs). Given the short DNA fragment reads obtained here, we could only reliably classify 28.5% of all sequences (28,413 sequences out of 99,661) using Blast. After filtering out genera not known to have nodulation genes we found that, despite *Bradyrhizobium* strains (the primary associates of acacias) being highly represented in both invaded and uninvaded soils, their abundances (number of sequencing reads) were generally lower in uninvaded soils (Figure S2). These differences were significant for Rustenburg and Vergelegen (*t* test: $t = 4.41$, $df = 4$, $p = .011$ and $t = 15.26$, $df = 4$, $p < .001$, respectively) but not Bilton (*t* test: $t = 1.66$, $df = 4$, $p = .17$). On the other hand, the relative abundances (%) of bradyrhizobia were not significantly different between all pairwise sampled invaded and uninvaded soils (*t* test on arcsine transformed percentages; Bilton: $t = 0.94$, $df = 4$, $p = .402$; Rustenburg: $t = 2.45$, $df = 4$, $p = .07$; Vergelegen: $t = -0.33$, $df = 4$, $p = .76$, Figure S3).

3.2 | Effect of soil legacy and inoculum on *Acacia* species' performance

Overall, the majority of rhizobia isolated from acacia root nodules represented strains of *Bradyrhizobium* (Table S2). We found a significant effect of soil status on at least one of the fitness correlates for all acacia species by site combinations, except for *Acacia longifolia* at Vergelegen (Table S4–S6). Specifically, acacias grown in invaded soils, irrespective of rhizobial inoculum, had significantly higher nodule numbers in five out of the seven comparisons (Figure 3). In four cases, inoculation with preferred rhizobia had a significantly positive effect on the number of nodules formed. Similarly, GRs were significantly higher for acacias grown in invaded soils in six instances, with rhizobium treatment having a significant impact only for *A. longifolia* at Bilton. Irrespective of inoculum treatment, R:S were significantly higher in uninvaded soils for three site x species combinations. A significant interaction between soil status and inoculum treatment was found for R:S at three sites (*A. saligna* from Bilton, Hopefield and Silwerstroom).

When we explored the overall effect of nodulation on acacia early growth kinetics, we found a significant effect of the number of nodules formed, soil type and their interaction on GR and R:S. Specifically, nodulation had a significantly positive effect on growth rate in both types of soils, but with a notably steeper slope in uninvaded soils (linear model: adjust. $R^2 = .54$, $p < .0001$; Figure 4). Number of nodules also significantly affected the root:shoot ratio for both types of soils. That is, as number of nodules increased the root:shoot ratio decreased, again at a faster rate (slope) in uninvaded soils (linear model: adjust. $R^2 = .31$, $p < .0001$; Figure 4). One outlier, *A. saligna* grown in uninvaded soils from Hopefield, was excluded from these analyses since trait measures were above 1.5 times the interquartile range.

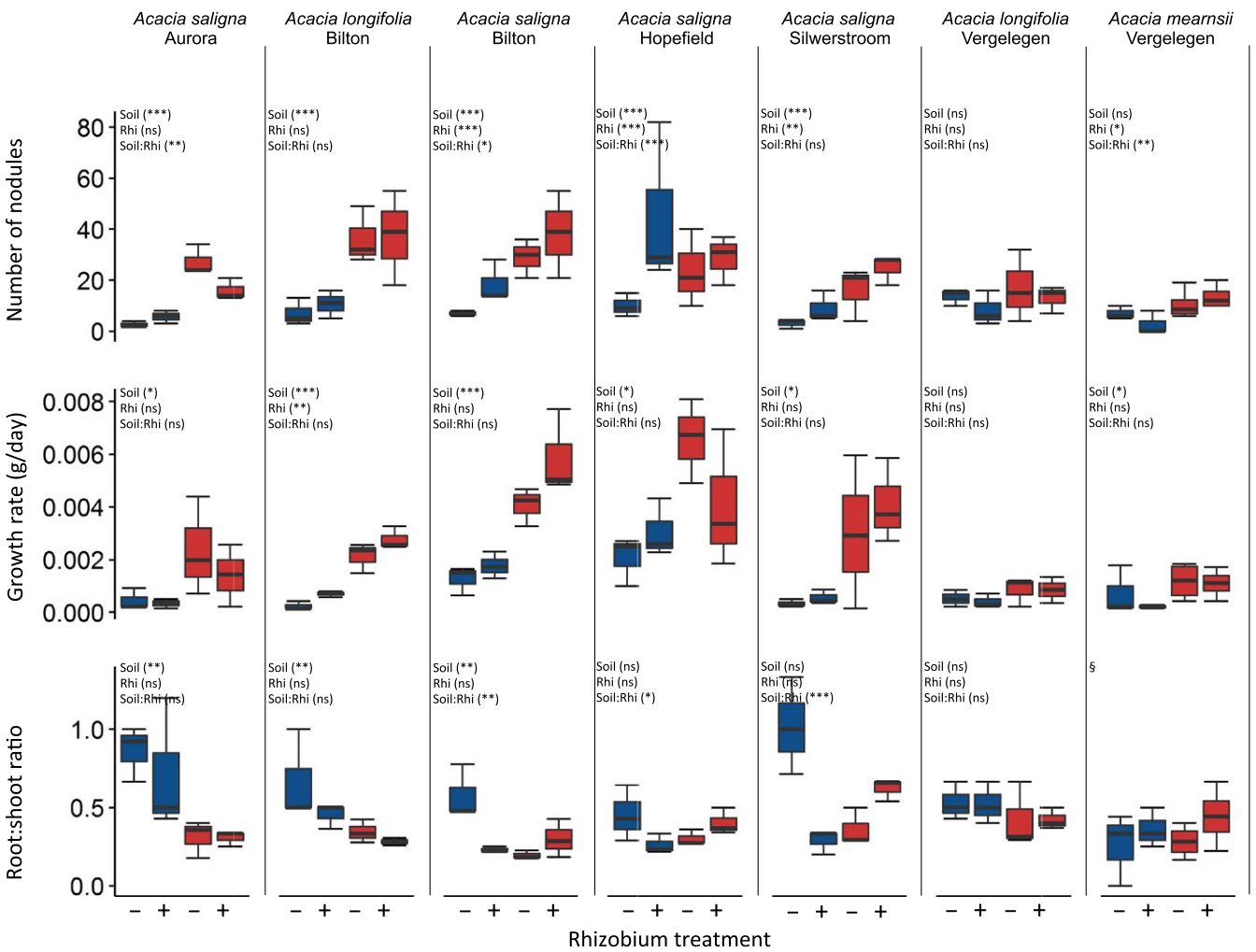

FIGURE 3 Boxplots illustrating the effects of soil conditions (invaded and uninvaded) and rhizobial inoculum treatment on the nodulation and growth kinetics of various *Acacia* species. Plots filled with red indicate treatments where plants were grown in invaded soils and those filled with blue plants grown in uninvaded soils. Rhizobial inoculum treatments are indicated by plus (+) and uninoculated treatments by minus (–) signs. Significance codes are indicated as *($p \leq .05$), **($p \leq .01$), ***($p \leq .001$). §not statistically analysed due to low replication [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Our data provide some support for our hypothesis that invasive Australian acacias reduce whole soil rhizobial community diversity and alter community composition. Changes in rhizobial composition and diversity following acacia invasions may be related to host plant-driven changes in the soil conditions. Such alterations can make conditions unsuitable for strains of native bacteria adapted to the low nutrient CCR environment to persist (Le Roux et al., 2017). At the same time acacia host plant densities may select for, and amplify, their preferred rhizobia. Similar to previous reports (Birnbaum et al., 2016; Keet, Ellis, Hui, & Roux, 2017; Le Roux et al., 2016; Ndlovu, Richardson, Wilson, & Le Roux, 2013; Rodríguez-Echeverría et al., 2011), rhizobia that we isolated from invaded soils and field-collected acacia root nodules, were predominantly from the genus *Bradyrhizobium*. Despite not being commonly associated with native CCR legumes (Lemaire et al., 2015), we identified bradyrhizobia in all soils, but generally in much higher abundances in invaded compared to uninvaded soils. Soil enrichment of bradyrhizobia by invasive acacias has been repeatedly shown from various parts of the world (Birnbaum et al., 2016; Kamutando et al., 2017; Slabbert, Jacobs, & Jacobs, 2014). The presence of relatively abundant bradyrhizobia in uninvaded CCR soils suggests that the availability of compatible rhizobia may not represent a significant threshold to acacias prior to becoming invasive. Enrichment of bradyrhizobia in invaded soils might reflect direct effects of acacias on bacterial population sizes. However, it is also conceivable that soil changes in response to acacia invasions, through indirect mechanisms like leaf litter deposition and decomposition, favour the performance and efficiency of existing and compatible rhizobia that are better able to cope with the novel abiotic conditions created by acacias (Le Roux et al., 2017).

Soil rhizobial communities in uninvaded areas were compositionally more dissimilar than those from acacia-invaded soils,

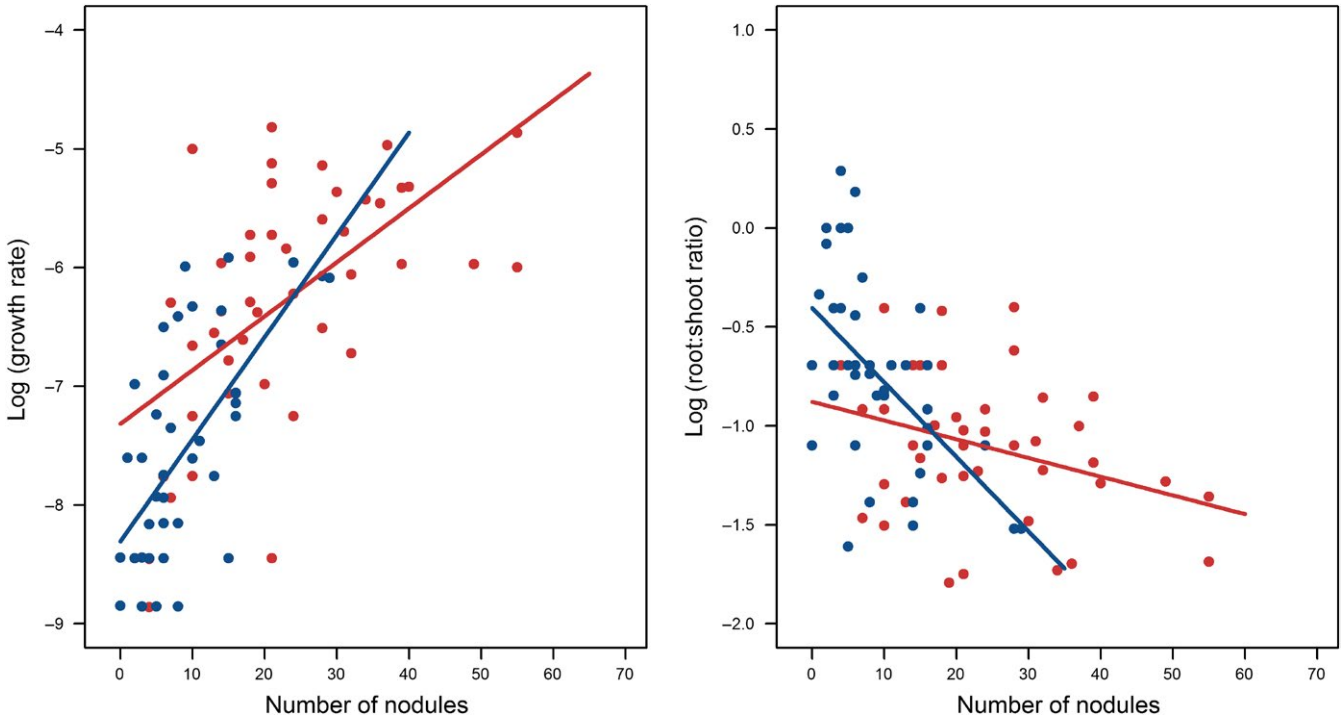

FIGURE 4 The effects of nodulation on the growth kinetics of various *Acacia* species grown in invaded and uninvaded soils, irrespective of rhizobial inoculum treatment (linear model: growth rate, adjust. R^2 = .54, p < .0001; root:shoot ratio, adjust. R^2 = .31, p < .0001). Red symbols and lines indicate invaded soils and those in blue uninvaded soils [Colour figure can be viewed at wileyonlinelibrary.com]

both within and across sites. These results indicate that acacia invasions have a "homogenizing" effect on rhizobial communities. Again, extreme dominance by one or two acacia species within invaded sites could drive this pattern through strong host selection for compatible rhizobia, exacerbated by lower native legume host diversity and abundance. In line with this, Keet et al. (2017) recently found 19 different invasive acacia species from a wide geographical sample in South Africa to share a few, highly abundant, bradyrhizobial strains. Such strong selection for compatible rhizobia by acacias may also explain Le Roux et al.'s (2016) observations that rhizobia associated with native CCR legumes show strong compositional turnover between acacia-invaded and -uninvaded sites.

In the CCR, the structure of plant communities and the underlying soil microbial community are closely related (Slabbert, Kongor, Esler, & Jacobs, 2010), where native CCR legumes have been reported to nodulate mainly with strains from the genera *Mesorhizobium* and *Burkholderia* (Lemaire et al., 2015). Consequently, native plant displacement by acacia invasions will have an impact not only on the above-ground plant community but also on their associated soil microbial communities (Keet et al., 2017). We found an increased representation of *Bradyrhizobium* at two of the invaded sites, and a shift in the second most abundant genus (*Mesorhizobium*) at the third site (Vergelegen). Additional to plant–plant competition, *Bradyrhizobium* strains in association with invasive acacias can have direct competitive effects over other native rhizobia, also inhibiting their nodulation ability (e.g. Barrett, Bever, Bissett, & Thrall, 2015). It is therefore expected that the disruption of native mutualistic interactions by acacia invasions will have a significant effect on the performance of native legumes, particularly those with highly specialized symbiont requirements (Le Roux et al., 2016, 2017). Here we provide evidence that changes in rhizobial community structure following acacia invasion may result from strong host selection on available rhizobia in synergy with altered soil conditions. The contributions of factors like interspecific rhizobial competition or native plant displacement by the invasive legumes in explaining changes in these communities deserves further attention.

We found a strong overall soil legacy effect, whereby heavily invaded soils enhanced nodulation and early growth responses for most acacias, irrespective of rhizobial addition. Poor performance of invasive acacias grown in previously uninvaded soils in New Zealand were thought to reflect the low availability of compatible rhizobia (Bakhoum et al., 2012; Wandrag et al., 2013). However, we found lower nodulation and more biomass investment in roots when acacias were grown in uninvaded soils, irrespective of rhizobial addition. The high prevalence of bradyrhizobia in uninvaded soils therefore suggests a lack of abiotic conditions condusive for optimal nodulation. That is, for successful nodulation, acacia-induced changes to soil conditions may be more important, or work in concert with rhizobial availability, than the immediate availability of rhizobia alone.

While the effects of rhizobial inoculation on acacia performance in invaded and uninvaded soils were less clear, the overall relationships between numbers of root nodules formed and early growth kinetics found here indicate a stronger influence of nodulation on

growth performances for acacias grown in uninvaded soils compared to invaded soils (Figure 4). Therefore, rhizobial availability, irrespective of soil abiotic conditions, may still be critical during the initial establishment phase of newly introduced legumes, especially under nutrient-poor soil conditions.

5 | CONCLUSIONS

Our study shows that invasion by Australian acacias impacts on soil nitrogen-fixing microbial community diversity, enriching strains commonly associated with, and preferred by, them. The community composition of rhizobia also differed between invaded and uninvaded areas, with evidence for acacias having a "homogenizing" effect within and between invaded sites. The relative high abundance of bradyrhizobia in uninvaded CCR soils suggests that acacia-induced changes to soil conditions represent a more critical functional threshold than immediate mutualist availability alone to plant performance. That is, as acacia densities increase, they change soil abiotic conditions in ways that may positively feedback into their associations with rhizobia and their performance. However, despite nodulating less under uninvaded soil conditions, a stronger relationship between nodulation and early growth kinetics under these conditions compared to invaded soils illustrates the importance of mutualist availability during the establishment phase of acacias. Our results confirm that soil conditions may represent a key functional threshold that needs to be overcome during acacia invasions and demonstrate how positive feedbacks may emerge after crossing such thresholds.

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AUTHORS' CONTRIBUTIONS

The ideas and methodology were devised by J.J.L.R. and A.G.E.; L.-M.v.Z., N.D.H. and J.-H.K. performed the experiments and collected data; F.A.Y., J.J.L.R. and J.-H.K. analysed the data; J.J.L.R. and F.A.Y. led the writing of the manuscript. All authors significantly contributed to previous drafts of this manuscript and gave final approval for publication.

DATA ACCESSIBILITY

The datasets generated during this study are available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.b47g160 (Le Roux, 2018).

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