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RESEARCH ARTICLE

Arbuscular mycorrhizal communities respond to nutrient enrichment and plant invasion in phosphorus-limited eucalypt woodlands

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Abstract

1. Arbuscular mycorrhizal fungi (AMF) facilitate ecosystem functioning through provision of plant hosts with phosphorus (P), especially where soil P is limiting. Changes in soil nutrient regimes are expected to impact AMF, but the direction of the impact may depend on context. We predicted that nitrogen (N)-only enrichment promotes plant invasions and exacerbates their P limitation, increasing the utility of AMF and promoting AMF diversity. We expected that enrichment with N, P and other nutrients similarly promotes plant invasions, but decreases the benefit and diversity of AMF because P is readily available for both native and exotic plants.
2. We tested these hypotheses in eucalypt woodlands of south-western Australia, that occur on soils naturally low in P. We evaluated AMF communities within three modified ground-layer states representing different types of nutrient enrichment and associated plant invasions. We compared these modified states to near-natural reference woodlands.
3. AMF richness varied across ground-layer states. The moderately invaded/N-enriched state showed the highest AMF richness, while the highly invaded/NP-enriched state showed the lowest AMF richness. The reference state and the weakly invaded/enriched state were intermediate. AMF richness and colonisation were higher in roots of exotic than native plant species.
4. AMF community composition differed among ground-layer states, with the highly invaded/NP-enriched state being most distinct. Distinctions among states were often driven by family-level patterns. Reference and moderately invaded/N-enriched states each supported distinct groups of zero-radius operational

taxonomic units (zOTUs) in Acaulosporaceae, Gigasporaceae and Glomeraceae, whereas Gigasporaceae and Glomeraceae were nearly absent from the highly invaded/NP-enriched state. Further, Diversisporaceae and Glomeraceae were most diverse in the moderately invaded/N-enriched state.

5. *Synthesis*. Both the nature of soil nutrient enrichment and plant provenance matter for AMF. N-only enrichment of low-P soils increased AMF richness, likely due to the introduction of AMF-dependent exotic plant species and exacerbation of their P limitation. In contrast, multi-nutrient enrichment, decreased AMF richness potentially due to a decrease in host dependence on AMF, regardless of host provenance. The changes in AMF community composition with nutrient enrichment and plant invasion warrant further research into predicting the functional implications of these changes.

KEYWORDS
arbuscular mycorrhizal fungi, ecological restoration, fertilisation, Glomeromycotina, Mediterranean-climate eucalypt woodlands, Mucoromycotina, nutrient enrichment, plant invasion

1 | INTRODUCTION

To help reverse the rapid decline in biodiversity and ecosystems worldwide, the Convention on Biological Diversity adopted the Kunming-Montreal Global Biodiversity Framework, aiming to 'ensure that by 2030 at least 30% of areas of degraded terrestrial, inland water, and marine and coastal ecosystems are under effective restoration' (Convention on Biological Diversity, 2022). To optimise restoration outcomes, we must first understand the mechanisms impeding recovery. Exotic plants and nutrient enrichment are often important limits to recovery (Corbin & D'Antonio, 2012), and these are often interrelated (Standish et al., 2006). Thus, restoration efforts that address plant–soil feedbacks between exotic plants and abiotic and biotic soil legacies may increase chances of success (Eviner & Hawkes, 2008).

Biotic soil legacies can include changes in the abundance and composition of beneficial soil microorganisms such as arbuscular mycorrhizal fungi (AMF). Arbuscular mycorrhizal fungi are plant mutualists that can increase plant phosphorus (P) and water uptake (Smith & Read, 2010), offer pathogen defence (Veresoglou & Rillig, 2012), improve soil physicochemical properties (Willis et al., 2013) and contribute to nutrient cycling (Powell & Rillig, 2018). They are a key component of ecosystem health, and the re-introduction of native AMF has been shown to promote vegetation recovery after degradation (Neuenkamp et al., 2019).

Nutrient enrichment is a form of degradation in native ecosystems, whereby soils become nutrient-enriched compared with their inherent levels. This can occur, for example, through fertilisation, global nitrogen (N) deposition or release of nutrients from disturbed vegetation, often leading to exotic plant invasions and loss of native diversity (Prober & Wiehl, 2012). These changes can be associated with the loss of key AMF or the arrival of 'weedy' AMF, both of which in turn may impede ecosystem recovery (Albornoz et al., 2023). We define 'weedy AMF' as those that arrive and persist in, or are promoted in degraded ecosystems, regardless of whether they are native or exotic.

It has been proposed that soil and plant N:P stoichiometry could determine the effect of nutrient enrichment on AMF (Johnson et al., 2013), based on Liebig's law of the minimum (von Liebig 1840). If native ecosystems are naturally P-limited or P and N co-limited, N enrichment is expected to exacerbate P limitation, potentially increasing the reliance of plants on their symbionts and promoting AMF. Alternatively, in ecosystems where P is readily available and N is limiting, N enrichment should alleviate plant nutrient co-limitation, potentially decreasing the plant's need to allocate carbon (C) to AMF (Johnson et al., 2013). Accordingly, in a global study of 25 grasslands, Leff et al. (2015) showed that N and P fertilisation decreased AMF relative abundance. Under high levels of N and P availability, plant diversity also decreases (Seabloom et al., 2021), potentially further limiting AMF due to low host diversity.

In extremely P-poor ecosystems, the proportion of non-AMF plant species tends to be higher due to the competitive advantage of other P-acquisition strategies that have evolved on these infertile soils (Lambers et al., 2011), potentially reducing host diversity and abundance for AMF. Hence, plant invasions, promoted by N enrichment, can increase host availability, further promoting AMF. In a recent study, for example, we showed that N enrichment indirectly increased the richness of AMF via increased exotic plant cover in P-limited eucalypt woodlands (Albornoz et al., 2023). These findings suggest that soil N enrichment (as a form of ecosystem degradation) and plant invasions could be synergistically linked to the promotion of weedy AMF. We expand on Johnson et al. (2013) to propose that an interaction between soil nutrient availability and changes in plant

provenance (i.e. native vs. exotic) could determine the trajectory of AMF associated with nutrient enrichment.

The hypothesised responses of AMF to nutrient enrichment proposed by Johnson et al. (2013) relate to C allocation and AMF abundance. Plant C allocation can mediate competitive interactions among AMF species, with repercussions for their diversity (Knegt et al., 2016; Liu et al., 2015). Hence, consistent with Albornoz et al. (2023), we propose that this hypothesised dependence on N versus P limitation could also apply to AMF species richness. Other nutrients such as potassium (K) could also influence AMF, as they can increase K uptake, potentially for osmotic adjustments as a counterion for P uptake (Garcia & Zimmermann, 2014). However, the effects of K or other micronutrients on AMF communities remain unresolved.

Here, we evaluate the effects of different types of nutrient enrichment and associated plant invasions on AMF in forb-rich York gum (*Eucalyptus loxophleba* subsp. *loxophleba*)—Jam (*Acacia acuminata*) woodlands, an extensive but threatened ecological community of the south-western Australian wheatbelt that is in widespread need of ecological restoration. Soil P is naturally extremely limited, and N is limited, in these woodlands (Prober & Wiehl, 2012). We used a state-and-transition approach to compare York gum woodland ground layers in a close-to-reference (near-natural) state with three modified states: (1) weakly invaded by exotic annuals and weakly enriched in N and P (hereafter 'weakly invaded/enriched'), (2) moderately invaded by exotic annuals and enriched in N (hereafter 'moderately invaded/N-enriched'), and (3) highly invaded by exotic annuals and enriched in P, N and other nutrients (hereafter 'highly invaded/NP-enriched'). We hypothesised:

H1. AMF richness is highest where N enrichment promotes exotic plant invaders and exacerbates P limitation. In contrast, AMF richness is lowest where N- and P enrichment reduces host reliance on AMF for nutrient acquisition.

H2. AMF richness and colonisation are higher in exotic than in native plant species due to exotics' reliance on AMF for nutrient acquisition.

H3. Changes in AMF communities reflect changes in soil nutrients and shift from native-dominated to exotic-dominated vegetation.

2 | MATERIALS AND METHODS

2.1 | Site selection

Sampling was centred on two nature reserves with areas of York gum (*Eucalyptus loxophleba* subsp. *loxophleba*)—Jam (*Acacia acuminata*) woodlands (hereafter York gum woodlands) in close to reference condition. The reserves are located in the central wheatbelt, Western Australia: Mount Caroline (31°45′25.3″ S, 117°38′38.3″ E) and Namelkatchem Nature Reserves (31°10′47.9″ S, 117°11′18.1″ E), and are ~70 km apart (Figure 1). These reserves have a history of minimal livestock grazing and plant invasion, permitting the persistence of large areas of diverse understoreys dominated by native perennial and annual forbs and grasses (Prober & Wiehl, 2012). The climate is Mediterranean type, with long-term (1990–2022) mean annual temperature and rainfall, respectively, of 17.8°C and 321 mm at Mt Caroline and 17.6°C and 333 mm at Namelkatchem (BoM, 2023).

Our experimental design involved four ground-layer states representing different levels of nutrient enrichment and plant invasions as described above: a reference (control) state in near-natural conditions with naturally low soil P and N, and three degraded states that we sought from different parts of the landscape: a weakly invaded/enriched state, a moderately invaded/N-enriched, and a highly invaded/NP-enriched state (Table 1; Figure 1). We chose the term 'ground-layer state' to be consistent with previous research (Albornoz et al., 2023; Prober et al., 2014). 'Ground-layer' is used rather than 'understorey' to distinguish the herbaceous ground-layer from a shrub layer that can occur in the woodland understorey.

Two areas within each of the two nature reserves with few-to-no exotic plant species were selected to sample the 'reference' state. Each reserve included localised patches invaded by exotic annuals, that likely arose historically due to localised disturbances (e.g. by introduced rabbits). These patches were used to represent the 'moderately invaded/N-enriched state', given that other studies have demonstrated that such exotic-invaded states of York gum woodlands are typically N-enriched (Prober & Wiehl, 2012).

Because P-enriched areas rarely occur inside the nature reserves, to represent the 'highly invaded/NP-enriched' state we sampled four fertilised 2 m × 2 m plots from a pre-existing long-term nutrient addition experiment located ~1.5 km from Mount Caroline Nature reserve (31°46′56.43″ S, 117°36′41.61″ E; Figure 1). The experiment was established in a grazed York gum woodland remnant in 2009, as part of the global Nutrient Network (NutNet) experiment (Borer et al., 2014). We also sampled four unfertilised plots from the same experiment, representing the 'weakly invaded/enriched' state that arose through historical sheep grazing, resulting in some plant invasion and N and P enrichment. To match the NutNet design, plots of 2 m × 2 m were established at both nature reserves for the reference and moderately invaded/N-enriched states. Plots were at least 20 m apart, with four replicate plots of each ground-layer state. Remnants of York gum woodlands are rare in the landscape; hence, we chose the two closest nature reserves near the NutNet experiment. This was done to incorporate as much of the natural variation within Reference sites as possible. We note that a previous study found little variation in AMF communities among remnants of York gum woodlands across 200 km distance (Prober et al., 2015).

The experimental plots from NutNet were arranged in a randomised complete block design with four blocks. Nutrients were added annually in autumn to plots representing the highly invaded/NP-enriched state, as 10 g N per $m^2 \cdot year^{-1}$ of timed-release urea, 10 g P per $m^2 \cdot year^{-1}$ as triple superphosphate and 10 g K per $m^2 \cdot year^{-1}$ as potassium sulphate. These plots also received a once-off addition

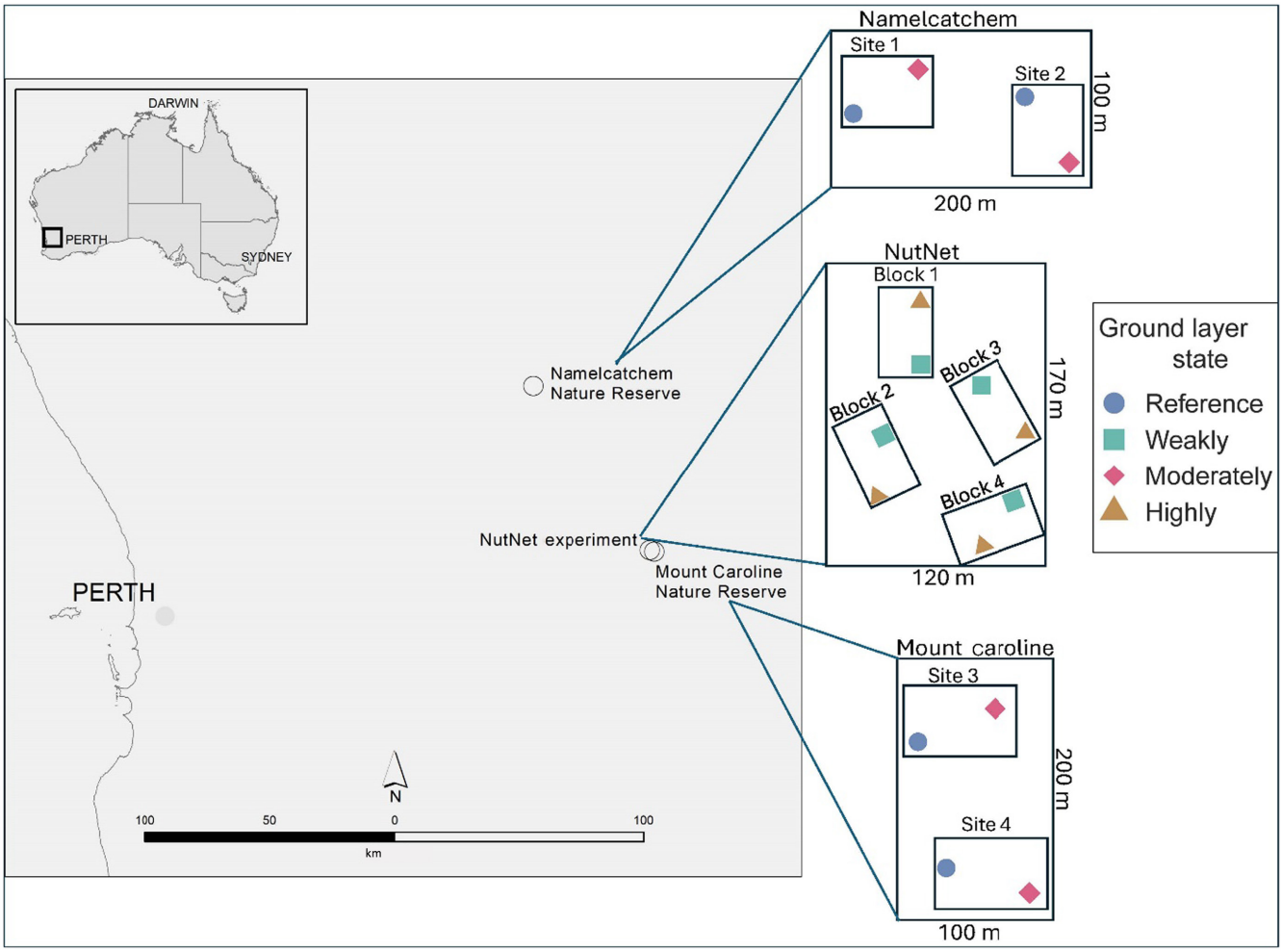

FIGURE 1 Location of the study sites and experimental design within each site. Namelcatchem and Mount Caroline Nature reserves were used for sampling reference and moderately enriched/invaded plots, and the Nutrient Network (NutNet) experiment was used for sampling weakly and highly enriched/invaded plots.

of other macro- and micronutrients in 2009: 100 g per m^{-2} of a mix containing iron (15%), sulphur (14%), magnesium (1.5%), manganese (2.5%), copper (1%), zinc (1%), boron (0.2%) and molybdenum (0.05%). Weakly invaded/enriched plots were dominated by native plant species and highly invaded/NP-enriched plots were dominated by exotic plant species. The experimental plots had been open to livestock grazing since European colonisation (1860s) until 2015.

2.2 | Sample collection and processing

Sampling occurred in August 2021 during the growing season. In each plot, plant community composition and cover were recorded. Then, rhizosphere soil and roots were collected from two plants of the six most abundant plant species. Samples from the two plants per species were pooled for a total of six samples per plot. Soils were thoroughly mixed in a sealable bag, collecting a subsample for DNA analyses. Roots were immediately stored in 98% ethanol pending processing. Soil and root samples were stored in 15-mL tubes and placed in dry ice during sampling and transport to the laboratory. Samples were stored at −80°C thereafter. Root samples were thoroughly cleaned with deionised water, and fine roots of <2 mm were retained. Clean root samples were split in two: one part for DNA analyses and one for root colonisation assessment. Two plots had only five plant species, resulting in 94 soil and 94 root samples (Table S1). No permit was needed to access and sample plots from the NutNet experiment. Permits to access and sample the two nature reserves were granted by the Department of Biodiversity, Conservation and Attractions of Western Australia (Licence: FT61000839; Regulation 4: CE006388).

2.3 | Soil chemical analyses

Soil samples were sent to CSBP Laboratories (Bibra Lake, Western Australia) for nutrient analyses. Plant-available P and K were measured using the Colwell test (Colwell, 1963; Rayment & Higginson, 1992). Organic carbon (OC) was determined according to Walkley and Black (1934). Ammonium-N, nitrate-N and total N were measured as per Searle (1984). Soil pH was measured in $CaCl_2$ in a solution ratio of 1:5 (Rayment & Lyons, 2012).

TABLE 1 Soil and floristic properties of each ground-layer state: Reference, weakly invaded/enriched, moderately invaded/N-enriched and highly invaded/NP-enriched states.

	Ground-layer state			
	Reference	Weakly	Moderately	Highly
Native plant cover (%)	38 ± 6^{b}	46 ± 4^{b}	32 ± 4^{b}	10 ± 5^{a}
Exotic plant cover (%)	3 ± 2^{a}	30 ± 4^{b}	52 ± 8^{c}	88 ± 6^{d}
Native plant richness	14 ± 1^{c}	11 ± 1^{c}	8 ± 1^{b}	2 ± 0^{a}
Exotic plant richness	2 ± 1^{a}	7 ± 0^{b}	5 ± 1^{b}	6 ± 1^{b}
Ammonium-N (mg/kg)	3.9 ± 0.2^{a}	5 ± 0.2^{b}	6.8 ± 0.4^{c}	5.6 ± 0.5^{bc}
Nitrate-N (mg/kg)	0.2 ± 0.1^{a}	0.2 ± 0.1^{a}	1.2 ± 0.3^{b}	0.3 ± 0.1^{a}
Total N (%)	0.06 ± 0.01^{a}	0.06 ± 0.01^{a}	0.11 ± 0.01^{b}	0.09 ± 0.01^{b}
Colwell P (mg/kg)	1.8 ± 0.2^{a}	4.2 ± 0.3^{b}	3.4 ± 0.3^{b}	196.1 ± 11.0^{c}
Colwell K (mg/kg)	121 ± 9^{a}	184 ± 4^{b}	114 ± 10^{a}	288 ± 16^{c}
Organi^{c} carbon (%)	0.94 ± 0.04^{a}	0.8 ± 0.04^{a}	1.68 ± 0.14^{b}	0.94 ± 0.04^{a}
Soil pH ($CaCl_2$)	4.53 ± 0.03^{a}	4.6 ± 0.02^{a}	4.84 ± 0.08^{b}	4.53 ± 0.04^{a}

Note: Values are mean ± standard error and different letters represent statistical differences among treatments ($p \leq 0.05$). For nutrients, $n = 24$; for vegetation variables, $n = 4$.

2.4 | Root colonisation

Root subsamples allocated for measurement of root colonisation were cleared in 1 M KOH and stained with ink in vinegar (5% v/v) as described by Vierheilig et al. (1998). Colonisation by AMF, including Glomeromycotina–AMF and Mucoromycotina–AMF, was scored using the line intercept method (McGonigle et al., 1990). One hundred intercept points were scored for each sample, and the percentage of root length colonised by AMF was calculated.

2.5 | DNA extraction and sequencing

Root samples allocated for DNA analyses were cut into 5 mm pieces, homogenised and ground with beads. DNA was extracted from 20 mg of root and 250 mg of soil material using the DNeasy Plant Pro kit and DNease PowerSoil Pro kit, respectively (Qiagen, Carlsbad, USA). PCR amplification and sequencing were performed by the Australian Genome Research Facility. For each sample, 15 ng DNA were used to amplify the 18S rRNA gene using the AMF primer set AMV4.5NF and AMDGR (Sato et al., 2005). These primers accurately retrieve a wide range of AMF taxa, including both Glomeromycotina and Mucoromycotina subphyla (Albornoz et al., 2022; Orchard et al., 2017). Thermocycling was completed with an Applied Biosystem 384 Veriti and using Platinum SuperFi II mastermix (Life Technologies, Australia) for the primary PCR. Thermocycling consisted of an initial denaturation at 98°C for 30 s followed by 30 cycles of 98°C for 10 s, 60°C for 10 s and 72°C for 30 s. The final extension was at 72°C for 5 min. The first stage PCR was cleaned using magnetic beads, and samples were visualised on 2% Sybr Egel (Thermo Fisher). A secondary PCR to index the amplicons was performed with the same conditions with Platinum SuperFi II mastermix (Life Technologies, Australia). The resulting amplicons were cleaned using magnetic beads, quantified by fluorometry (Promega Quantifluor) and normalised. The eqimolar pool was cleaned a final time using magnetic beads to concentrate the pool and then measured using a High-Sensitivity D1000 Tape on an Agilent 2200 TapeStation. The pool was diluted to 5 nM, and molarity was confirmed using a Qubit High-Sensitivity dsDNA assay (Thermo Fisher). This was followed by sequencing on an Illumina MiSeq (San Diego, CA, USA) with a V3, 500 cycle kit (2 × 250 base pairs paired-end).

2.6 | Bioinformatics

Following sequencing, adapters were trimmed using 'cutadapt' (Martin, 2011), retaining only sequences that contained primers. After trimming, further quality checks and sequence processing were done in VSEARCH v2.14.1 with default parameters (Rognes et al., 2016). Trimmed paired-end sequences were merged with a minimum of 10 bp overlap. Merged sequences were filtered using a maximum error rate of 0.1 and a minimum length of 200 bp. Filtered sequences were dereplicated at 100% identity and singletons were discarded. Unique sequences were clustered into zero-radius operational taxonomic units (zOTUs), chimeras were detected (*denovo*), and a zOTU abundance table was produced using the UNOISE3 algorithm in USEARCH v11 (Edgar, 2016). Finally, zOTUs were queried against the SILVA SSUref v138.1 database (Quast et al., 2013). Taxonomy was assigned to zOTUs with a threshold of >95% match and query cover of >90%. Sequences matching Glomeromycotina–AMF or Mucoromycotina–AMF were classified as AMF.

2.7 | Statistical analyses

Two samples failed to amplify DNA, resulting in 186 samples for statistical analyses. The initial denoised zOTU abundance table was rarefied to the smallest sequencing depth (4927 sequences) to avoid sequencing depth bias (Dickie, 2010; Figure S1).

All data were analysed, and figures were created, in R (R Core Team, 2016). To visualise variation in plant and AMF communities among ground-layer state, nonmetric multidimensional scaling with Bray–Curtis dissimilarity of the log-transformed matrix was used. To visualise species contributions to the ordination, two-way tables were constructed with the 'inkspot' function from the *rioja* package (Juggins & Juggins, 2020). Replicate plots were ordered on the *x*-axis by ground-layer states, while each individual zOTU was placed on the *y*-axis. Two-way tables summarise and present the raw community data as a powerful way of visualising the distribution of taxonomic units along environmental, spatial or temporal gradients.

To test for differences in community compositions among ground-layer states and source material (i.e. soil vs. roots), permutational multivariate analysis of variance were performed using 'adonis2' within the *vegan* package (Oksanen et al., 2017). To test which vegetation and soil variables correlated with plant and AMF communities among ground-layer states, vectors of maximum correlation were calculated with the vector-fitting procedure using 'envfit' (9999 permutations) within the *vegan* package (Oksanen et al., 2017). A biplot was drawn on the ordination to display the relationships between explanatory variables and ordination axes. Because floristic variables strongly covaried (Figure S2), principal component analyses (PCA) were used to distil covariates to PCA axes (Figure S3). An 'exoticPCA1' (i.e. the first axis of a PCA between exotic plant cover and richness) and a 'nativePCA1' (i.e. the first axis of a PCA between native plant cover and richness) indices were created to represent the difference in cover and richness for exotic and native plants, respectively.

Differences in plant cover, plant richness, rarefied zOTU AMF richness (hereafter 'AMF richness') and soil chemistry among ground-layer states were evaluated. These differences were analysed using linear mixed effect models using 'block' as random effect. To test how environmental attributes associated with transition among ground-layer states related to AMF richness and community composition, structural equation models (SEM) were built using the *lavaan* R package (Rosseel, 2012). To meet *lavaan*'s requirements, ground-layer state was transformed to an ordinal variable based on exotic plant abundance and levels of nutrient enrichment (reference = 0; weakly invaded/enriched = 1; moderately invaded/N-enriched = 2; highly invaded/NP-enriched = 3). To include a proxy of community composition in the SEMs, the first axis of a PCA of the log-transformed AMF community matrix was used. Due to high covariation among most soil variables (Figure S2), models were simplified to only include variables with a priori knowledge of being involved in state transitions in eucalypt woodlands. These variables were OC, nitrate-N, ammonium-N and available P, which are hypothesised to promote exotic annuals and AMF richness (Albornoz et al., 2023; Prober et al., 2014; Prober & Wiehl, 2012). Neither nitrate-N nor 'nativePCA1' were selected in the best model.

We acknowledge that feedbacks between soil chemistry and plants are likely. However, in our SEMs, we chose soil chemistry as the driver for exoticPCA1 for two reasons: first, there is a priori knowledge that N enrichment promotes plant invasions in these ecosystems (Prober et al., 2012). Second, the highly invaded/NP-enriched state was created by deliberate nutrient addition, meaning it was demonstrated experimentally that nutrient addition promoted the observed plant invasions, not the other way around.

Comparisons of AMF richness and communities between native and exotic plant species across treatments (i.e. Plant provenance × Ground-layer state) were not possible due to the absence of sufficient native plant species in the highly invaded/NP-enriched state, and exotic plant species from the reference state. Furthermore, for most samples, no root material was left after prioritising the subsample for DNA analyses, leaving 31 (32%) samples for scoring colonisation. Hence, AMF richness and root colonisation comparisons between native and exotic plants are presented using ground-layer state as a random effect.

3 | RESULTS

3.1 | Plant composition and soil chemistry

Floristic surveys and soil chemical analyses confirmed our expectations for mean levels of exotic plant invasion and nutrient enrichment in the sampled ground-layer states (Table 1). The reference state had an average of only 3% exotic plant cover and was lowest in all soil nutrients. The weakly invaded/enriched state had 30% exotic plant cover and slight elevations in ammonium-N and available P relative to the reference state. The moderately invaded/N-enriched state averaged 52% exotic plant cover and had elevated N levels similar to the highly invaded/NP-enriched state. The highly invaded/NP-enriched state had the highest cover of exotic plants (88%) and was highest in available P and K. Native plant richness decreased with increasing exotic plant cover, and exotic plant richness was significantly lower in the reference state than all other states. Other soil variables showed minor variations among ground-layer states (Table 1).

Community composition of native and exotic plants also differed significantly among ground-layer states (Figure 2a,b; Figure S4; Tables S2 and S3). Changes in native plant communities correlated with available P, K and N:P ratio, while exotic plant communities correlated with available P and ammonium-N (Figure 2a,b; Table S4).

3.2 | AMF overview

We obtained 639,148 sequences and 379 zOTUs belonging to AMF (Glomeromycotina and Mucoromycotina) across all samples. These zOTUs belonged to nine taxonomic families and there was a large proportion of unclassified AMF (18% of zOTUs). The most diverse families were Endogonaceae (29% of zOTUs) and Glomeraceae (20% of zOTUs), while the least diverse were Claroideoglomeraceae and Pacisporaceae (0.5% and 1% of zOTUs, respectively). The other families comprised between 3% and 10% of zOTUs.

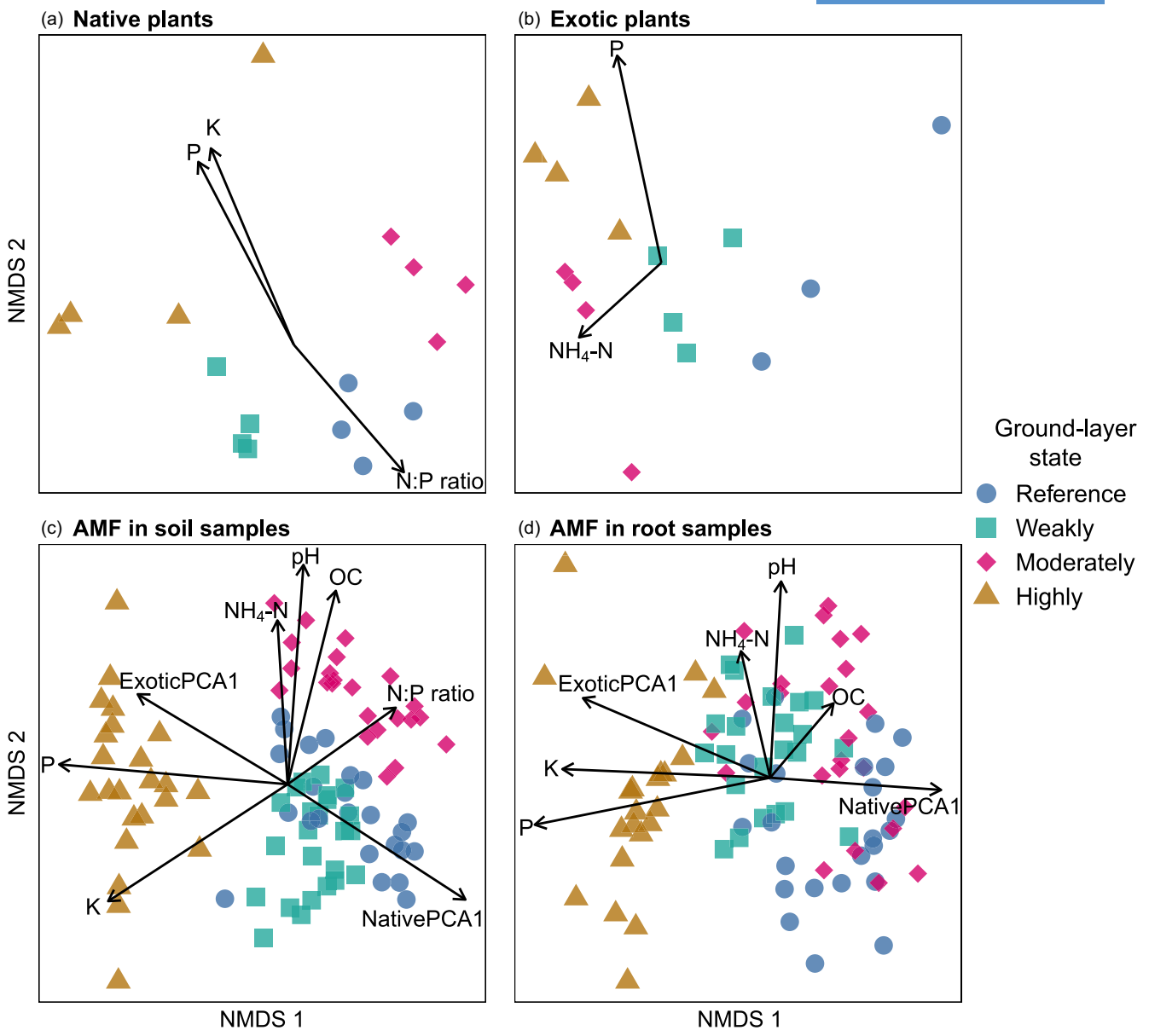

FIGURE 2 Nonmetric multidimensional scaling ordination showing differences in community composition of plants and arbuscular mycorrhizal fungi (AMF) among ground-layer sates: (a) native plants (3D stress = 0.12), (b) exotic plants (3D stress = 0.11), (c) AMF in soil samples (3D stress = 0.12) and (d) AMF in root samples (3D stress = 0.15). Only first two axes are shown. Shape and colour of symbols represent the four different ground-layer states: Reference, weakly invaded/enriched, moderately invaded/N-enriched and highly invaded/NP-enriched states. Vectors show variables selected from maximum correlation analyses (*Permutations* = 9999) correlated with ordination. Only vectors with $R^2 > 0.2$ are shown for simplicity. Vector length is proportional to their relative importance (Table S7).

3.3 | AMF community composition

AMF community composition differed among ground-layer states and source material (i.e. soil vs. root samples), with no interaction between the two factors (Table S5). Soil and root samples yielded similar results, but patterns among ground-layer states were clearer in soil samples.

Community composition of AMF in both soil and root samples differed among all pairwise comparisons of ground-layer states (Figure 2c,d; Table S6). The highly invaded/NP-enriched state showed the highest, while the weakly invaded/enriched state the lowest, dissimilarity in AMF community composition with the reference state (Figure 2c,d; Table S6). The unconstrained ordination showed that these differences in AMF communities correlated with several plant and soil variables (Figure 2c,d; Table S7). For both soil and root samples, the strongest correlation was with available P, K, soil pH, and both exoticPCA1 and nativePCA1 (i.e. the first axis of a PCA between plant cover and richness; Figure 2c,d; Table S7).

Changes in AMF communities among ground-layer states were driven by distinct patterns in the relative abundance and composition of several AMF families (Figure 3). For example, Acaulosporaceae, Gigasporaceae and Glomeraceae showed distinct groups of zOTUs unique to either the reference or moderately invaded/N-enriched

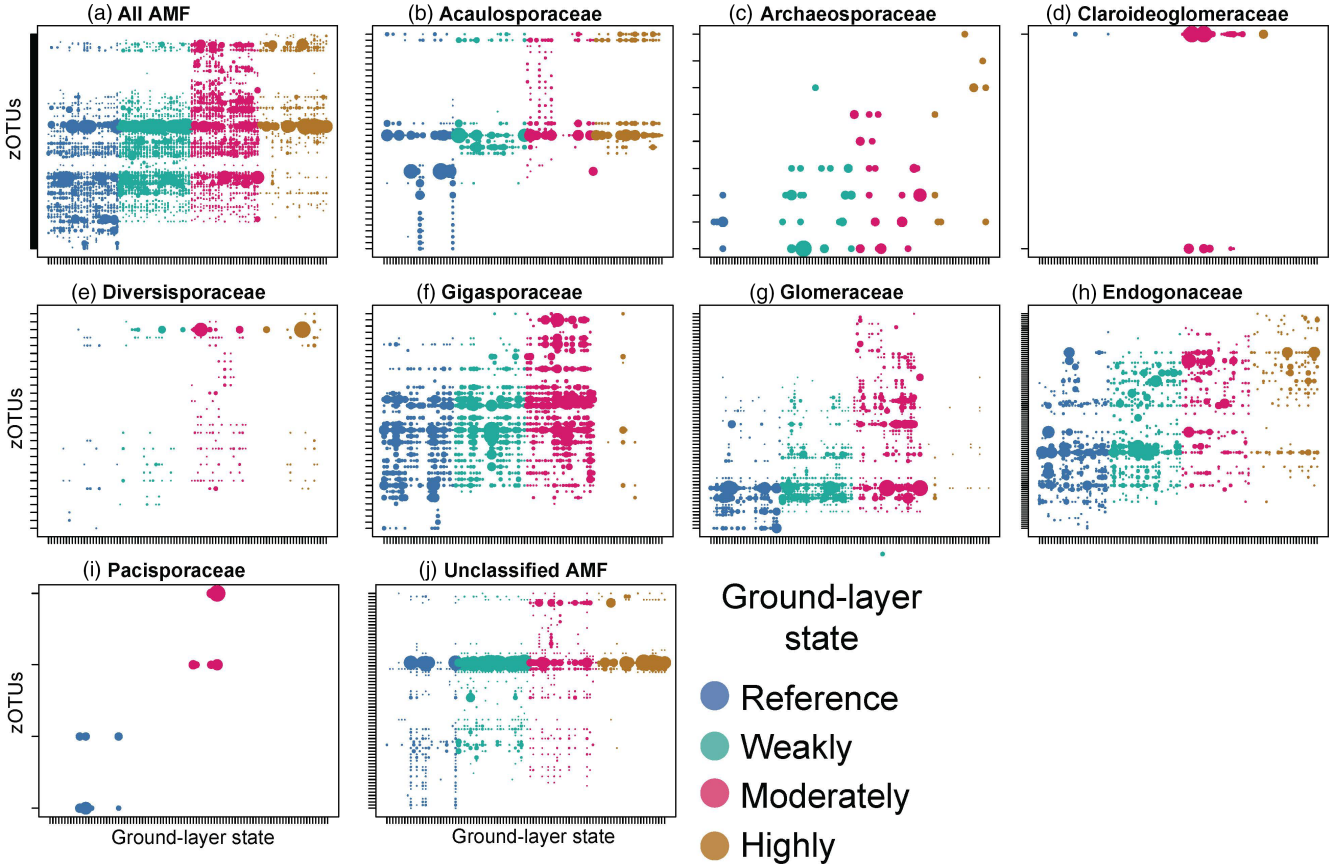

FIGURE 3 Two-way table of arbuscular mycorrhizal fungi (AMF) from soil samples. Samples are coloured and ordered in the *X*-axis by ground-layer state: Reference, weakly invaded/enriched, moderately invaded/N-enriched and highly invaded/NP-enriched states. *Y*-axis shows each zOTU but was compressed to show the full dataset on each panel. First panel (a) shows all AMF, while all other panels (b–j) show individual taxonomic families of AMF. Bubble size is proportional to sequence number within, but not among, panels within the figure.

states, while showing little to no unique zOTUs in weakly and highly invaded/NP-enriched states (Figure 3b,f,g). Gigasporaceae and Glomeraceae were almost completely absent from the highly invaded/NP-enriched state (Figure 3f,g). Archaeosporaceae, Glomeraceae and Diversisporaceae were most speciose in the moderately invaded/N-enriched state (Figure 3c–e). Endogonaceae was diverse in all ground-layer states, albeit most zOTUs being represented by <400 sequences (Figure 3h). Pacisporaceae was extremely rare with each zOTU being represented by <15 sequences, and only two zOTUs found only in reference and two zOTUs in moderately invaded/N-enriched states (Figure 3i). Similarly, Claroideoglomeraceae was represented by only two zOTUs found almost exclusively in the moderately invaded/N-enriched state (Figure 3d).

3.4 | AMF richness and root colonisation

Ground-layer ($\chi^2 = 284.1$; $p < 0.0001$) and source material ($\chi^2 = 18.3$; $p < 0.0001$) affected AMF richness, but there was no significant interaction (Ground-layer state × Material; $\chi^2 = 5.1$; $p = 0.17$). AMF richness was highest in the moderately invaded/N-enriched state, intermediate in the reference and weakly invaded/enriched states, and lowest in the highly invaded/NP-enriched state (Figure 4). Differences in AMF richness among ground-layer states were driven mostly by an increase in richness of Glomeraceae, Diversisporaceae, Gigasporaceae, Archaeosporaceae and unclassified AMF (Figure 3). The richness of AMF was higher in soil than in root samples, driven by higher richness of most AMF families, except Claroideoglomeraceae and Pacisporaceae (Figure S5). The main contributor, however, was Gigasporaceae (Figure S5).

Since there were only two native plant species in the highly invaded/NP-enriched state and two exotic plant species in the reference state, testing two-way interactions between ground-layer states and plant provenance was not possible. Hence, we compared AMF root colonisation and richness between native and exotic plants using ground-layer state as a random effect, noting that means from exotic species were drawn mostly from weakly invaded/enriched, moderately invaded/N-enriched, and highly invaded/NP-enriched states and native species mostly from reference, weakly invaded/enriched and moderately invaded/N-enriched states. AMF root colonisation was 48% higher in roots of exotic than native plants ($\chi^2 = 6.69$; $p < 0.01$; Figure S6a). AMF richness was 17% higher in exotic than in native plants ($\chi^2 = 14.87$; $p < 0.0001$; Figure S6b).

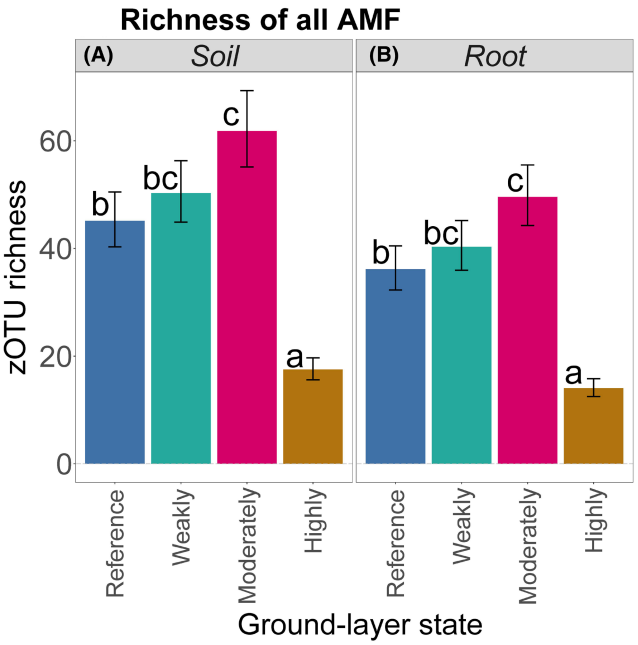

FIGURE 4 zOTU richness of arbuscular mycorrhizal fungi among ground-layer states for (A) soil samples and (B) root samples. Colour of bars represents four ground-layer states: Reference, weakly invaded/enriched, moderately invaded/N-enriched and highly invaded/NP-enriched. Error bars represent estimate ±95% CI from linear mixed effect models. Different letters represent statistical differences among ground-layer states using the post hoc Tukey test (alpha = 0.05).

The SEM linking ground-layer state, soil chemistry and exoticPCA1 with AMF communities in soil samples was well supported by the data ($\chi^2 = 5.32$; d.f. = 5; $p = 0.38$; RMSEA = 0.02; CFI = 0.99; Figure 5a). Ground-layer state was indirectly linked with AMF richness and community composition mediated by soil OC, ammonium-N, available P and exoticPCA1 (Figure 5a). Indeed, the higher OC, ammonium-N and available P found in the moderately invaded/N-enriched state was associated with higher exoticPCA1 (i.e. higher exotic plant cover and richness). In turn, higher exoticPCA1 was positively linked with AMF richness and community composition (Figure 5a). However, the extremely high levels of available P found in the highly invaded/NP-enriched state were negatively linked with AMF richness, irrespectively of exoticPCA1 (Figure 5a).

For root samples, the SEM linking ground-layer state, soil chemistry and exoticPCA1 with AMF communities was also well supported by the data ($\chi^2 = 7.2$; d.f. = 5; $p = 0.20$; RMSEA = 0.05; CFI = 0.98; Figure 5b). The model showed similar results as for soil samples, except for exoticPCA1 and available P showing a stronger and weaker effect, respectively, on AMF community composition than in soil samples (Figure 5b).

3.5 | Patterns of reference AMF

To explore potential losses of native AMF with nutrient enrichment and exotic plant invasion, we separately analysed trends of only zOTUs that were found in at least one plot representing the reference state (hereafter 'reference AMF'). Overall, 245 zOTUs occurred in at least one reference plot (i.e. reference AMF), but only 34 were unique to the reference state (Figure S7). The highly invaded/NP-enriched state had substantially lower richness of reference AMF, whereas all other states had similar richness (Figure S8). Community composition of reference AMF did, however, change across all ground-layer states and these changes were associated with the same soil and vegetation variables as for the analysis of all AMF (Figure S9).

From the 245 reference AMF, several key taxonomic trends were found to reflect the loss of zOTU richness in the highly invaded/NP-enriched state. From Gigasporaceae, almost all reference AMF were absent from the highly invaded/NP-enriched state (Figure S10). From the zOTUs that matched a known species of Gigasporaceae, all reference AMF that matched *Scutellospora crenulata* ($n = 4$) and *Scutellospora spinosissima* ($n = 3$) were absent from the highly invaded/NP-enriched state. Similarly, from the 24 zOTUs that matched *Scutellospora calospora*, 21 were absent from the highly invaded/NP-enriched state. From the three reference AMF that matched *Scutellospora aurigloba*, two were unique to the reference state, while the third was present in all ground-layer states. It is worth noting that the two *Scutellospora aurigloba* zOTUs unique to the reference state only matched *S. auribloga* at 97% identity, while the zOTU present in all ground-layer states matched at 100%. Hence, the two zOTUs unique to the reference state might be a different species of *Scutellospora*.

From the 24 reference AMF that matched Acaulosporaceae, 10 were unique to the reference state, and only five (all matching *Acaulospora laevis*) were present in the highly invaded/NP-enriched state (Figure S10). All five reference AMF that matched *Acaulospora longula* were unique to the reference state.

From the 36 reference AMF that matched Glomeraceae, 14 were absent from the highly invaded/NP-enriched state (Figure S10). However, only four were unique to the reference state. From the remaining reference AMF, no clear trends were found, or taxonomic resolution did not allow for such comparisons.

3.6 | Patterns of Glomeromycotina–AMF and Mucoromycotina–AMF

Because it has been previously suggested that the ecology of Mucoromycotina–AMF (i.e. Endogonaceae) differs from that of Glomeromycotina-AMF (G-AMF) (Albornoz et al., 2021, Albornoz et al., 2022), we compare the responses to changes in ground-layer states between the two subphyla of AMF. The richness of Endogonaceae was 45% and 55% lower in the highly invaded/NP-enriched state compared with the other states, for soil and root samples respectively, while there were no differences among the other states (Table S8). In contrast, the richness of G-AMF (or non-Endogonaceae AMF) was highest in the moderately invaded/N-enriched state, intermediate in the reference and weakly invaded/

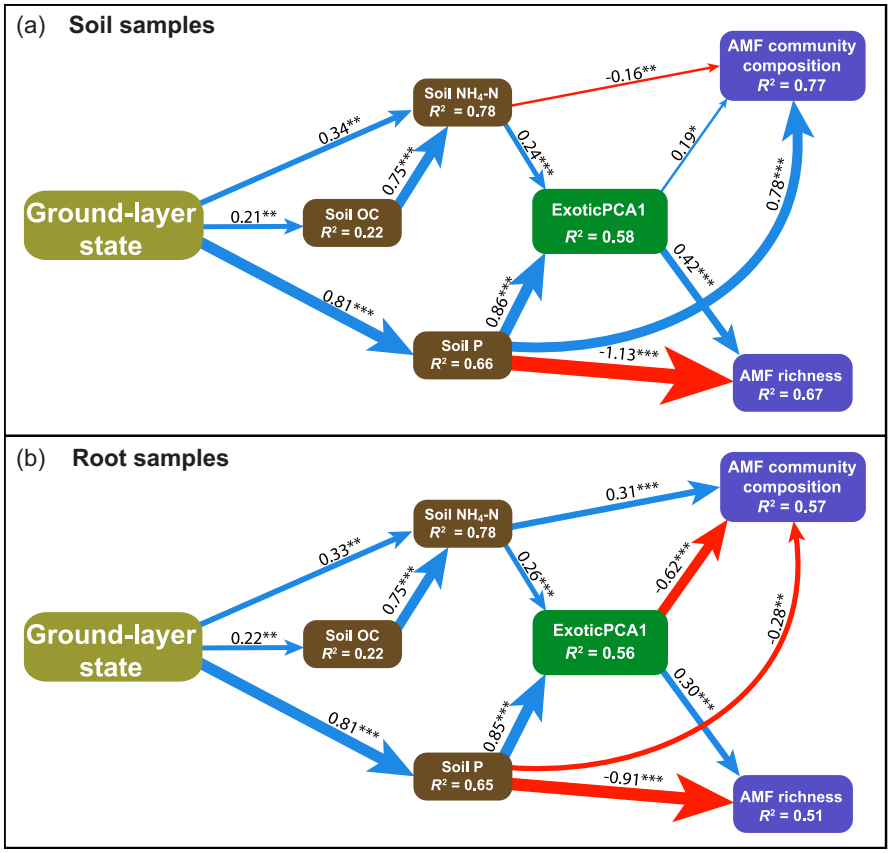

FIGURE 5 Multilevel pathway model showing the direct and indirect pathways by which ground-layer states influence communities of arbuscular mycorrhizal fungi in (a) soil and (b) root samples. Ground-layer states were transformed to an ordinal variable (reference = 0; weakly invaded/enriched = 1; moderately invaded/N-enriched = 2; highly invaded/NP-enriched = 3). Boxes represent soil chemistry (brown), exoticPCA1 (green) and fungal variables (blue). Arrows represent positive (blue) and negative (red) effects. For simplicity, nonsignificant effects are not shown. Standardised path coefficients are shown for each arrow, and arrow width is proportional to their standardised path coefficients The amount of variation explained by each variable in the model (R^2) is shown within each box. Asterisks indicate significant differences (**$p < 0.01$; ***$p < 0.001$).

enriched states and lowest in the highly invaded/NP-enriched state, for both soil and root samples (Table S8).

In soil and root samples, the community composition of both Endogonaceae and G-AMF also differed between ground-layer states, but the trend was less clear for Endogonaceae (Figure S11; Table S9). However, the community composition of Endogonaceae between moderately invaded/N-enriched and highly invaded/NP-enriched states were more similar to each other, while those of G-AMF showed the greatest dissimilarity (Figure S11).

4 | DISCUSSION

We found that AMF richness varied across ground-layer states in accordance with our hypotheses. Moderately invaded/N-enriched states showed the highest AMF richness, and highly invaded/NP-enriched states showed the lowest AMF richness, supporting our first hypothesis (H1) that AMF richness is highest where N enrichment promotes exotic plant invaders and exacerbates P limitation, and lowest where N- and P enrichment reduces host reliance on AMF for nutrient acquisition. We also found support for our second hypothesis (H2), that AMF richness and root colonisation are higher in exotic than in native plant species. Finally, we found strong support for our third hypothesis (H3), that AMF communities (i.e. richness and composition) differed across woodland ground-layer states, associated with changes in exoticPCA1 and soil nutrients.

4.1 | Trends in AMF richness

Our results support our predictions that different nutrient limitations, as well as plant provenance (i.e. native vs. exotic), affect AMF communities (i.e. richness, colonisation and community composition). Based on our SEM, soil P enrichment appeared to be the strongest driver, with a strong and direct negative effect on AMF richness (Smith & Read, 2010). This trend was found for all AMF and reference AMF, and suggests a loss of putative native AMF diversity (particularly of Gigasporaceae and Acaulosporaceae) with P enrichment. We note that available P was extremely high in our highly invaded/NP-enriched state, even compared with agricultural soils (Weaver et al., 2023), so further studies would be beneficial to explore at which concentrations of P such losses may begin to

occur. Furthermore, both P and K were higher in our highly invaded/NP-enriched state. We contend that our results reflect P rather that K availability, given the greater magnitude of differences in soil P concentrations, and that even though AMF can increase hosts' K uptake, it likely done as a counter ion for the accumulation of P to maintain homeostasis (Garcia & Zimmermann, 2014), indicating AMF can increase K uptake as a response to increased P availability. Nevertheless, it remains feasible that both P and K contributed to the observed effect.

After soil P, exoticPCA1 (i.e. exotic plant cover and richness) was the second most important mediator of AMF richness, based on our SEM. This concurs with other studies where the identity and provenance of plant species is strongly linked to the trajectory of AMF richness during ecosystem degradation (Albornoz et al., 2023; Lekberg et al., 2013). For example, Albornoz et al. (2023) showed that N enrichment and invasion by AMF-dependent exotic plants into native eucalypt woodlands can promote AMF richness. We found a parallel result of higher AMF and AMF–plant richness in the moderately invaded/N-enriched state of this study, corroborating the finding across these two ecosystems. Notably, the increase in AMF richness in the moderately invaded/N-enriched state did not occur when only focusing on reference AMF. This result, in addition to the fact that the moderately invaded/N-enriched state had more than double the amount of unique zOTUs than the reference state, suggests the increase AMF richness in this ground-layer state was due to the arrival of AMF not present in any of the reference plots.

This finding supports the proposal by Albornoz et al. (2023) that the moderately invaded/N-enriched state may have been invaded by 'weedy', potentially non-native, AMF. A key question arising from this is whether exotic plant–'weedy' AMF feedbacks promote the persistence of this moderately invaded/N-enriched state. If so, then restoration interventions to recover N-enriched York gum woodlands could include the removal of invasive plant hosts, which should weaken any stabilising plant–soil interactions and lead to a reduction in weedy AMF. This could be achieved through direct removal (e.g. herbicides), or manipulation to reduce available N (e.g. Prober et al., 2005), and it would be of interest to investigate whether these methods would facilitate recovery to reference levels of both AMF composition and available N. In the context of manipulation of AMF as a restoration tool, this approach contrasts with the more common recommendation to re-establish lost AMF species—we highlight that management of weedy AMF should also be considered. It remains an open question whether inoculation with the few missing AMF (e.g. *S. aurigloba* and *A. longula*) from our reference plots would hasten the restoration of moderately invaded/N-enriched states in York gum woodlands.

Our comparison of native and exotic hosts in this study showed that exotic plant species host greater richness and root colonisation of AMF than native species, indicating a mechanism for the increase in AMF in the moderately invaded/N-enriched state. Many Mediterranean-climate ecosystems harbour a great diversity of non-AMF plant species, which is attributed to their extremely low soil P availability, promoting the evolution of more efficient P-acquisition and P-use strategies that do not depend on AMF (Lambers et al., 2011). The fact that AMF root colonisation and richness were higher in roots of exotic than native plants in the present study suggests that native plants in extremely P-limited ecosystems rely less on AMF than exotic plant species.

Despite our conclusion that exotic plants (i.e. abundance and richness) contributed to an increase in AMF richness in the moderately invaded/N-enriched state, this pattern was not apparent in the highly invaded/NP-enriched state. The latter supported more exotic plants than all other ground-layer states but had the lowest AMF richness. Hence, the extremely low AMF richness found in the highly invaded/NP-enriched state was likely due to a decrease in host reliance on their symbionts, rather than a loss of hosts (Smith & Read, 2010). While the link between nutrient enrichment and plant invasion is well established, the links to soil biota are less so. Our results suggest that understanding the three-way interactions between soil nutrients, plant provenance, and AMF is paramount to describing ecosystem degradation and for designing effective restoration practices.

4.2 | Trends in AMF community composition

We found that all ground-layer states differed in AMF community composition, with the highly invaded/NP-enriched state showing the highest difference. This is consistent with Prober et al. (2015) who found that P was the strongest driver of AMF composition in York gum woodlands, although they did not find effects on species richness. In our study, compositional trends involved clear patterns at the family level. Notably, no AMF family was limited by N-only enrichment (i.e. the moderately invaded/N-enriched state), consistent with previous findings in other woodlands (Albornoz et al., 2023). However, all AMF families were limited or absent in the highly invaded/NP-enriched state.

Chagnon et al. (2013) proposed that Glomeraceae, Acaulosporaceae and Gigasporaceae follow a ruderal, stress-tolerator and competitor life strategy, respectively, and suggest a coupling of life strategies between host and symbiont (e.g. ruderal plants associate with ruderal AMF). We thus expected that Glomeraceae (i.e. ruderal AMF) to have thrived where ruderal plants (i.e. exotic annuals) and N were abundant (similar to early successional habitats; Chagnon et al., 2013). This was indeed the case for the moderately invaded/N-enriched state. A relatively large group of Glomeraceae zOTUs was unique to the moderately invaded/N-enriched state, with a smaller group occurring only in reference states, similar to findings of Prober et al. (2015) for these woodlands. On the contrary, Glomeraceae were almost completely absent from the highly invaded/NP-enriched state, which was heavily dominated by exotic ruderal plants. This is likely due to the highly invaded/NP-enriched state being a stressful habitat for AMF, since plants are limiting their C supply to their symbionts, regardless of

plant hosts being mostly ruderal. The fact that different groups of zOTUs were unique to contrasting ground-layer states reflect the functional diversity described for Glomeraceae (Klironomos, 2000) and suggest that part, rather than all, of the family have adopted a ruderal strategy.

There is evidence that Acaulosporaceae can tolerate stress because it has been shown to be common in acid soils or other conditions where plant C fixation is constrained (Albornoz et al., 2023; Chagnon et al., 2013). In the present study, this trend was less clear. Acaulosporaceae relative abundance was greatest in the reference and highly invaded/NP-enriched states, while zOTU richness did not differ between the reference and moderately invaded/N-enriched states. Nevertheless, there were two clear groups of zOTUs that were unique to either the reference or moderately invaded/N-enriched states. From the reference AMF, zOTUs that matched *A. longula* were unique to the reference state, while only zOTUs that matched *A. laevis* were present the highly invaded/NP-enriched state. This suggests the two Acaulosporaceae species may both be adapted to limited C supply, due to differing underlying drivers. *A. longula* might be adapted to limited plant C fixation due to extreme low nutrients and hosts, while *A. laevis* might be able to tolerate low C supply from plants with little to no nutrient limitations (Chagnon et al., 2013).

Gigasporaceae was relatively stable across all ground-layer states, except in the highly invaded/NP-enriched state, where their richness and relative abundance were drastically lower. Gigasporaceae have been classified as competitors due to their high P-benefits to their hosts (Chagnon et al., 2013). This would explain why Gigasporaceae was almost completely absent from the highly invaded/NP-enriched state, as the P-benefits provided to their hosts might no longer be needed due to high soil fertility. This is consistent with Prober et al. (2015), who found Gigasporaceae, including *Scutellospora* species, were most common in reference York gum woodlands compared with NP-enriched, grazed woodlands. However, all AMF families were less diverse in the highly invaded/NP-enriched state, suggesting that plants were no longer allocating C to their symbionts, hindering all AMF, regardless of their life strategy. In terms of diversity loss with nutrient enrichment and exotic plant invasion, two Gigasporaceae (*S. auribloga* and *S. longula*) were the only identifiable species unique to the reference state. However, these two species comprise only 20% of the total zOTUs unique to the reference state, while the remaining zOTUs unique to the reference state could not be assigned taxonomy to the species level. Further exploration of the Chagnon et al. (2013) framework is warranted as it could be oversimplified for other families too (e.g. Acaulosporaceae).

The only AMF family that persisted in the highly invaded/NP-enriched state was Endogonaceae (i.e. Mucoromycotina–AMF). More importantly, community composition between moderately invaded/N-enriched and highly invaded/NP-enriched states were the most similar in Endogonaceae, but the most distinct for G-AMF. This result is consistent with Albornoz et al. (2022) who demonstrated that this group thrives in agricultural systems compared with co-occurring G-AMF. However, even though Endogonaceae richness was lower in the highly invaded/NP-enriched state, the decline was far less than what was observed for G-AMF. This decline might reflect the high levels of P in the highly invaded/NP-enriched state (196 mg/kg), while the average P level found in farms near eucalypt woodlands in Albornoz et al. (2022) was 84 mg/kg. This niche difference compared with other AMF families highlights the importance of incorporating Mucoromycotina–AMF in future studies. Even though they have been acknowledged as AMF since 2017 (Orchard et al., 2017), only a handful of studies have attempted to include them in mycorrhizal studies (e.g. Mansfield et al., 2023).

5 | CONCLUSION

We conclude that the Johnson et al. (2013) hypotheses regarding AMF response to N:P stoichiometry are supported, and future AMF research must also account for plant provenance. We also propose that overall AMF richness is not a good measure of ecosystem health as increase richness might be due to the arrival of 'weedy' AMF. Paying attention to changes in community composition or focusing on reference AMF might be more informative. For example, whether the re-introduction of the loss taxa within Gigasporaceae can promote ecological restorations of York gum woodlands without the need of managing weedy AMF remains to be tested. By viewing ecosystems from a holistic perspective including soil nutrient availability, plant species provenance and AMF characteristics of the reference ecosystem, we can better predict the effects of different types of degradation on AMF. By doing so, we can test assumptions and fully explore the potential for AMF to facilitate restoration outcomes rather than merely follow above-ground interventions (Harris, 2009).

AUTHOR CONTRIBUTIONS

Felipe E. Albornoz, Rachel J. Standish and Suzanne M. Prober developed the conceptual framing with feedback from all other co-authors. Felipe E. Albornoz led field sampling, processed samples and conducted DNA extraction. Felipe E. Albornoz analysed the data and led the manuscript writing. Rachel J. Standish, Suzanne M. Prober and Andrew Bissett provided feedback and guidance on statistical analyses. All co-authors contributed to data interpretation, manuscript writing and conceptual synthesis.

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CONFLICT OF INTEREST STATEMENT

None of the authors have a conflict of interest.

DATA AVAILABILITY STATEMENT

Data have been archived in Dryad: https://doi.org/10.5061/dryad.c866t1gfw (Albornoz, 2024) and DNA sequences in GeneBank (accession number: PP924141–PP925575).

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SUPPORTING INFORMATION

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

Table S1. Summary of sampling design indicating the four sampling scales.
Table S2. PERMANOVA for community composition among ground-layer states of native and exotic plants.
Table S3. PERMANOVA for native and exotic plant communities among pairwise ground-layer states.
Table S4. Squared correlation coefficient and significance of correlation with 3D-NMDS for plant communities.
Table S5. PERMANOVA for AMF community composition among ground-layer states and between source material.
Table S6. PERMANOVA for AMF communities among pairwise ground-layer states.
Table S7. Squared correlation coefficient and significance of correlation with 3D-NMDS for AMF communities.
Table S8. Summary table of zOTU richness among ground-layer state and source material of Glomeromycotina-AMF and Endogonales.
Table S9. PERMANOVA for G-AMF and Endogonaceae communities among pairwise ground-layer states.
Figure S1. Rarefaction curves of unrarefied zOTU matrix and rarefied zOTU matrix.
Figure S2. Correlation plot among floristic and soil chemistry variables.
Figure S3. Principal components analysis showing the two axes of vegetation variables.
Figure S4. Two-way table of plant species found across all ground-layer states.
Figure S5. Comparison of zOTU richness of individual families of AMF for soil versus root samples.
Figure S6. AMF root colonisation and zOTU richness between native and exotic plant species.
Figure S7. Observed number of unique zOTUs among ground-layer states.
Figure S8. zOTU richness of 'reference AMF' among ground-layer states.
Figure S9. NMDS of community composition of 'reference AMF' among ground-layer states.
Figure S10. Two-way table of 'reference AMF' across ground-layer states.
Figure S11. NMDS of community composition of Endogonaceae and G-AMF among ground-layer states.

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