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Road Disturbance Shifts Root Fungal Symbiont Types and Reduces the Connectivity of Plant-Fungal Co-Occurrence Networks in Mountains

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ABSTRACT

Roads are currently one of the most disruptive anthropogenic disturbances to mountain ecosystems worldwide. These disturbances can have a profound effect on roadside soil properties and vegetation, typically favouring fast-growing and ruderal species. However, their effect on plant-associated fungal communities and plant-fungal interactions remains largely unknown. In this study, we examined the changes in root-associated fungal communities as well as plant-fungal and fungal-fungal co-occurrence networks along mountain roads from four biogeographical regions. We found that roadsides consistently altered plant and fungal community composition, generally favouring arbuscular mycorrhizal fungi and putative plant pathogens at the expense of ectomycorrhizal fungi. Moreover, roadsides consistently reduced the complexity of plant-fungal and fungal-fungal co-occurrence networks (with 66%–95% and 40%–94% reduction in total edge density, respectively), even though the richness of fungal communities was not reduced and many of the naturally occurring highly connected taxa were still present. Our findings suggest that altered and transient conditions in the roadsides may favour more generalist symbionts like AMF and pathogens with low fidelity for particular hosts as opposed to surrounding natural vegetation which is dominated by symbionts with higher

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specificity for the host (like ectomycorrhizal fungi). We conclude that road disturbance may have a consistent negative imprint on connectivity between plants and fungi; a consequence that deserves attention as it could render mountain roadside systems unstable and vulnerable to further pressures such as climate change and invasive species.

1 | Introduction

Mountain ecosystems, with their complex relief and heterogeneous microclimates, often host an exceptional diversity of habitats and communities (Körner and Spehn 2002). However, these ecosystems are also highly vulnerable to environmental perturbations such as climate change, land-use changes, and other anthropogenic disturbances as well as to the spread of invasive alien species (Körner and Spehn 2002; Pauchard et al. 2009; Haider et al. 2022). Over the last decades, mountain ecosystems have seen an increase in anthropogenic pressures, among others due to significant land use change, intensification of road network use, and expansion of tourist activities (Lembrechts et al. 2017; Haider et al. 2022). Roads do not only cause direct mechanical disturbances; they can also alter microclimatic, hydrological, and soil biochemical conditions (e.g., increase soil pH and nutrient content) along roadsides (Forman and Alexander 1998; Müllerová et al. 2011). With their particular environmental conditions, roadsides favour the establishment of new plant communities, typically with a higher abundance of ruderal and stress-tolerant species with great regenerative capacity (Vítková et al. 2012) as well as of non-native species (Watkins et al. 2003). Additionally, roads act as dispersal corridors for both native and locally non-native, potentially invasive, species (Lembrechts et al. 2017). If non-native species spread into the natural vegetation in mountains, aided by climate change, they have the potential to further alter mountainous plant communities with a cascading effect on ecosystem functioning (Daehler 2005; Haider et al. 2022).

The effects of roads on plant–plant interactions and their implications for mountain plant communities have been the focus of recent studies (Lembrechts et al. 2014, 2017; Haider et al. 2018). However, much less information is available on the interaction between plants and their root-associated fungi in roadsides (Clavel et al. 2021) and particularly the interactions among these fungi. Plant roots harbour rich fungal communities that form symbiotic, parasitic, or pathogenic associations which can strongly influence plant diversity, productivity, co-existence, and invasiveness in communities (Klironomos 2002; Bonanomi et al. 2005; Bever et al. 2010). Mycorrhizal fungi, for example, can facilitate the establishment of their hosts by helping them acquire limiting nutrients and providing protection against environmental stress and pathogens (van der Heijden et al. 2008), while plant pathogens maintain plant diversity through trade-offs between competitive ability and resistance to pathogens (Bever et al. 2015). Therefore, fungi can determine which plant species can gain a competitive edge and successfully establish themselves under certain environmental conditions (Bever et al. 2015; Tedersoo et al. 2020).

Recurrent disturbance events along roadsides might disrupt natural plant-fungal interactions favouring particular taxa that in turn promote the development of their partners. For instance, a study in northern Norwegian mountains showed that roadside conditions promoted the establishment of arbuscular mycorrhizal fungi (AMF) and their host plants while the surrounding vegetation was dominated by ericaceous plants associating with ericoid mycorrhizal fungi (Clavel et al. 2021). Clavel et al. (2024) also showed that the cover of AMF-associated plants, often ruderal herbs, is higher in roadsides compared to surrounding vegetation at different mountainous regions across the globe, while the opposite was found for ecto- and ericoid mycorrhizal plants, many of which are more conservative, woody species. Additionally, the presence of roads is known to increase the levels of nutrients in the roadsides compared to surrounding undisturbed soil, which can reduce the colonisation of roots by mycorrhizal symbionts (Gryndler et al. 2006) or select for less mutualistic mycorrhizae (Johnson 1993), thereby reducing the overall connectivity between plants and fungi. Finally, fast-changing conditions on the roadsides may lead to unstable and transient interactions between plants and fungi that may not consistently persist across elevation gradients as is possible in the adjacent natural communities. Unravelling the effect of road disturbances on fungal communities and their associations with plants may bring us closer to understanding the mechanism by which roads shape mountainous roadside communities, particularly in light of ongoing environmental changes and the increased pressure of invasive species.

Most studies rely on analysing community composition and diversity of individual groups (e.g., plants and fungi) to explore the effect of environmental changes on biota. However, the ecological properties that are thus assessed may not be able to comprehensively capture the complex interplay that exists between plants, fungi, and their environmental settings. Recent statistical tools, such as joint species distribution models (Ovaskainen and Abrego 2020), have increased the capability to estimate the true covariances that are observed among these groups of variables and calculate network properties describing the full range of co-occurrence patterns. Co-occurrence networks may not always represent true biotic interactions, but they can help understand community complexity (Barberán et al. 2012; Freilich et al. 2018; Ramirez et al. 2018) and how such complexity might change in response to environmental factors. This can result in identifying highly connected—keystone—taxa, which might have a particularly important role in maintaining community structure and function (Banerjee et al. 2019).

To examine the effect of roads on plant and fungal community complexity and their potential interaction patterns, we surveyed plants and root-associated fungi along mountainous roads in four regions exposed to contrasting climatic conditions. As part of the Mountain Invasion Research Network (MIREN) (Haider et al. 2022), the study encompasses regions with in-depth knowledge of plant community structures. In each region, one of the most canonical ecological pressures–disturbance – (in this

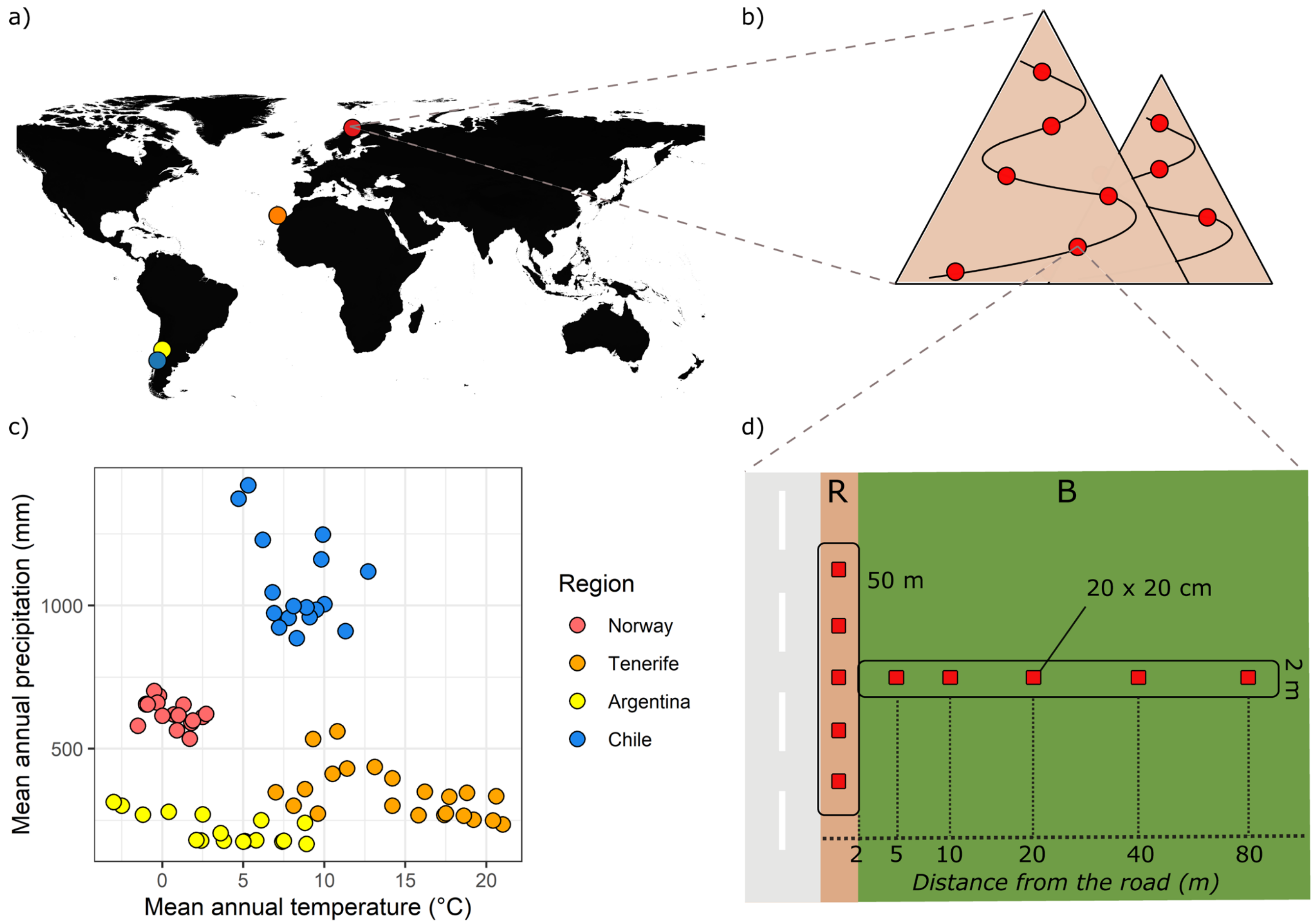

FIGURE 1 | (a) The location of the four study regions; (b) Sampling sites within each region were located along two to three mountain roads at different, evenly spaced elevations; (c) Long-term averages (1979–2913) of the mean annual temperature and precipitation for each sampling site across the four study regions, extracted from CHELSA (Karger et al. 2017); (d) Sampling design within each site. One large plot was located parallel to the road (roadside, R) while the other was perpendicular to it and extended 80 m into background vegetation (B). Red squares represent the 20 × 20 cm subplots surveyed. The distances are not scaled.

study, a complex set of disturbances imposed by roads) is studied along several elevational gradients used as replicates. These ecosystems thus present a model to test the roadside effects on plants and their associated fungi along elevation gradients and examine the consistency of findings across contrasting climatic zones and vegetation types.

We hypothesized that road disturbance would significantly reduce root-associated fungal richness and alter plant and fungal community composition along roadsides compared to the surrounding vegetation in all regions. More specifically, roadsides were expected to harbour an increased abundance of arbuscular mycorrhizal fungi at the expense of ectomycorrhizal fungi. We also expected that plant-fungal and fungal-fungal co-occurrence networks in the roadsides would be less connected because (1) plants may be less dependent on mycorrhizal symbionts and (2) they may associate non-specifically and transiently with generalist fungal symbionts, or (3) due to a loss of fungal richness and alteration of fungal communities, there may be a lack of particular keystone taxa in the roadsides responsible for maintaining the network structure in the surrounding vegetation.

2 | Methods

2.1 | Experimental Design and Sampling

All sampling sites are part of the global long-term vegetation monitoring of the Mountain Invasion Research Network (MIREN) (Haider et al. 2022) and are located along mountain roads in four distinct and distant regions: Norway, Spain (Tenerife), Chile, and Argentina (Figure 1, Figure S1, Table S1). The Norwegian sites are located in an area with a largely subarctic climate along three roads stretching from 13 to 683 m a.s.l. (sites located at 6–7 elevation points per road; 20 sites in total), which turn from paved roads at low elevations to gravel roads at higher elevations. The dominant vegetation along the elevation gradients consists of birch forest with an understory of ericaceous shrubs at lower elevations, which changes into largely dwarf-shrub-dominated tundra vegetation at higher elevations (Clavel et al. 2021). The sites in Tenerife are located along three paved roads stretching from 24 to 2377 m a.s.l. (7–8 elevation points per road; 22 sites in total) on the southern flanks of the Teide volcano. The climate is predominantly warm and arid. The vegetation consists of thermophilous scrubs until 1000 m a.s.l., which transition into a forest of Canary pine up to 2000 m a.s.l., followed by high mountain scrubland (Kühn et al. 2021). Chilean sites are located along two paved roads stretching from 378 to 1645 m a.s.l. (sites located at 8 and 11 elevation points per road; 19 sites in total). The climate is predominantly Mediterranean. The low-elevation areas are dominated by Mediterranean and temperate deciduous forests, which turn into Andean resinous forests and scrublands at higher elevations (Fuentes-Lillo et al. 2021). Finally, the Argentinean study region consists of three gravel roads stretching from 1755 to 3782 m a.s.l. (6–7 elevation points per road; a total of 20 sites). The climate is cold and dry. Low elevations are dominated by shrublands and herbaceous steppes and the higher elevations by sparse vegetation of cushion shrubs and perennial herbaceous plants (Aschero et al. 2021).

The experimental setup was designed as an expansion of the MIREN road survey. The MIREN road survey is a global monitoring initiative established to study the long-term dynamics of plant species composition along mountain roads (Seipel et al. 2012; Haider et al. 2022). Each sampling site in our study (81 in total) consists of two large plots organised in a T-shape, with one plot parallel to the road (2 m × 50 m) and the other perpendicular to the road and extending from 2 to 102 m (i.e., 50 m + 50 m; see Haider et al. (2022)) from the road and into the background vegetation (Figure 1d). In this study, the plots following the road are referred to as roadside (R) plots and those extending into the background vegetation as background (B) plots (Table S1). Within each of these two large plots, five subplots of 20 cm × 20 cm were selected, approximately 10 m from each other in the roadside plots and 5, 10, 20, 40, and 80 m from the road for background plots. Due to practical constraints, in Norway, up to four subplots (10, 20, 40, and 80 m from the road) were sampled instead of five. Altogether, the expected number of samples was 810 (81 sites × 2 plots × 5 subsamples), but given that it was not always possible to sample vegetation in five subplots and some sequencing samples failed (see below), the total number of samples with matching plant and fungal data was 673 (Table S1).

Vegetation surveys were performed during the peak of the growing season in 2017 for Norway, in 2019 for Tenerife, and in 2018–2019 for Argentina and Chile. At each site, the presence/absence and percentage cover of all plant species within the subplots were recorded. At the time of the surveys, a random subset of plants from the subplots was harvested together with their roots. The collective root sample from each subplot (roots from all plants pooled together) was washed with ionised water and stored in Ziplock bags at 4°C before being shipped to the University of Antwerp for the analysis of root-associated fungi. We specifically chose small-sized subplots to ensure that we could harvest the roots of most plant species observed aboveground. This allowed us to link plant-associated fungi with their hosts as closely as possible and ensured that observed plants and fungi were likely interacting.

2.2 | Analyses of Microbial Communities

2.2.1 | Sample Preparation and Sequencing

Root samples were cleaned in demineralised water over a 2-mm sieve to remove the soil material, after which fine roots were cut into 1 cm pieces for DNA analyses. A subset of 10 randomly selected 1-cm root pieces per subsample was lyophilised and pulverised with sterile tungsten beads. DNA from roots was isolated using the DNeasy PowerSoil Kit according to the manufacturer's protocol (Qiagen, Venlo, the Netherlands). The fungal ITS1 region was amplified using general fungal primers ITS1f (Gardes and Bruns 1993) and ITS2 (White et al. 1990) modified according to Smith and Peay (2014). Each 25 μL reaction mixture contained 1 μL of the sample, 0.2 μM of forward and 0.2 μM of reverse primer, 1× PCR buffer, 200 μM dNTPs, and 1 U Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). The settings of PCR conditions were as follows: initial denaturation at 98°C for 60 s; followed by 35 cycles of denaturation at 98°C for 30 s; annealing at 55°C for 30 s; extension at 72°C for 30 s; and an additional extension of 72°C for 10 min. The success of amplification was tested on a 1.5% agarose gel. For the samples that did not amplify successfully, amplification was attempted again with a modified mixture that contained 2 μL of the sample and 0.4 μM of forward and reverse primer. Successful PCR products were diluted 50-fold, and a second PCR was performed using dual barcoded primers with Illumina adapters (2.5 μL of diluted PCR products and 0.1 μM of each primer). The conditions were 98°C for 60 s; followed by 12 cycles at: 98°C for 10 s; 63°C for 30 s; 72°C for 30 s; and 72°C for 5 min. PCR products were run on an agarose gel, and successful amplicons were pooled in equimolar concentrations, purified using the QIAquick Gel Extraction Kit (Qiagen, Venlo, the Netherlands), and quantified using qPCR (KAPA Library Quantification Kits, Kapa Biosystems, Wilmington, MA, USA). The sequencing was performed at the Centre of Medical Genetics of the University of Antwerp using the Illumina MiSeq platform (Illumina Inc.; San Diego, CA, USA) with 2 × 300 cycles for forward and reverse reads.

2.2.2 | Quality Filtering and Bioinformatics Analyses

The sequences were analysed using the USEARCH (v8.1.1861) and VSEARCH (Rognes et al. 2016) software following the UPARSE pipeline (Edgar 2013). After trimming to 250 bp, the paired-end reads were merged, and primers were removed. Merged sequences were quality-filtered using the expected number of errors (E) as a measure of read quality, with a threshold of Emax = 0.5. This yielded 10,985,494 good-quality reads. Following singleton removal, the sequences were clustered into operational taxonomic units (OTUs) based on 97% similarity, using the UPARSE-OTU algorithm (Edgar 2013) which also filters out chimaeras. Filtered reads were then mapped to the OTUs with an identity threshold of 0.97, yielding a table of fungal OTUs. Representative OTUs were aligned to the UNITE database (Kõljalg et al. 2005) (release date 2.2.2019), using the *sintax* command in USEARCH with a 0.8 cut-off. Any non-fungal sequences were removed from the OTU table. All raw sequences are deposited in NCBI—SRA databases with the accession number PRJNA1182238.

Further steps were performed using the R software (R Core Team 2020). The number of reads per sample was rarefied to 1151 reads using the *rrarefy* function in the vegan package (Oksanen et al. 2020), as rarefaction curves showed that the number of taxa was levelling off for many samples at this depth (Figure S2). Samples that had fewer sequences than this threshold were removed, and the final number of high-quality fungal samples (from single 20 × 20 cm subplots) was 673.

To annotate sintax-assigned fungal sequences to known genera based on the UNITE database, we used NCBI's BLAST algorithm with default settings. OTUs were then assigned to particular taxa if they had a maximum *E*-value of 10^{-36}, and from this, the lowest *E*-value hit with a known genus was selected. If there were none, the genus level was left unassigned. OTUs were subsequently assigned to functional groups using two methods: (1) according to FUNGuild (Nguyen et al. 2016) and (2) by matching the genera with known lifestyles in the studies by Tedersoo et al. (2014) and Liu et al. (2016) (hereafter referred to as the Tedesoo-Liu database). The databases were used to assess the potential functional status of fungal taxa, focusing particularly on plant symbionts: arbuscular mycorrhizal fungi, ectomycorrhizal fungi, and plant pathogens. Ericoid mycorrhizal fungi were poorly represented and were thus not analysed as a group. The first database is more conservative, especially for plant pathogens, and it was used to assess the change in fungi whose primary lifestyle type is plant pathogen, while the second database (particularly the part based on Liu et al. 2016) includes a larger list of fungi with known plant pathogenic potential and thus allows for incorporating a wider range of possible pathogens compared to FunGuild.

2.3 | Statistical Analyses

2.3.1 | Community Composition and Fungal Functional Groups/Genera

We averaged the subplot relative abundances of plant species and fungal OTUs per sampling plot and used PERMANOVA analysis (function adonis2 in the vegan package) to examine the effect of disturbance (factor variable with two levels: R vs. B) on overall plant and fungal community composition for each region including the variable ‘sampling site’ as ‘strata’ to account for the fact that different sites have different communities. The effects of road disturbance per region were also analysed and visualised using the *dbrda* function (controlling for the ‘sampling site’ nested in ‘mountain road’) on Bray-Curtis (BC) distances calculated after the Hellinger transformation. PERMANOVA analyses were used also to test the effect of elevation on plant and fungal community composition per region, using ‘road’ as strata (‘site’ was not used in this case as each site is located at a different elevation). We calculated the fungal richness of each plot and tested if they were influenced by roads, elevation, or their interactions across regions using generalised linear mixed-effects models with the package glmmTMB (‘site’, nested in ‘road’ nested in ‘region’ as a random intercept term) adopting a negative binomial distribution (nbinom2) suitable for count data.

We used generalised mixed effect models (‘site’ nested in ‘road’ nested in ‘region’) adopting a beta distribution (package glmmTMB) to analyse the influence of plot type (B and R) on the relative abundances of fungal functional groups: (1) ectomycorrhizal fungi (both those assigned as exclusively and putatively ectomycorrhizal), (2) arbuscular mycorrhizal fungi, (3) putative plant pathogens according to FUNguild, and (4) putative plant pathogens according to Tedersoo-Liu. AMF and (uniquely) ectomycorrhizal fungi from both databases matched perfectly, while, as expected, deviations were found between putative plant pathogens. Given that the inclusion of the full random effect structure accounting for the hierarchical design of the study often produced non-convergent models or those with higher AIC values compared to the simpler model, we finally retained only two random effects: ‘region’ and ‘sampling site’ in all cases. This simplification of the model did not notably affect the results. We dealt with the presence of zeros by transforming the data according to (Cribari-Neto and Zeileis 2010) using the formula $y \cdot ((n-1)+0.5)/n$; where y is the response variable and n is the number of samples. Adopting the same approach, we analysed the effect of plot type on the 35 most dominant fungal genera in the dataset implementing a Benjamini-Hochberg correction of *p*-values. Model assumptions of homoscedasticity and overdispersion were tested using the DHARMa package (Hartig 2018). In a few cases where the assumptions of homoscedasticity were not fully met, family ‘*tweedie*’ with default settings was used instead of ‘beta’, to confirm the pattern observed in initial models. No substantial impacts on model results were found.

2.3.2 | Co-Occurrence Networks

Networks of co-occurrence patterns between plant and fungal taxa and among fungal taxa were constructed for background and roadside plots in each of the four regions, yielding a total of eight co-occurrence networks. This was done by estimating the species-to-species association matrices using hierarchical modelling of species communities (HMSC), a method based on hierarchical Bayesian joint species distribution modelling (Ovaskainen et al. 2017). Given the small sizes of subplots, we focused on the presence-absence model examining which plants and fungi and fungi—fungi co-occurred within the subplots, and we included only those plant species and fungal OTUs observed in $>7\%$ and 10% of roadside or background subplots, respectively, within a region. This is done because the rare taxa are not sufficiently informative to enable fitting species-specific models (Ovaskainen and Abrego 2020). The threshold for plants was lowered compared to the one typically used for microbial community data (Odriozola et al. 2021) because using a 10% threshold on the patchier occurrence of plants relative to fungi at our scale of sampling resulted in the omission of too many (and in case of Tenerife all) potentially informative plant species. Thus, the 7% threshold currently used was the highest threshold that retained at least three plant species per region and thus the highest possible threshold that could be used. The number and identity of plant species and fungal OTUs that were retained in the analysis per region and plot type are shown in Table S2.

We fitted a binomial HMSC model using the Hmsc package in R, assuming the default prior distributions (Ovaskainen and Abrego 2020) with presence–absence plant and fungal data as a response variable, elevation as an environmental covariable, and type of organism (plant or fungi) as a trait. The model was fitted using a probit link function and included two random intercept terms: ‘road’ and ‘sampling site’. We sampled the posterior distribution with four Markov Chain Monte Carlo (MCMC) chains, each of which was run for 112,500 iterations, of which the first 37,500 were removed as burn-in. The iterations were thinned by 500 to yield 150 posterior samples per chain and thus 600 posterior samples in total. We chose the above-described parameters after examining MCMC convergence (Gelman and Rubin 1992) for species-to-species associations and finding that potential scale reduction was generally lower than 1.1 (Figure S3). We concluded that MCMC convergence was satisfactory and that increasing the number of iterations, while computationally much more intensive, would not produce a substantially better convergence. Co-occurrence networks between taxa were calculated by residual 'species-to-species' association matrices at the level of the 'sampling site', where only the associations with a support level higher than 0.95 were retained (Ovaskainen and Abrego 2020). The community-level random effect represents covariation among the species after partialling out the effect of elevation (and with it largely the climate) and is thus either a result of true biotic interactions among species or a result of their common responses to other environmental covariates not included in the model (Ovaskainen and Abrego 2020).

Network properties were calculated using the package *igraph* (Csardi and Nepusz 2006) in R. The edge density (number of observed links compared to the total possible number of links) was used as an estimate of the level of network connectedness. Using the 'cluster_waktrap' and 'modularity' functions in the *igraph* package, we calculated the overall weighted network modularity which demonstrates the extent to which the network is divided into modules or clusters; where higher values indicate stronger clustering (i.e., dense connections within and sparse connections between the clusters).

The 'evcent' function in *igraph* was used to calculate the eigenvector centrality of each taxon in the network. Eigenvector centrality ranges from 0 to 1 and is a measure of the influence of a node in a network, which was used to identify the highly connected—keystone or core—taxa (here defined as those with eigenvector centrality ≥ 0.8). We visualised the eigenvector centrality of the core taxa from the background networks along the eigenvector centrality of the same taxa in roadside networks (if they were present) to examine to what extent the changes in the network structure in the roadsides were due to the absence of core taxa observed in the plots away from the roads.

Finally, to analyse whether the edge density of networks was significantly different between B and R plots and whether they were correlated with elevation and temperature, we created subnetworks for each plot similarly as in Wagg et al. (2019). Briefly, from the main network of each region, we created subgraphs per site including all the plant and fungal taxa that appear at that site and the links between them. We then calculated the edge density of positive links for these site-level subnetworks and correlated them with temperature and elevation for B and R plots separately to examine if edge density across regions could be explained by these environmental factors. We used mixed-effect models (glmmTMB function) using both beta distribution and tweedie distribution (accounting for zero inflation) with 'road' nested within 'region' as a random effect to quantify the effect of plot type (B and R) on site-level plant-fungal and fungal-fungal positive edge density.

2.3.3 | Testing the Effect of the Design

To test if our hierarchical, yet asymmetrical, sampling method (regularly spaced samples in roadside plots as opposed to background plots with increasing distances from the road) captured a similar range of community variation in background and roadside plots, several tests were performed; we analysed (1) whether Bray-Curtis distances among communities within each sampling site were different between roadside and background plots; (2) whether the within-site beta dispersion for plant and fungi differed in B and R plots (betadisper function); and (3) whether the community composition systematically changed with increasing distance to the road in background plots using PERMANOVA on subplot data (i.e., not aggregated per site). We also tested the effect of distance to the road on plant and fungal richness using mixed-effect models on subplot data. We found no consistent evidence that the communities in B plots had higher levels of community heterogeneity than R plots due to their larger plots extending from the road nor that distance from the road had a consistent effect on community turnover or richness in B plots (Figure S4; Table S3, Table S4; Table S5; Annex S1). It is thus unlikely that the different sizes of B and R plots substantially affected the co-occurrence patterns analyses in different regions, but care should be taken in the case of fungi in Tenerife, which had lower dissimilarity among roadside communities and lower beta dispersion in roadsides compared to the background (Annex S1).

3 | Results

3.1 | Community Composition and Richness

Roadside plant and fungal communities were significantly different (PERMANOVA; $p < 0.05$) from background communities in all the regions, but a particularly clear difference was observed in Norway ($R^2 = 0.18$ and $R^2 = 0.14$, for plants and fungi, respectively; Figure 2). Elevation had a significant effect on both plant and fungal community composition in all the regions (except for fungi in Norway) with the strongest effect in Tenerife ($R^2 = 0.12$ and $R^2 = 0.14$, for plants and fungi, respectively) (Table S6), which also had the longest elevation gradient. Fungal species richness was not affected by plot type, elevation, or their interaction across all regions (Table S7). There was thus a clear consistent effect of roadside disturbance on plant and fungal community composition across regions, while the effect on fungal richness was largely absent.

Mixed-effect models demonstrated a significant increase in AMF in the roadsides across regions and a significant decrease in ectomycorrhizal fungi, both when looking at exclusively ectomycorrhizal fungi or those that are known to be both saprotrophic and ectomycorrhizal (Figure 3; Table S8). The relative abundance of putative plant pathogens (as assigned by FunGuild) was as a whole not significantly different between B and R plots, but putative plant pathogens according to the combined Tedersoo-Liu database were significantly enriched in R compared to B plots (Figure 3).

On the level of genera, seven out of 35 were significantly different in B compared to R plots after the correction for multiple

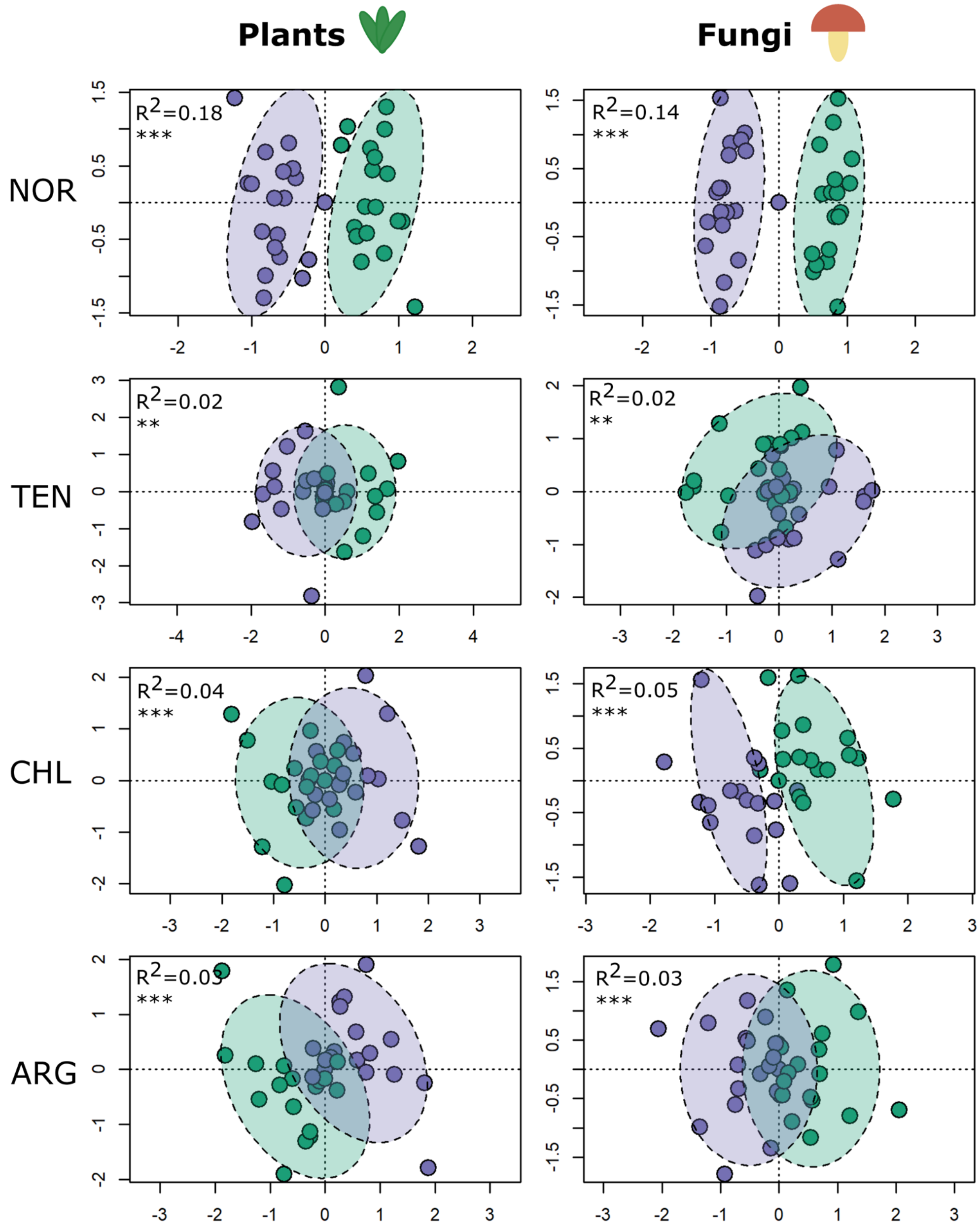

FIGURE 2 | Partial distance-based redundancy ordination of plant and fungal community composition in four different regions for background (green) and roadside (purple) plots, constrained by the variables 'site' within 'mountain road'. NOR—Norway, TEN—Tenerife (Spain), CHL—Chile, and ARG—Argentina. The differences in community composition were tested using PERMANOVA analyses (Tables S6).

testing (adj.$p<0.05$). *Penicillium*, *Mycena*, *Cladophialophora*, *Mortierella*, and *Meliniomyces* significantly decreased in R plots, while the genus *Phoma* and *Naganishia* significantly increased. Moreover, the genus *Phialocephalia* showed a near-significant decrease, while *Cadofora* and *Serendipita* showed an increasing trend in R plots (adj.$p<0.1$) (Figure 4).

3.2 | Co-Occurrence Networks for Background and Roadside Plots

To recreate co-occurrence patterns of plant-fungal and fungal-fungal associations, we used HMSC, a method based on hierarchical Bayesian joint species distribution modelling (Ovaskainen et al. 2017). This analysis showed that plant-fungal co-occurrences were substantially more abundant in background plots than in roadsides (Figure 5; Figure S5), for all four studied regions and both positive and negative links, even though roadside networks shared a substantial number of taxa with background networks (Figure S6). In the roadsides in Chile, the total plant-fungal edge density was reduced by 95% compared to the background plots. In Norway and Argentina, no plant-fungal links that met the criteria (i.e., co-occurring for reasons other than a preference for the same elevational belt) were detected in the roadsides. The weakest effect of roadsides on plant-fungal edge density was found in Tenerife with a decrease of 66%. The reduction of fungal-fungal edge density of both positive and negative links was also present in all the regions with 94% and 93% reduction in Norway and Chile; 40% reduction in Tenerife and no links present in the Argentinian

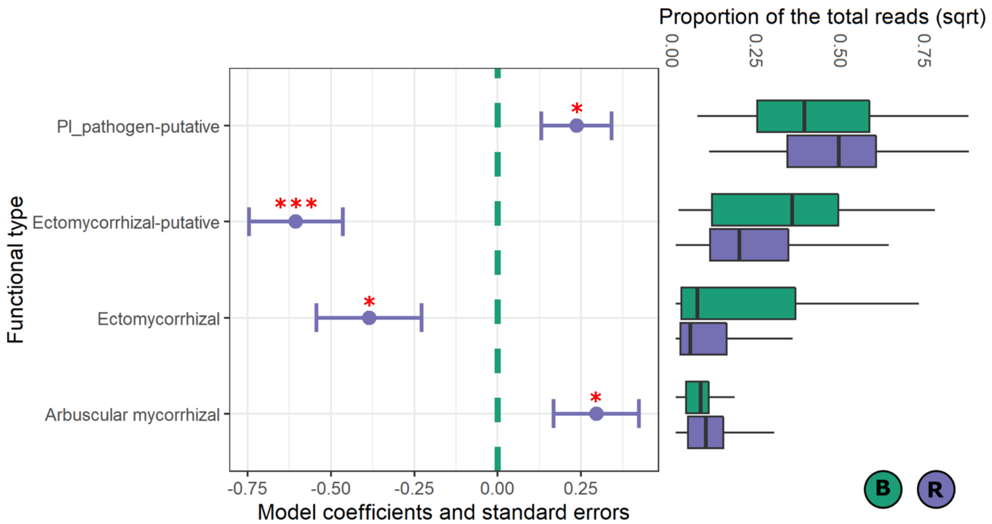

FIGURE 3 | The results of mixed-effect models analysing the difference in fungal functional types (plant symbionts) in roadsides compared to the background. The left part demonstrates model coefficients with standard errors (average difference in the relative abundance of fungal functional types) at roadsides compared to the reference background (green dashed line at 0). Asterisks represent the p values of the model: * ($p < 0.05$); *** ($p < 0.001$). The boxplots on the right demonstrate the proportion of total reads that were assigned to a particular group for background (B) compared to roadside (R) plots.

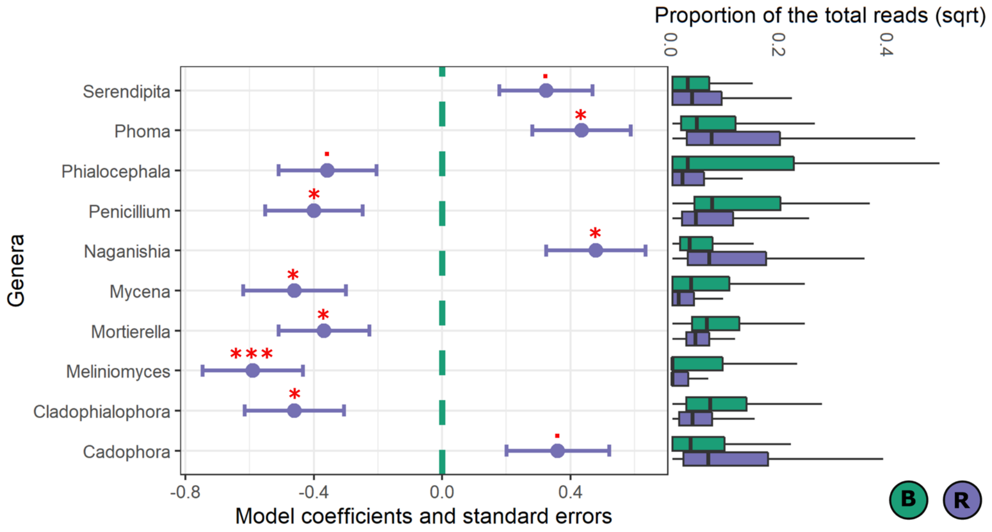

FIGURE 4 | The results of mixed-effect models analysing the difference in 35 dominant fungal genera at roadsides compared to the background. Only the genera that showed significant (<0.05) or near-significant (<0.1) differences after p-value corrections were shown here. The left part demonstrates model coefficients with standard errors average difference in the relative abundance of fungal genera at roadsides compared to the reference background (green dashed line at 0). Asterisks represent the p values of the model: . ($p < 0.1$); * ($p < 0.05$); *** ($p < 0.001$). The boxplots on the right demonstrate the proportion of total reads that were assigned to a particular genus for background (B) compared to roadside (R) plots.

roadsides. Generalised mixed effect models confirmed that there was an overall significant decrease in positive plant-fungal and fungal-fungal edge density (Figure 5; Table S9) in roadsides across regions.

While the smallest effect of roads was found in Tenerife, the total positive network modularity was still reduced in the roadside compared to the background plots (0.30 vs. 0.43, respectively) in this region, showing that the structure of the background network (clustering into different groups of co-occurring taxa) has been disrupted in the roadsides. The other regions had too few links in the roadsides to effectively compare network modularity between background and roadside plots.

The number and the identity of the core (highly connected) taxa in the networks were also generally altered in the roadside compared to the background plots (Figure 6). In Norwegian background plots, highly connected taxa were predominantly from the Ericaceae family (for plants) and from the Helotiaceae, Hyaloscyphaceae, Serendipitaceae, and Archaeorhizomycetaceae families for fungi, but the few links that remained in the Norwegian roadsides were formed between entirely different taxa. 15% of highly connected background taxa

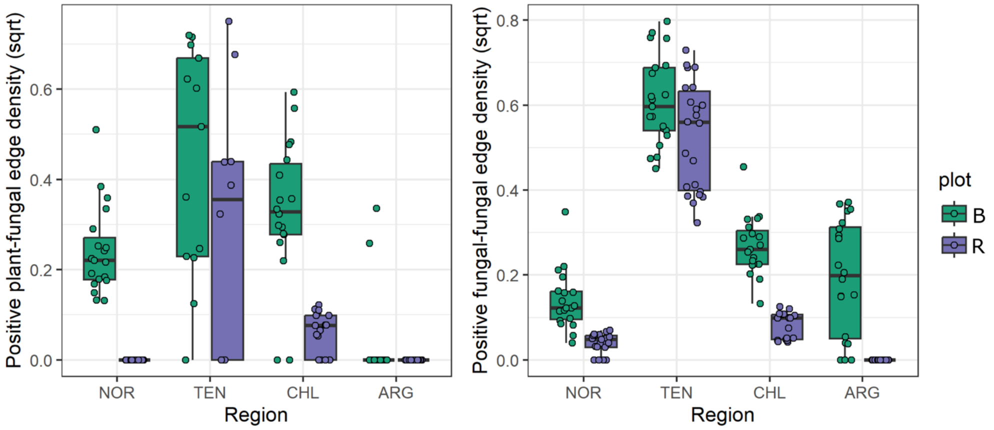

FIGURE 5 | The boxplots depicting positive plant-fungal (left) and fungal-fungal (right) edge density of plot-based subnetworks from the background (B) vs. roadsides (R) for each of the four regions.

were present in the roadside networks, but none formed observable links. In Tenerife, 68% of the highly connected taxa in the background plots were present in the roadside networks, most of which (57% of the total) formed connections. These highly connected taxa include *Hyparrhenia hirta* for plants and members of Nectriaceae and Pleosporaceae families for fungi. On the other hand, in Chile, 50% of the core background taxa occurred in the roadside networks, but only 14% formed observable links. Aspergillaceae and Herpotrichiellaceae were the fungal families with the highest number of core taxa in the background plots. In Argentina, 40% of the background core taxa were found in the roadsides, but none of those formed any links. Several of the fungal OTUs forming links in background plots were from the Glomeraceae family (AMF). Nonetheless, the same taxa failed to form links in the roadsides.

4 | Discussion

Our findings consistently point towards a change in plant and associated fungal community composition along roadsides compared to background vegetation, with an increased relative abundance of arbuscular mycorrhizal fungi and potential plant pathogens and a decrease in ectomycorrhizal fungi. Moreover, we found a strong reduction in the plant-fungal and fungal-fungal network complexity along roadsides in mountains from four biogeographic regions, even though fungal richness was largely unaffected and many of the background core taxa were present. The existing literature shows how mountain road disturbances alter plant community composition and species distributions in mountain regions across the globe (Lembrechts et al. 2014, 2017; Haider et al. 2018). Our findings add an important belowground component to this: the effects of mountain roadside disturbance are reflected and even enhanced in belowground network complexity.

4.1 | Roadside Disturbance Shifts the Abundance of Plant-Associated Fungi

Our findings indicate that road disturbance might promote arbuscular mycorrhizal fungi while reducing the abundance of ectomycorrhizal fungi across different regions. Moreover, we found a strong decrease in the genus Meiliniomyces which could be associated with ericoid (or ecto-) plants and potentially can form ericoid mycorrhizal associations (Hambleton and Sigler 2005). Our study confirmed the indirect evidence found in Clavel et al. (2024) which examined 11 MIREN regions focusing on the effect of roadsides on plant mycorrhizal types. Based on these findings, it can thus indeed be concluded that roadside conditions, typically characterised by faster and more extreme environmental changes as well as increased loads of nutrients, tend to favour grasses and more ruderal plants which are usually associated with AMF (Müllerová et al. 2011) while reducing the abundance of more perennial plants which thrive in more stable environments such as many woody and ericaceous species associated with ecto- and ericoid mycorrhizal fungi (Clavel et al. 2024).

Furthermore, we found an increase in putative plant pathogens in the roadsides as well as an increase in dominant fungal genera *Phoma* and *Cadofora*, both containing species that are known endophytic root colonisers with plant pathogenic potential (Maciá-Vicente et al. 2020; Salmaninezhad et al. 2022). Particularly, the genus *Phoma* contains prominent plant pathogens. A significant increase in the roadsides was also observed for the genus *Naganishia*, which includes members characteristic of high elevations and are described as flexible "opportunitrophs" tolerant to harsh environments because they can grow during short periods of favourable conditions (water from melting snow and influx of nutrients) (Schmidt et al. 2017). Interestingly, it appears that transient roadside conditions promote the members of this genus capable of thriving under unstable conditions.

Besides Meiliniomyces, other dominant genera that were abundant in surrounding vegetation but declined significantly in the roadsides were *Mycena*, *Penicillium*, *Mortierella*, and *Cladophialophora*. *Mycena* is considered a common saprotrophic genus often containing generalist root invaders, with a yet unknown effect on the plants (Harder et al. 2023). *Penicillium* and *Mortierella* are also a widespread saprotrophs in soil, containing species that can interact positively with plant roots and enhance plant growth by supplying nutrients and plant growth hormones and by producing antibiotics against plant pathogens (Srinivasan et al. 2020; Ozimek and Hanaka 2020). The

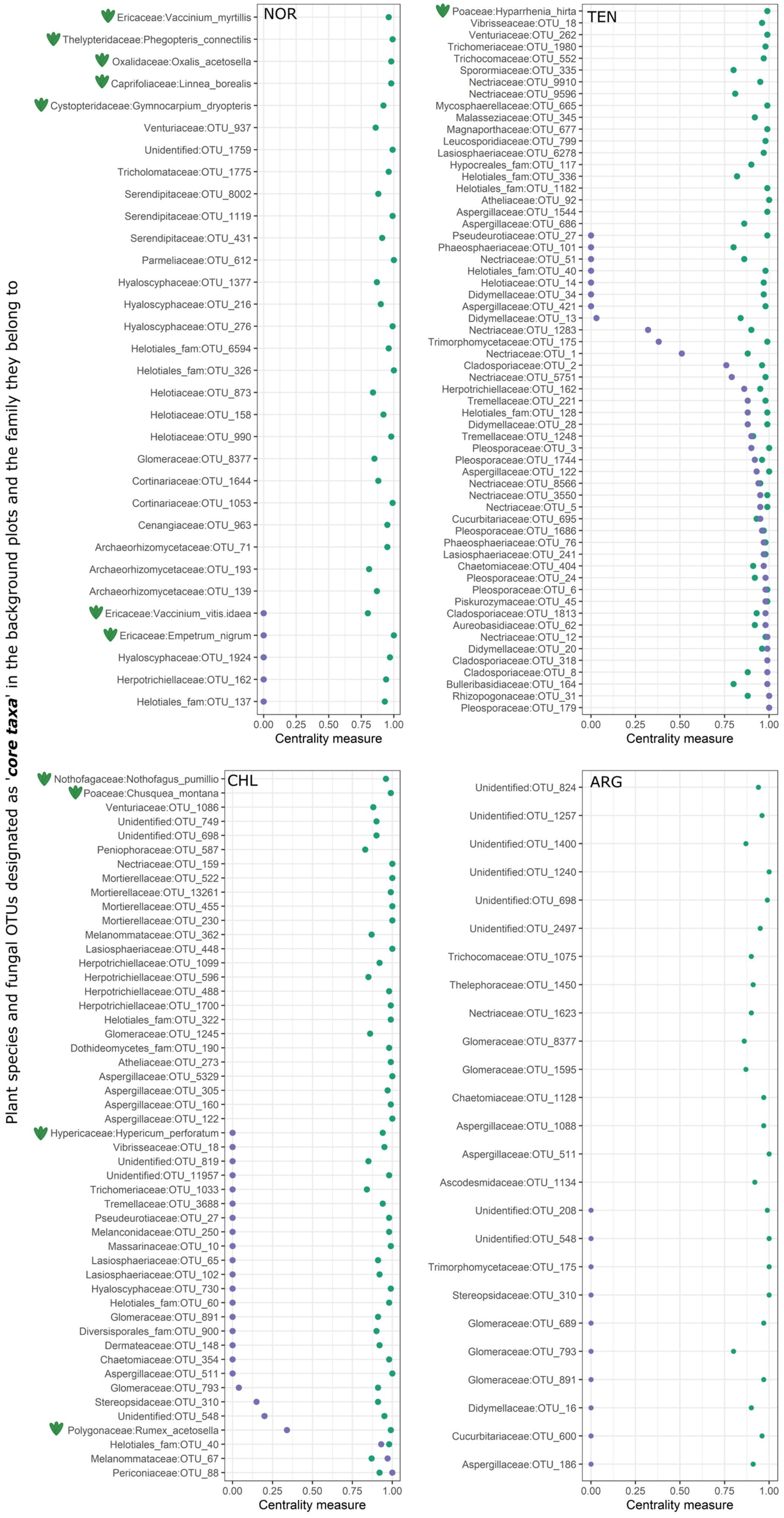

FIGURE 6 | Legend on next page.

FIGURE 6 | Eigenvector centrality of the core taxa (ec > 0.8) in the background networks (green) for the four different study regions, compared to the eigenvector centrality of the same taxa in the roadsides (purple), when present in the R network. The green symbol represents plant species, all the other taxa are fungal OTUs.

reduction of these important fungi in the roadside root microbiome could thus have negative effects on plant growth, fitness, and tolerance to pathogens.

It is not currently clear whether roadsides tend to promote AMF plants which then accumulate more AMF or vice versa, but the shift to higher AMF abundance could have important implications for the further development of the roadside systems in terms of plant community assembly, species redistribution patterns, and the invasion by non-native species. Root-associated fungi and their plant hosts can reinforce each other's development thereby further promoting the conditions that are suitable for their growth while outcompeting other types of plant and fungal communities (Radujković et al. 2020). For instance, many non-native species have been shown to associate with AMF in the roadsides of different mountainous regions (Clavel et al. 2024), and these species could suppress other mycorrhizal fungi and reduce the success of native species, while promoting their own soil communities and further facilitating invasion by non-native species (Vogelsang and Bever 2009). Therefore, these altered interactions with mycorrhizal fungi could play an important role in the invasions of mountainous roadsides by non-native species and ruderal species that are typically outcompeted under natural conditions. These conditions could be particularly detrimental for ecto- and ericoid-mycorrhizal plants which may experience a decline in their fungal symbionts while potentially being exposed to an increased pressure of generalist plant pathogens promoted by the roadside conditions.

4.2 | Roadside Disturbances Decrease Co-Occurrence Network Complexity

Different regions had different total network edge densities in background vegetation likely as a result of the differences in their regional climatic conditions, vegetation types, species diversity, and environmental turnover along elevation gradients. For instance, in Argentina, much fewer connections were found in background vegetation than in the other regions possibly due to a very high turnover in plant species composition along the elevation gradient (Aschero et al. 2021), reducing our capacity to detect significant co-occurrences between taxa. Moreover, we showed that the number of links across regions was closely correlated with the mean annual temperature, where the region with higher temperatures (Tenerife) had many more links than colder regions (Norway and Argentina) (Figure S7).

Regardless of this difference in the absolute edge density, in three out of four regions, roadside vegetation lost virtually all of the links present in the background vegetation and saw them replaced with weaker co-occurrence networks consisting of links between a different and smaller set of plant and fungal taxa (Figure S5). Surprisingly, the reduction of network complexity along roadsides was apparent even though the roadsides had a similar fungal richness as background (Table S7) and roadside networks contained many of the same taxa found in the background networks (Figure S6, Figure 6). The changes in co-occurrence networks along roadsides were thus not always due to the absence of particular taxa per se but the result of reductions and changes in the myriad of different positive and negative co-occurrence patterns that jointly shape a complex network. Co-occurrence networks thus appear to be more sensitive to roadside disturbances than are species richness alone. It is worth noting that previous studies have reported an increase in plant species richness (Lembrechts et al. 2014) and wider distributional ranges (Lembrechts et al. 2017) in roadside areas compared to nearby vegetation. However, if the co-occurrence network complexity is indicative of ecosystem stability (Okuyama and Holland 2008), our findings caution that these apparent benefits may be temporary and insufficient to establish stable populations of the expanding species.

The road effect on plant and fungal communities was most apparent in Norway, where the specific roadside conditions, such as physical disturbance, increased soil nutrient loads, and increased soil pH, are known to be especially unsuitable for naturally dominant ericaceous vegetation and their fungi (Tybirk et al. 2000; Hein et al. 2021). In the background plots, the naturally occurring ericoid dwarf shrubs (e.g., *Empetrum nigrum*, *Vaccinium* sp.) were the most connected plant species, while Archaeorzhiomycetaceae, Helotiaceae, Serendipitaceae, and Hyaloscyphaceae, known as putative symbionts of ericaceous plants (Vohník 2020; Radujković et al. 2020; Griffin and Kernaghan 2022) were the most connected fungal families. In roadside networks, some of these taxa were still present, but they formed almost no observable links. Very similar patterns were observed in Chile and Argentina. On the other hand, in Tenerife, many identical links and fungal core taxa were found in roadsides and background conditions—for example, members of the Nectriaceae family that comprise many plant pathogens (Lombard et al. 2015). This suggests that the level of disturbance imposed by roads in Tenerife mountains may be lower compared to other regions potentially because of lower levels of traffic-induced pollution and nutrient depositions. Alternatively, naturally occurring dry vegetation and long-lived shrubs present in this region (Kühn et al. 2021) may be less sensitive to disturbance, as previous findings demonstrated no effect of roads on plant functional diversity (Ratier Backes et al. 2021). Nonetheless, here as well, there was a clear reduction in network edge density along roadsides, as well as a change in network structure, as demonstrated by a decrease in the degree of clustering of co-occurring taxa (i.e., reduced network modularity). Even though only three plant species were maintained in the analyses for Tenerife, which is presumably not representative of plant-fungal networks in this region, fungal-fungal networks followed a similar trend as observed in other regions.

It has previously been found that a range of physical disturbances, such as erosion (Qiu et al. 2021), increased amounts of soil nutrients (Ai et al. 2015; Huang et al. 2019), atmospheric

N deposition (Ma et al. 2022), agricultural intensification (Banerjee et al. 2019), increased soil pH (Yang et al. 2022), and drought (Boczoń et al. 2021) are all associated with reduced soil microbial network complexity and/or weakened plant–soil interactions. Many of the same environmental pressures (mechanical disturbance, increased soil pH, increased nutrient deposition, altered hydrology) are present in the roadsides, which could explain the substantial reduction in community network complexity observed across the studied biogeographical regions. Furthermore, roadsides are often susceptible to colonisation by non-native species (Seipel et al. 2012; McDougall et al. 2018) which can alter fungal community composition by disrupting plant–fungal interactions (Grove et al. 2017). These findings collectively demonstrate that human-induced environmental changes often result in reduced complexity of co-occurrence networks among soil microbes as well as between soil microbes and plants.

4.3 | Potential Implications for the Functioning of Mountain Roadside Systems

Co-occurrence analyses have the potential to reveal which species respond in similar ways to ecosystem conditions and unearth non-random co-variation patterns which may reflect community organisation—such as direct interactions or a shared environment (Berry and Widder 2014). In the current study, the links between taxa were not due to shared environmental conditions associated with a particular elevation, such as mean temperature and precipitation (the effect of elevation was removed by the model), but could still reflect other shared environmental conditions. Nonetheless, given that this study focuses specifically on root-associated fungi and our small plot size ensured a close spatial connection between plants and fungi, many of the observed links may truly represent effective interactions between plants and fungi.

Regardless of whether the co-occurrence networks represent the interactions between taxa or shared environmental niches, the fact that the road effects lead to a consistent reduction of network complexity and alterations of network structure might have important consequences for ecosystem functioning and the response of communities to environmental changes (de Vries et al. 2018). For instance, using a mesocosm experiment, Wagg et al. (2019) have shown that reduced co-occurrence network complexity within and among fungal and bacterial communities was linked to reduced ecosystem multifunctionality, such as decomposition and nutrient uptake. They concluded that the decline of complex ecological interactions below ground can impair ecosystem functioning. Similarly, it has been found that soil network complexity enhances the link between biodiversity and multifunctionality in agricultural systems (Jiao et al. 2022) and that complex networks with greater connectivity are related to a more efficient carbon uptake (Morriën et al. 2017). Moreover, the processes of carbon, nitrogen, and phosphorus cycling were found to be correlated with microbial co-occurrence patterns (Banerjee et al. 2018).

Currently, the mechanisms with which the complexity of plant-fungal and particularly fungal-fungal co-occurrence networks could be related to ecosystem functioning are not entirely clear. However, if plant-fungal co-occurrences in our study partially represent plant-fungal interactions, then our results point to either a decline in plant-fungal interactions or non-consistent interactions between particular plant and fungi taxa along roadsides. Given that there was an increase in the relative abundance of arbuscular mycorrhizal fungi and putative plant pathogens in the roadsides, it is unlikely that the reduction in links represents a lack of interactions between plants and fungi. It is more likely that these fungi form largely generalist and random interactions with plants compared to the background vegetation. Indeed, some AMF taxa were found to be highly connected in the background while failing to form consistent connections in the roadsides (e.g., in Argentina, Figure 6). It is thus possible that the roadsides, with their highly unstable and altered conditions, do not necessarily only increase the relative abundance of AMF but also disrupt the interaction patterns that these fungi form under non-disturbed conditions. Moreover, changes in fungal-fungal interaction patterns could also have consequences for the ecosystems because mycorrhizal fungi and some other root-inhabiting fungi (such as members of the genus *Penicillium*) can negatively affect the colonisation of roots by fungal pathogens; either directly by competing for space and nutrients or indirectly by increasing host vigour and improving plant growth or by producing antibiotics (Borowicz 2001; Yang et al. 2014). Yet again, in the roadsides, these connections seem to be disrupted, indicating that the formation of consistent and stable interactions between taxa could be hampered by transient roadside environmental conditions.

5 | Conclusions

Despite the inevitable regional differences, our results demonstrate that the roadside effects consistently lead to an erosion and structural change of plant-fungal and fungal-fungal co-occurrence patterns across mountainous regions. Roads also induce a decline of ecto- and potentially certain ericoid mycorrhizal fungi in favour of arbuscular mycorrhizal fungi and putative plant pathogens, indicating that even with the increase of particular plant symbionts in the roadsides, the consistency of the potential interactions they form is lower. The sensitivity of co-occurrence networks to roadside effects found in this study, and to other anthropogenic stressors as found in previous studies, suggests that reduced network complexity is a consistent consequence of human-induced disturbances which may lead to a reduction in ecosystem stability and functionality. These findings emphasise the need for further research into the mechanistic links between the complexity of plant–soil co-occurrence networks and ecosystem functioning as well as the effect of anthropogenic disturbances, climate change, and invasions on mountainous ecosystems.

Author Contributions

J.J.L., D.R., E.V., and I.N. conceived the study. D.R., J.J.L., and J.C. performed the lab analyses. D.R. performed the statistical analyses and wrote the first draft of the paper together with J.J.L. and with the input of E.V., D.R., J.J.L., and E.V. interpreted the data. J.J.L. and all other co-authors collected and provided root samples and plant data. All co-authors provided significant input to the final draft of the manuscript.

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Disclosure

Benefit sharing: A research collaboration was developed with scientists from the countries providing root samples and plant data. All collaborators are included as co-authors, and the results of the research have been shared with them.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw sequences are deposited in the NCBI – SRA database with the accession number PRJNA1182238. All other data and codes are deposited in the Dryad database with the link https://doi.org/10.5061/dryad.ht76hdrsk

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.