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## The role of arbuscular mycorrhizal fungi in non-native plant invasion along mountain roads

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### Summary

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- Accepted Article
- Plant associated mutualists can mediate invasion success by affecting the ecological niche of non-native plant species. Anthropogenic disturbance is also key in facilitating invasion success through changes in biotic and abiotic conditions, but the combined effect of these two factors in natural environments is understudied.
  - To better understand this interaction, we investigated how disturbance and its interaction with mycorrhizas could impact range dynamics of non-native plant species in the mountains of Norway. Therefore, we studied the root colonisation and community composition of arbuscular mycorrhizal (AM) fungi in disturbed vs. undisturbed plots along mountain roads.
  - We found that roadside disturbance strongly increases fungal diversity and richness while also promoting arbuscular mycorrhizal fungal root colonisation in an otherwise ecto- and ericoid-mycorrhiza dominated environment. Surprisingly, AM fungi associating with non-native plant species were present across the whole elevation gradient, even above the highest elevational limit of non-native plants, indicating that mycorrhizal fungi are not currently limiting the upward movement of non-native plants.
  - We conclude that roadside disturbance has a positive effect on AM fungal colonization and richness, possibly supporting spread of non-native plants, but that there is no absolute limitation of belowground mutualists, even at high elevation.

Keywords:

Arbuscular mycorrhizal fungi, plant invasion, elevation gradient, anthropogenic disturbance, range shifts, roads, sub-arctic, soil microbiota

## Introduction

The mutualistic association between non-native plants and mycorrhizal fungi, both native and introduced, is suspected to play a substantial role in the successful spread of non-native plant species (defined here as species which originated from outside the region and were introduced by anthropogenical means). A better understanding of this interaction could be crucial to improve our insight into invasion patterns (Bever *et al.*, 2010; Dickie *et al.*, 2017). Mycorrhizal associations occur in the majority of terrestrial plants (Wang & Qiu, 2006) and are increasingly recognized as fundamental determinants of plant community composition and ecosystem functioning (Klironomos *et al.*, 2011; Wagg *et al.*, 2014; Neuenkamp *et al.*, 2018). Previous research on non-native plant invasion success has shown both mycorrhizal limitation and facilitation across a variety of ecosystems (Pringle *et al.*, 2009; Dickie *et al.*, 2017; Policelli *et al.*, 2019). Furthermore, the impact of non-native plant species on the native mycorrhizal fungal community and subsequent effects on native flora also varies between studies from stimulation, through no observable effect, to disruption of mutualism (Mummey & Rillig, 2006; Stinson *et al.*, 2006; Dickie *et al.*, 2017; Grove *et al.*, 2017; Urcelay *et al.*, 2017). With such a diversity of possible responses, it is clear that a better understanding of the underlying processes is crucial to predict how mycorrhizal associations will affect the invasion process, and whether they may be drivers or passengers of non-native plant success (Zobel & Opik, 2014). Recent studies have moved in this direction, and identified mycorrhizal status (Menzel *et al.*, 2017) and overlap in mycorrhizal associations with native vegetation (Bunn *et al.*, 2015) as potential predictors of invasion success of non-native plant species.

Apart from mycorrhizal associations, anthropogenic disturbances are another important determinant of non-native plant invasion (Hobbs & Huenneke, 1992; Jauni *et al.*, 2015; Lembrechts *et al.*, 2016). The effects of disturbance on plant competition (Biswas & Mallik, 2010), nutrient availability (Davis *et al.*, 2000; Blumenthal, 2006) and propagule and colonization pressure (Vilà & Ibáñez, 2011; Blackburn *et al.*, 2020) are all known to facilitate the invasion success of non-native plants. We hypothesize that changes in belowground mutualist interactions caused by disturbances could also play a significant role during the invasion process. Until now, the effect of physical disturbance per se on mycorrhizal fungal communities has been primarily studied in the context of tillage effects on arbuscular mycorrhizal (AM) fungi in lowland agricultural landscapes, showing reduced fungal diversity and root colonisation (Goss & De Varennes, 2002; Kabir, 2005; Schnoor *et al.*, 2011). However, less is known about how disturbance in natural ecosystems influences mycorrhizas, where it may modulate AM fungi community and facilitate invasion

success by AM associated plants. In this study, we aim to bridge this gap by investigating the effects of disturbance in an otherwise natural setting on belowground interactions and whether these could play a role in regulating non-native plant invasions.

To achieve this goal, we studied the abundance and diversity of mycorrhizal fungi along mountain roads in the northern Scandinavian mountains (the Scandes) in Norway. The crucial role of disturbance in facilitating invasion success in mountain systems is well known (Pauchard *et al.*, 2009; Alexander *et al.*, 2016; Lembrechts *et al.*, 2016), making them ideal ecosystems in which to assess how mycorrhizas could mediate this role. Roads in particular offer a clear juxtaposition of disturbed and undisturbed conditions and have been shown globally to facilitate the upward expansion of non-native plant species (Müllerová *et al.*, 2011a; McDougall *et al.*, 2018). The upward expansion dynamic of non-native plant species along roadsides has been repeatedly observed in local studies, for example in the Himalayas (Bhattarai *et al.*, 2014), northern China (Zhang *et al.*, 2015) and the Rocky Mountains in the USA (Pollnac *et al.*, 2012). Furthermore, a global survey of non-native plant species in relation to mountain roads showed that the number of non-native plant species was found to be higher along roadsides than in the natural vegetation, leading in turn to a more homogenized flora along roadsides (Haider *et al.*, 2018). Similar patterns have been observed in the northern Scandes which are still in an early stage of invasion with non-native plant species increasing their elevation range along roads, yet currently remaining largely restricted to the roadsides, suggesting a crucial role of the disturbed environment in their range expansion (Lembrechts *et al.*, 2014). Candidate causes for roadside affinity of non-native plants in high latitude acidic-soil ecosystems such as the northern Scandes, are the physical modification of the environment and alteration of chemical properties of soils, for example with alkaline building materials enhancing soil pH (Müllerová *et al.*, 2011b)

Non-native plants occurring in this system are typically associated with AM fungi, as opposed to the natural vegetation which mostly associates with ecto- and ericoid mycorrhizal fungi, especially at high elevations (Wang & Qiu, 2006; Newsham *et al.*, 2009; Lembrechts *et al.*, 2014). These native mycorrhizal fungi are better adapted to low temperatures, low soil pH and slow cycling of nutrients locked up in recalcitrant litter compared to AM fungi (Read, 1991; Soudzilovskaia *et al.*, 2015). We therefore expect the previously mentioned changes caused by road disturbance, such as increased soil pH and nutrient availability, to lead to a more suitable environment for AM fungi and in turn for non-native AM associated plant species which would benefit from the increased AM fungi availability. Conversely, a lack of appropriate AM fungi in the natural vegetation might constrain the expansion of non-native plants away

from roadsides. Furthermore, we know from previous studies (Lembrechts et al., 2014) that non-native plant species richness in our study system decreases with increasing elevation, with no non-native species currently present above the tree line. This pattern coincides with the globally observed decline in non-native plant species richness along elevation gradients (Alexander et al., 2011). We hypothesize that this could be in part caused by a lack of adequate mycorrhizal fungal partners, as the harsher conditions at high elevations are likely to be less suitable for AM fungi (Bueno *et al.*, 2017). Finally, the presence of the non-native plant species could lead, independently of the direct road effect, to a further increase in AM fungi colonisation in their surrounding vegetation, as observed in other systems (Stinson et al., 2006; Lekberg et al., 2013).

In this study, we assessed the distribution of AM fungi in the roots of three non-native AM plant species invading the northern Scandes: *Trifolium pratense* L.; *Trifolium repens* L.; and *Achillea millefolium* L. These are the three most common non-native plant species in the region (Lembrechts et al., 2014), but have yet to reach a state in which they could be considered as invasive, as their impact on the ecosystem is currently minimal. We also assessed AM fungi in the roots of the surrounding native vegetation where the non-native species are mostly absent. Sampling was performed along three elevational gradients from sea-level to the alpine zone above the treeline at around 700 m a.s.l. to test the following hypotheses:

**H1:** There is a positive correlation between road disturbance and AM fungal abundance and diversity, which plays a role in the success of non-native plant species spread along roadsides.

**H2:** AM fungal abundance and diversity diminish towards higher elevations, which might limit the upward expansion of non-native plant species.

**H3:** The presence of non-native plant species along disturbed roadsides correlates with increased presence of AM fungi in their surrounding roadside environment.

## Materials and Methods

### *Study region*

The study sites are located in the northern Scandes, 220 km north of the Arctic Circle in the vicinity of the city of Narvik, Norway (68°26'N, 17°25'E). Three mountain roads were selected, hereafter called R1, R2 and R3 (see Fig. **1a**), reaching respectively from sea level up to 609, 697 and 633 m a.s.l. across lengths of 7.1, 26.4 and 20 km. The roads are made of asphalt at lower elevations, turn to gravel upon reaching higher elevations and are flanked by drainage systems (see pictures Fig. **S1**). These roads were built in the

1980s, are maintained through yearly mowing and gravel addition approximately every three year and are used regularly in summer by cars and trucks for tourism and to access high elevation hydropower plants for two of the roads. The elevational gradients crossed by these roads allow us to observe the impact of roadside disturbances on mycorrhizal fungal communities across a wide climatic range. Vascular plant communities along these roads have been monitored since 2012 in the framework of a global long-term study on native and non-native plant species distributions in mountain ecosystems (The Mountain Invasion Research Network, MIREN, [www.mountaininvasions.org](http://www.mountaininvasions.org)), which revealed the communities to be in an early stage of colonisation by non-natives species (Lembrechts *et al.*, 2014). The vegetation along the roads transitions from birch dominated forests with pines and willows at low elevations, with an understory of *Vaccinium spp.* and *Empetrum hermaphroditum* Hagerup, towards alpine shrublands at higher elevation mainly composed of a range of ericaceous dwarf shrubs (Lembrechts *et al.*, 2014). These vegetation types are dominated by ecto- and ericoid mycorrhizal plant types (Bueno *et al.*, 2017). However, AM fungi are still likely to be present in association with native forbs and grasses such as *Solidago virgaurea* L. or *Calamagrostis purpurea* Trin., and various mosses, which occur along the whole elevational gradient in the study system.

We studied the distribution and mycorrhizal associations of the three most common non-native plant species spreading towards higher elevations along the mountain roads in the region (Lembrechts *et al.*, 2014). These are *Achillea Millefolium* L., *Trifolium repens* L., and *Trifolium pratense* L., all three being AM associated plant species (Wang & Qiu, 2006). As a native reference species, we included *Solidago virgaurea* L., which is the most common native AM plant species found along the whole studied elevational gradient, both along the disturbed roadsides and inside the undisturbed natural vegetation. The native and non-native status of these species was previously assessed in the study by Lembrechts *et al.*, 2014, with non-native plant species being defined as species having been introduced into the northern third of Norway from another region after 1492. *A. millefolium* is known as "facultative" AM plant species (known to have non-mycorrhized occurrences), while *T. repens*, *T. pratense* and *S. virgaurea* are known to be an "obligate" AM plant species (Wang & Qiu, 2006), although these delimitations should be interpreted with caution (Brundrett & Tedersoo 2019).

#### *Sampling design*

The three studied roads were each divided into segments with intervals of, on average, 111 m of elevation. One transect was established at each segment junction, resulting in 7 transects along roads R2 and R3, and 5 transects along the shorter road R1, for a total of 19 transects covering the three

elevational gradients (Fig. 1b). Each of those transects was then further divided into two 2 m × 50 m plots organized in a T-shape, with one plot following the road and the other perpendicular to the first plot, extending from the road to 50 m into the undisturbed vegetation (Fig. 1c). This specific T-shaped set-up follows the MIREN design, aimed at the long-term survey of plant species composition along mountain roads as initiated in the region in 2012 (Seipel *et al.*, 2012; Lembrechts *et al.*, 2014). The presence or absence and estimated cover of each of the focal plant species was recorded in each of these plots in parallel with the sampling for mycorrhizal analysis. The sampling was done over a period of a month from July to early August 2017. To reduce the potential confounding effect of the difference in phenology between the start and the end of our field season, uneven numbered transects were surveyed and sampled at the start of the fieldwork period, while even transects were sampled at the end.

Four root samples (henceforth referred to as background samples) were taken for AM fungal measurement (see below) in each of the 19 transects. These four background samples were split between 2 disturbed vegetation samples and 2 undisturbed vegetation samples. All background samples consisted of three pooled topsoil cores of 5 cm diameter and 5 cm depth taken inside a 20 cm × 20 cm square which included a random assortment of roots from the surrounding vegetation (See Fig. 1). The two natural vegetation samples were taken at medium (10 m) and far (40 m) distances from the road to verify if there was a difference caused by proximity to the disturbance as Lembrechts *et al.* (2014) showed that the roadside disturbance effect on vegetation did not extent further than 25 m from the road, yet personal observations indicated that any roadside effect on the community was no longer observable around 5 m. Initially, five background samples were taken regularly along each roadside, although practical constraints kept us from individually processing all five samples. We kept one of those samples and pooled the remaining four, resulting in two disturbed vegetation samples per transect. We found no difference in colonisation or diversity between the pooled and non-pooled samples and thus decided to keep this pooling approach in the analysis.

In addition to these background samples, up to four root samples per transect were taken for each of the focal plant species, when present in one of the plots, and subjected to AM fungal measurements. For those focal plant species, a sample consisted of the roots of one individual excavated from inside the transect (See Table S1 for the list of all background and focal plant species samples). Among the focal plant species, only *S. virgaurea* and *A. millefolium* (rarely) were found and sampled in the undisturbed vegetation and therefore the majority (89 % of non-native species, and 75 % for *S. virgaurea*) of focal plant species' root samples originated from the disturbed vegetation plots.



All root samples from both background and focal plant species samples were cleaned in demineralised water over a 2 mm mesh size sieve to remove the soil material, after which fine roots were cut into 1 cm pieces for further analysis of AM fungal colonisation and community composition. Finally, two soil samples were taken in each transect in the same way as the root background samples, one taken in the disturbed vegetation and the other in the undisturbed vegetation at 40 m from the road. With these two samples, we measured soil pH (using KCl-extractions), available P (using P Olsen (Olsen *et al.*, 1954)) and mineral N ( $\text{NH}_4^+$  and  $\text{NO}_3^-$ , using KCl extractions) to assess the abiotic differences between disturbed and undisturbed vegetation (Table S2).

#### *Arbuscular Mycorrhizal fungal root colonisation and molecular analysis*

Both AM fungal root colonisation and community composition were measured for all background and focal plant species samples. The root colonisation rate of AM fungi was measured by counting mycorrhizal structures (aggregating hyphae, arbuscules and vesicles) under the microscope using the gridline and intersection method described in McGonigle *et al.* (1990). This method obtains the proportion (%) of root length colonised by AM. For this purpose, root samples were cleared using a 5 % KOH solution and cut into on average twenty pieces of 1 cm before being stained using a solution of 10 % Schaeffer black ink and 10 % acetic acid, as described by Vierheilig *et al.* (2005).

For DNA-based barcoding of the AM fungi community of each sample, a subset of ten randomly selected 1 cm root pieces was lyophilised and pulverized with sterile tungsten beads in a grinder that holds Eppendorf tubes, vigorously shaken for 60 s, after which the DNA was extracted using DNeasy PowerSoil Kit following the standard protocol (Qiagen, Venlo, the Netherlands). We targeted the AM fungi 18S rDNA using the primer pair AMV4.5NF/AMDG (Sato *et al.*, 2005; Van Geel *et al.*, 2014), augmented with multiplexing barcodes and sequencing adapters in a second polymerase chain reaction (PCR) step. The first PCR was performed in 25  $\mu\text{l}$  volumes using 1  $\mu\text{l}$  of template, 400 nM of both primers, 1X PCR buffer, 200  $\mu\text{M}$  of each dNTP and 1 unit of polymerase from the Phusion High-Fidelity DNA Polymerase kit (New England Biolabs, Ipswich, USA). The PCR conditions were: initial denaturation at 98 °C for 30 s; 30 cycles of denaturation at 98 °C for 30 s; annealing at 65 °C for 30 s and extension at 72 °C for 30 s; and a final step at 72 °C for 10 min. Successful amplification was confirmed using agarose gel electrophoresis and samples that failed to produce PCR products were run again for 40 cycles. Samples that did not successfully produce PCR products after the second attempt (about 19 % of the samples) were excluded. The second PCR used 1  $\mu\text{l}$  of a 1:100 dilution of product of the first PCR, 200 nM for both forward and reverse barcoded primers and was otherwise identical to the former PCR mix. The PCR conditions were: initial denaturation at 98 °C for 30 s; 10 cycles of denaturation at 98 °C for 10 s; annealing at 63 °C for 30 s and

extension at 72 °C for 30 s; and a final step of 72 °C for 10 min. Again, successful amplification was confirmed using agarose gel electrophoresis. The resulting 163 PCR products were purified and equalized using sequalprep plates (Thermo Fisher Scientific, Waltham, USA) before being pooled into a single library. A gel extraction was performed on the pooled library to ensure absence of primer-dimers, and further purified using QIAquick Gel Extraction Kit (Qiagen, Venlo, Netherlands). The library was then quantified using real-time PCR (KAPA Library Quantification Kit, Kapa Biosystems, Wilmington, MA, USA) and sequenced using the Illumina MiSeq platform (Illumina Inc; San Diego, CA, USA) with 300 cycles for forward and reverse reads and double indexing. The raw sequences were deposited in the National Center for Biotechnology Information's (NCBI's) Sequence Read Archive database under the accession no. PRJNA663438sky.

Note that AM fungi were studied in roots only, and not in soil samples. While some additional AMF taxa absent from root samples could have been picked up in soil samples, these would be non-associative and inactive AMF taxa which are not relevant to our study. We accounted for the possibility that different individuals and species would not include all AMF taxa present in the background vegetation by pooling roots from multiple individuals and species whenever present.

#### *Bioinformatics*

The USEARCH software was used following the UPARSE pipeline (Edgar, 2013) for the first steps of the bioinformatic analysis. Sequences were trimmed to 200 bp, paired end reads were merged, and primer sequences were removed. After quality filtering with a maximum expected error of 0.5, about 418,439 high quality sequences were kept. These reads were dereplicated and clustered into Operational Taxonomic Units (OTUs) using a threshold of 97 % similarity (Öpik et al. 2010; Lekberg et al. 2014). Chimera filtering resulted in the removal of 4.2 % of reads, leaving a total of 432 distinct OTUs. The resulting OTUs were then aligned against the AM fungi specialised MaarjAM database (Öpik *et al.*, 2010). Out of the resulting hits, only the ones with an identity score higher than 90 % were retained. Those sequences were then aligned against the Silva database, specialised in small and large subunits of ribosomal RNA (Yilmaz *et al.*, 2014) as well as against the full NCBI database (O'Leary *et al.*, 2016). Sequences which had lower E-values for non-AM sequences in SILVA or NCBI compared to their AM fungi alignment in MaarjAM were discarded as likely not being AM fungal sequences. The remaining 43 AM fungal OTUs (Table **S3**) were then rarefied to 200 reads per sample which has previously been shown to adequately cover AM fungal communities in roots (Van Geel *et al.*, 2018).

#### *Statistical analyses*

## 1) Arbuscular mycorrhizal fungal root colonisation

Models were made to test for the effects of both elevation, road disturbance and their interaction on the AM fungal root colonisation rate of background samples ( $N = 69$ ). Since AM root colonisation was measured as a proportion of discrete counts, we used beta regressions, following transformation of the response variable (i.e. proportion data) to avoid extreme values of 0 and 1 (Cribari-Neto & Zeileis, 2010) and using the `glmmTMB` package (Brooks *et al.*, 2017). As explanatory variables, we used elevation and disturbance (a two-level factor including disturbed vs undisturbed vegetation backgrounds) as well as their interaction term. The two-leveled disturbance variable was preferred over the three leveled variable including road, medium (10 m) and far (40 m) distance from the road as we tested for the effect of medium vs far amongst undisturbed vegetation and found no difference (GLMM,  $N=35$ ,  $R^2=-0.004$ ,  $P=0.65$ ) between the two distances. Undisturbed samples taken at 10 and 40 m were henceforth treated as repeated samples in the same plot. A random intercept term of plot nested in transect nested in road was included to account for our hierarchical sampling design. Model analysis was performed through model selection by comparing candidate models with all possible combinations of fixed effects derived from the full model and retaining only candidate models with a  $\Delta AICc$  (Akaike Information Criterion, corrected for small sample sizes) of less than 2 units compared to the best candidate model (Zuur *et al.*, 2009).

A similar approach was used to test for the effect of elevation and species identity on focal species' root colonisation rate ( $N = 92$ ). In this case, we used focal species AM root colonisation rate as a response variable, with species identity and elevation and their interaction as explanatory variables, with the same random intercept term as above. Disturbance was not included here due to the low number of observations of non-native species in the undisturbed natural vegetation (11 %).

To explore how disturbance (disturbed vs undisturbed) and elevation influence abiotic soil conditions, we ran linear mixed-effects models (lmer, Bates *et al.*, 2015) with soil pH, N and P as response variables, as a function of disturbance and elevation ( $N = 69$ ). Additionally, we tested the residuals of the background sample AM root colonisation models against soil pH, N and P to investigate whether these factors had an additional impact separate from the direct disturbance effect. Residual normality and homoscedasticity was first tested using the `DHARMA` package (Hartig, 2020) and all models showed residual normality and homoscedasticity. Then the aggregate residuals were obtained by weighted averaging of the residuals of each independent retained model ( $\Delta AICc < 2$ ). The latter residuals were then tested with linear mixed-effects models (lmer, Bates *et al.*, 2015) against soil pH, N and P, with the same random structure as

before. Similarly to the AM fungal root colonisation model, model selection was done by selecting all models with a  $\Delta\text{AICc}$  smaller than 2 from the best model.

## 2) Root fungal community composition

To test for the effects of elevation and disturbance on the OTU community composition of the background samples, PERMANOVAs were performed ( $N = 144$ ) using the `adonis` function from the R package `vegan` (Oksanen *et al.*, 2019). To consider the nested nature of our design and avoid pseudo-replication we then ran this PERMANOVA one thousand times, each time randomly dropping one of two replicates from our dataset (i.e. one of the disturbed vegetation samples and one of the undisturbed vegetation samples). We then assessed the distribution of  $R^2$ 's and P values across the thousand replicates to infer trends in the OTU community composition.

## 3) Relationship between non-native plant species presence and AM fungal root colonisation

To further disentangle whether non-native plant species presence influences rates of AM root colonisation independently of the direct effect of disturbance, we tested for the effect of non-native plant species presence/absence and soil pH, as a proxy for abiotic soil factors, on AM root colonisation rate of disturbed vegetation background samples, using the same approach as described in section 1 ( $N = 38$ ).

Additionally, we applied a variance partitioning procedure to determine the proportion of variance in disturbed vegetation background AM fungal root colonisation explained by both soil pH and the absence/presence of non-native plant species. To achieve this, we fitted LMMs with each explanatory variable (log-transformed) independently and one model including both together but without their interaction, using these to calculate the independent explained variance ( $R^2$  calculated using the method described in Nakagawa & Schielzeth, 2013) for both factors (variance explained by factor A = variance of the full model – variance of the model with only factor B), as well as their shared explained variance. We could not use the above-mentioned beta regression models for this variance partitioning approach, as calculating  $R^2$ -values for beta regression mixed models is not supported. Results from the variance partitioning procedure thus have to be interpreted with caution.

## Results

### 1) Arbuscular mycorrhizal fungal root colonisation

We found disturbance to be the strongest predictor of AM fungal root colonization rate in the background samples (Table 1a), with a higher colonization rate in the disturbed plots. This pattern was reinforced by the higher proportion of background samples in which AM fungi were found by visual examination of

stained roots in the disturbed vegetation compared to the adjacent undisturbed vegetation (76 % vs. 50 %). There was also a small decrease in the rate of AM fungal root colonisation with increasing elevation (Table 1a, Fig 2a), as well as an interaction between elevation and disturbance, showing AM fungal root colonisation rate to diminish less strongly with elevation in the undisturbed vegetation (maintained in one of the three best models only, however). Similarly, there was a slight decrease in AM fungal root colonisation rate with elevation amongst focal plant species samples (Table 1b, Fig 2b). The *Trifolium* species had much higher colonisation rates than the other two focal plant species (Fig. 2b). In line with their obligatory mycorrhizal status, the *Trifolium* species had much higher colonisation rates (100 %) than the other two focal plant species (Fig. 2b), as opposed to only 78 % in *A. millefolium*. Contrary to expectation from the literature, we found *S. virgaurea* was a facultative species for AM fungi colonisation as only 66 % of its samples were colonised by AM fungi (Table S1). *T. pratense* was found in 17% of the disturbed vegetation plots and *T.repens* in 39% but neither of the *Trifolium* species was ever observed in the undisturbed vegetation, while *A. millefolium* occurred in 5 % of all the undisturbed vegetation plots, compared to 37 % of the disturbed vegetation plots, and *S. virgaurea* was found in 70 % of the undisturbed vegetation plots and 97 % of the disturbed vegetation plots.

Soil pH was higher along the disturbed roadside compared to the undisturbed vegetation, while we found no difference for soil P and N (Table S4). Variation in soil pH was also the strongest abiotic predictor of remaining variation in the residuals of the background samples models, while soil P and N had a much weaker correlation with the residuals (Table 1c).

## 2) Root AM fungal community composition

Disturbed vegetation background samples showed a higher total richness of AM fungal OTUs than the undisturbed vegetation. A total of 34 OTUs was found in the disturbed vegetation, of which 23 were exclusive to this habitat type, as opposed to only 14 in the undisturbed vegetation backgrounds (3 unique, Fig. 3b). We found that OTU specificity was low across the focal plant species, with only 1 of the 15 most frequent OTUs across all samples (focal species and background samples) not present in each of the focal plant species (Fig. 5a). Six additional OTUs were found in the focal plant species roots that did not previously occur in the background samples, bringing the total to 43. The few OTUs restricted to one focal plant species were all rare, with the most common one occurring in only 20 % of its associated species samples and thus unlikely to be critical for that plant species' establishment.

The results of the whole dataset PERMANOVA showed AM fungal community composition to not change with elevation across all background samples combined (PERMANOVA,  $R^2 = 0.018$ ,  $F_{1,55} = 0.98$ ,  $P=0.46$ ),

or when considering road backgrounds (PERMANOVA,  $R^2 = 0.036$ ,  $F_{1,29} = 1.05$ ,  $P=0.41$ ) or undisturbed vegetation backgrounds (PERMANOVA,  $R^2 = 0.044$ ,  $F_{1,25} = 1.09$ ,  $P=0.36$ ) separately. It also showed a significant difference in AM fungal community composition between the disturbed and undisturbed vegetation backgrounds (PERMANOVA,  $R^2 = 0.346$ ,  $F_{1,55} = 1.94$ ,  $P=0.016$ ).

The random sampling approach across a thousand replications (Table S5) also shows little effect of elevation with less than 1 % of replicates resulting in a  $P$ -value inferior to 0.05. The same approach, when looking at the effect of disturbance, showed around 40 % of all replicates resulting in a  $P$ -value inferior to 0.05. This does denote a tendency for the AM fungal community composition to differ between the disturbed and undisturbed vegetations as we would expect only 5 % of replicates to have  $P$ -values inferior to 0.05 if there was no difference between two environments.

The elevation range of most OTUs extended from the lowest elevations upwards (Fig. 4, dots on the red line); only few, typically infrequent, OTUs were present exclusively at higher elevations (Fig. 4, dots below the red line). Furthermore, the most common OTUs were also those with the largest elevation range and were mostly found all the way up to the highest elevations, with 18 OTUs being found across the whole elevation gradient (Fig. 5b). Additionally, the distribution of many of the infrequent OTUs seemed similar to the distribution of the non-native plant species, with their maximum observed elevation range being mostly similar or slightly lower than that of the non-native plant species (Fig. 4, dots on the red line).

### 3) Relationship between non-native plant species presence and AM fungal root colonisation

We found that the rate of AM fungal colonisation in background samples was higher in transects where non-native plant species were present than in transects where no non-native plant species were found meaning that the vegetation was only composed of native plant species (Table 1d). We confirmed that this effect was not due to the former background samples potentially including non-native plant species roots as there was no difference in the rate of AM fungal root colonisation between background samples in proximity of non-native plants (i.e. non-natives occurred in accompanying vegetation survey of sample within plot, which was true for 35 % of all background samples) and the ones that did not ( $t$ -test,  $df= 18$ ,  $t(18) = 0.42$ ,  $p = 0.68$ ). Our variation partitioning approach showed a higher degree of variance in the rate of AM fungal root colonisation being explained by the presence of non-native plant species ( $R^2 = 0.297$ ) than by soil pH (as a proxy for soil factors,  $R^2 = 0.195$ ), while the two factors' shared variance was  $R^2 = 0.093$ .

## Discussion

Mycorrhizal fungi and other soil biota are increasingly recognized as key determinants of plant invasions and subsequent ecosystem transformations (Dickie *et al.*, 2017; Waller *et al.*, 2020). Disturbance along mountain roads is known to facilitate non-native plants, but whether mycorrhizal fungi play a decisive role in this process is hitherto unknown. Our results indicate a strong correlation between mountain road disturbance and AM fungal distribution, with higher AM fungi occurrence, a more diverse AM fungal community (Fig. 3b), and higher root colonisation rate in the disturbed vegetation (Fig 2b, Fig. 3a) compared to the undisturbed natural vegetation, which is in line with our first hypothesis (H1). This difference most likely results from the striking contrast in biotic and abiotic conditions between the two environments (Müllerová *et al.*, 2011a). Arctic forests and heathlands such as found in the Scandes are known for slow nutrient cycling, high organic matter content and low soil pH conditions favourable to ecto- and ericoid-mycorrhizal species, while AM fungi and AM plants tend to be more abundant in environments with faster nutrient cycling and are less tolerant of low soil pH conditions (Soudzilovskaia *et al.*, 2015; Steidinger *et al.*, 2019b). As expected from the literature (Müllerová *et al.*, 2011a), we did find a clear relationship between road disturbance and changes in soil pH which was higher in the disturbed vegetation (Fig. S2). That difference in soil pH should lead to a more benign environment for AM fungi compared to the undisturbed vegetation (Van Aarle *et al.*, 2002), and help explain our observed pattern of AM fungal distribution. This is reinforced by the results of the models testing our measured abiotic factors against the residuals of our initial models (respectively Table 1a and 1c) which show soil pH to be a strong additional predictor of increased AM fungal root colonisation rate whereas soil N and P play only a marginal role. The effect of disturbance on AM fungi is also illustrated by an increased abundance of native ruderal species known to associate with AM fungi in the disturbed vegetation compared to the undisturbed vegetation, which is dominated by plants typically associated with ecto- and ericoid-mycorrhizal fungi (Lembrechts *et al.*, 2014).

This strong contrast between disturbed and undisturbed conditions, combined with the reliance of the non-native plant species on their AM fungal symbionts, suggest that a lack of, and unsuitable conditions for, AM fungi are likely to be an overlooked barrier to the spread of non-native plant species from roadsides towards the undisturbed vegetation (McDougall *et al.*, 2018). The fact that out of our three non-native focal plant species, only the facultative *A. millefolium* was observed in the undisturbed vegetation as opposed to the two obligatory mycorrhizal *Trifolium* species is another observation supporting this argument. It is however difficult through observational data alone to infer the importance of AM fungal limitation amongst other factors preventing non-native success in the undisturbed vegetation (Lembrechts *et al.*, 2016; McDougall *et al.*, 2018). For example, we observe higher soil

temperatures along roadsides in this region (unpublished data in the context of the SoilTemp project (Lembrechts *et al.*, 2020)) which could lead to a faster phenological cycle compared to the undisturbed vegetation and partly explain the distribution patterns of non-native plants. Further factors such as reduced biotic interactions between plant species, or shorter growing seasons in the undisturbed vegetation could also play a role in limiting the success of non-native plant species away from the disturbed roadsides. Disentangling all these possible explanations behind the observed patterns will however require further experimental effort. Nevertheless, the coincidence between the observed current distribution of non-native plants and root colonization intensity by AM fungi, combined with the strong positive correlation between road disturbance and root colonization rate by AM fungi, suggests that the impact of disturbance on belowground symbiosis plays an important role in driving the plant invasion patterns that were observed in our system.

Contrary to our expectation (H2), there was little effect of elevation on AM fungal distribution. The rate of AM fungal root colonisation only slightly diminished with elevation, in both disturbed and undisturbed vegetation (Table 1a, Fig 2a). This shows that AM fungi are already present above the current upper limit of the studied non-native plant species' elevational ranges thanks to their association with native AM plant species, such as for example *S. virgaurea*, which are widely present, but not dominant, in the natural vegetation. Furthermore, there was no effect of elevation on AM fungi community composition amongst both disturbed and undisturbed background samples. Importantly, we also found all our focal plant species to associate with any of the most common AM fungal OTUs which we found to be already present across the whole elevation gradient, including above the current upper elevational range limit of the non-native plant species (Fig. 4, 5b). These observations indicate that an absence of suitable AM fungi is currently not a limiting factor for the upward spread of non-native plants in the region, as has also been concluded by others, for example by Oehl & Körner (2014) in the Swiss Alps and by Kotlínek *et al.* (2017) in the Himalayas. The spread of non-native plant species to the higher elevation disturbed roadsides is thus more likely to be limited by climatic factors (for example colder temperatures leading to reduced winter survival (Haider *et al.*, 2011)), weaker propagule pressure or even reduced efficiency of AM fungi mycorrhizal symbiosis due to the slow decomposing litter types most found under arctic climates (Steidinger *et al.*, 2019a), rather than by the unavailability of mycorrhizas themselves (Ruotsalainen *et al.*, 2004; Alexander *et al.*, 2016; Lembrechts *et al.*, 2016).

Finally, we observed a pattern of overall higher AM fungal colonisation rates in disturbed vegetation plots when non-native plant species were present (Fig. 3c). This could have multiple cause: high rates of AM fungal colonisation being a driver of non-native plant success, AM fungi being passengers by following



changes in non-native plant distribution, or a third factor – for example disturbance – positively affecting both AM fungi colonisation and non-native plant species success in a concomitant manner (Zobel & Opik, 2014). Our observation that non-native presence is a better predictor of AM fungal colonisation rate than soil pH, even though the latter is likely to be a dominant environmental filter in this system, suggests that the presence of non-native plant species is likely to be driving increases in AM fungal colonisation rate. Neither explanations are however mutually exclusive, and both non-native plant species promotion of associated mycorrhizas and the mycorrhizal facilitation of non-native plant species success have been previously observed across different habitats (Richardson *et al.*, 2000; Reinhart & Callaway, 2006; Shah & Reshi, 2009; Yang *et al.*, 2018). Of note, we also observed a number of less frequent AM fungi OTUs which happen to have similar ranges as the non-native plants. It is tempting, though speculative, to suggest that the higher AM plant densities brought about by invasion could lead to a richer AMF community by recruiting from co-dispersing AM fungi or from rare locally present AM fungal taxa (Chaudhary *et al.*, 2020), explaining the matching distribution patterns. This would mean that these OTUs matching non-native plant species distribution could be a sign of further changes in mycorrhizal background, potentially also facilitating further non-native plant success (Thakur *et al.*, 2019). Regardless, it is not possible to conclusively determine which of these mechanisms are at play in our system without access to a time series of AM fungal root colonisation rate and AM fungi community composition or experimental data. This should be an important avenue for future research, as a non-native driven positive effect on AM fungi could be self-reinforcing by facilitating the invasion success of other non-native plant species. This could increase our understanding of invasion dynamics and help develop successful intervention methods.

## Conclusion

Our results align with a possible facilitating role of mycorrhizal fungi on the establishment success of non-native plants through disturbance along roads, because (1) AM fungal abundance was elevated along the disturbed roadsides, to which non-native plants are largely restricted, along the whole elevation gradient in the northern Scandinavian mountains, and (2) increased AM fungal abundance correlated with high abundance of non-native plants within these roadsides. We conclude however that the movement of non-native plant species to higher elevation is not limited by mycorrhizal fungal presence per se as AM fungi occurred along the whole elevation range, including the AM fungal taxa the non-native plants were found to interact with most. Our results represent a crucial first step in understanding the combined effects of disturbance and mycorrhizal interactions on non-native plant species invasions and offer us new insights into the potential self-reinforcing effect of non-native species through their fungal interactions, which will require further research to be fully disentangled.

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## **Author contributions**

JC, JLembrechts, EV and IN designed the research. JC and JLembrechts conducted fieldwork. JC, JLembrechts and EV analysed data. JC wrote the manuscript with significant contributions from JLembrechts, EV, IN, JA, SH, JLenoir, AM, MN and AP.

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**Supplementary material:**

**Fig S1:** Pictures of disturbed roadside taken along our study roads.

**Fig S2:** Soil pH variation with elevation in the disturbed and undisturbed vegetation.

**Table S1:** List of all analysed root samples.

**Table S2:** Soil pH, available P and mineral N for each plot.

**Table S3:** List of retained OTUs identified as arbuscular mycorrhiza.

**Table S4:** Model selection for abiotic factors.

**Table S5:** Result distribution of randomized PERMANOVAs for arbuscular mycorrhiza fungal community composition.



## Figures and Tables:

**Figure 1: Sampling design along three mountain roads in the northern Scandinavian mountains.** **a)** Location of the studied mountain roads in the northern Scandinavian mountains, near Narvik, Norway, with map showing mean annual temperature from CHELSA (Karger *et al.*, 2017) for Scandinavia **b)** Transects were spread with fixed elevation steps along the whole elevation gradient covered by each road. **c)** Each transect was subdivided into 2 plots (white rectangles) following the MIREN protocol (Seipel *et al.*, 2012). Each plot was 2 x 50 m, the first plot following the road covered the area impacted by the road disturbance while the second plot extended into the undisturbed natural vegetation perpendicular to the road. For each plot, the presence and total cover of focal plant species (*Achillea millefolium*, *Trifolium repens*, *Trifolium pratense* and *Solidago virgaurea*) was measured. Five disturbed vegetation background root samples (orange squares) were taken in the roadside plot in randomly chosen locations and two undisturbed vegetation background samples (green squares) were taken 10 m and 40 m away from the road. Each background sample was composed of three pooled soil cores of 5 cm diameter by 5 cm depth (blue circle) taken in a 20 x 20 cm square. Roadside samples were further pooled (see Methods section). When present, up to four root samples of focal plant species were taken in the roadside (purple stars) for each focal plant species present. Two additional soil samples were taken in the roadside and at 40 m into the undisturbed vegetation for soil pH, P and N analysis (red circles). Figure adapted from Lembrechts *et al.* (2014).

**Figure 2:** Elevation effect on the percentage of arbuscular mycorrhizal (AM) fungal root colonisation along mountain roads in the northern Scandinavian mountains across a 700 m elevation gradient for background samples (**a**) including undisturbed vegetation background (green) and disturbed vegetation background (brown), as well as for four focal plant species (**b**): two obligatorily mycorrhizal non-native plant species *Trifolium repens* (blue) and *Trifolium pratense* (purple), one facultative mycorrhizal non-native plant species *Achillea millefolium* (green) and one facultative mycorrhizal native species *Solidago virgaurea* (orange). See Table 1 for the coefficients of the relationships.

**Figure 3: Effects of road disturbance on arbuscular mycorrhizal fungi (AMF) distribution along roadsides.** **a)** Violin plots (boxplot-like plots with horizontal width depending on number of samples at that specific percentage) of background AMF colonisation in the disturbed vegetation and in the undisturbed vegetation. **b)** Venn-diagram of AMF operational taxonomic unit (OTU) overlap between disturbed vegetation background (brown) and undisturbed vegetation background (green) communities. **c)** Violin plots of background AMF colonisation in the disturbed vegetation for plots with or without presence of non-native plant species.

**Figure 4: Elevation range of arbuscular mycorrhiza OTUs and focal plant species.** Relationship between elevation range and maximum elevation for each AM fungi OTU (circles; both background and focal samples combined) and each focal plant species (triangles; *Achillea millefolium* L., *Trifolium repens* L., *Trifolium pratense* L., *Solidago virgaurea* L.). OTUs and plant species close to the red line are found along the whole gradient from the lowest elevation up to their maximum elevation of occurrence. Exceptions are likely caused at least in part by limited

sample sizes, as illustrated by the colour gradient. The 18 most common OTUs (green, note some overlap of points) were present across most of the elevation gradient and above the current maximum elevation of non-native plant species.

**Figure 5: Pattern of OTU occurrence by focal plant species and background types.** The colour scale represents for each focal plant species (columns, named at the top, AM: *Achillea millefolium*, TP: *Trifolium pratense*, TR: *Trifolium repens* and SV: *Solidago virgaurea*) and background type (DB: Disturbed vegetation background and UB: undisturbed vegetation background) the percentage of samples in which each of 43 OTUs was found (ranging from 0 % to 100 %). a) OTUs ordered from low to high total occurrence following the direction of the arrow over the whole dataset. All the focal plant species associated with the most common OTUs (in green) and those OTUs are present in both undisturbed and disturbed vegetation backgrounds. b) OTUs ordered by their elevation range, from OTUs found in only one transect (i.e. a range of 0 m) to a range of 700 m, following the direction of the arrow. The most common OTUs (in green) are found across the largest elevation range.

**Table 1: Selected models explaining percentage root length colonised by Arbuscular Mycorrhizal Fungi:**

Coefficients (and their p-values, \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ ) for **a)** background samples, **b)** focal plant species, **c)** abiotic factors (in this case the response factor was the residuals from **a)** instead of AM fungal root colonisation), **d)** the effect of non-native plant species presence.

a) Background samples

Model	Intercept (Undisturbed vegetation)	Elevation	Disturbed vegetation	Elevation x Disturbed vegetation	AICc	$\Delta$ AICc
1	-2.376*** ( $P < 0.001$ )		0.689** ( $P = 0.002$ )		-164.6	0
2	-2.393*** ( $P < 0.001$ )	-0.132 ( $P = 0.231$ )	0.704** ( $P = 0.002$ )		-164	0.6
3	-2.396*** ( $P < 0.001$ )	-0.014 ( $P = 0.936$ )	0.700** ( $P = 0.002$ )	-0.193 ( $P = 0.392$ )	-162.7	1.9

b) Focal plant species

Model	Intercept ( <i>Achillea</i> <i>millefolium</i> )	Elevation	<i>Solidago</i> <i>virgaurea</i>	<i>Trifolium</i> <i>pratense</i>	<i>Trifolium</i> <i>repense</i>	AICc	$\Delta$ AICc
1	-1.417*** ( $P < 0.001$ )		-0.386 ( $P = 0.149$ )	1.597*** ( $P < 0.001$ )	1.998*** ( $P < 0.001$ )	-108.2	0
2	-1.486*** ( $P < 0.001$ )	-0.131 ( $P = 0.323$ )	-0.216 ( $P = 0.499$ )	1.583*** ( $P < 0.001$ )	2.019*** ( $P < 0.001$ )	-107.2	1

c) Abiotic factors

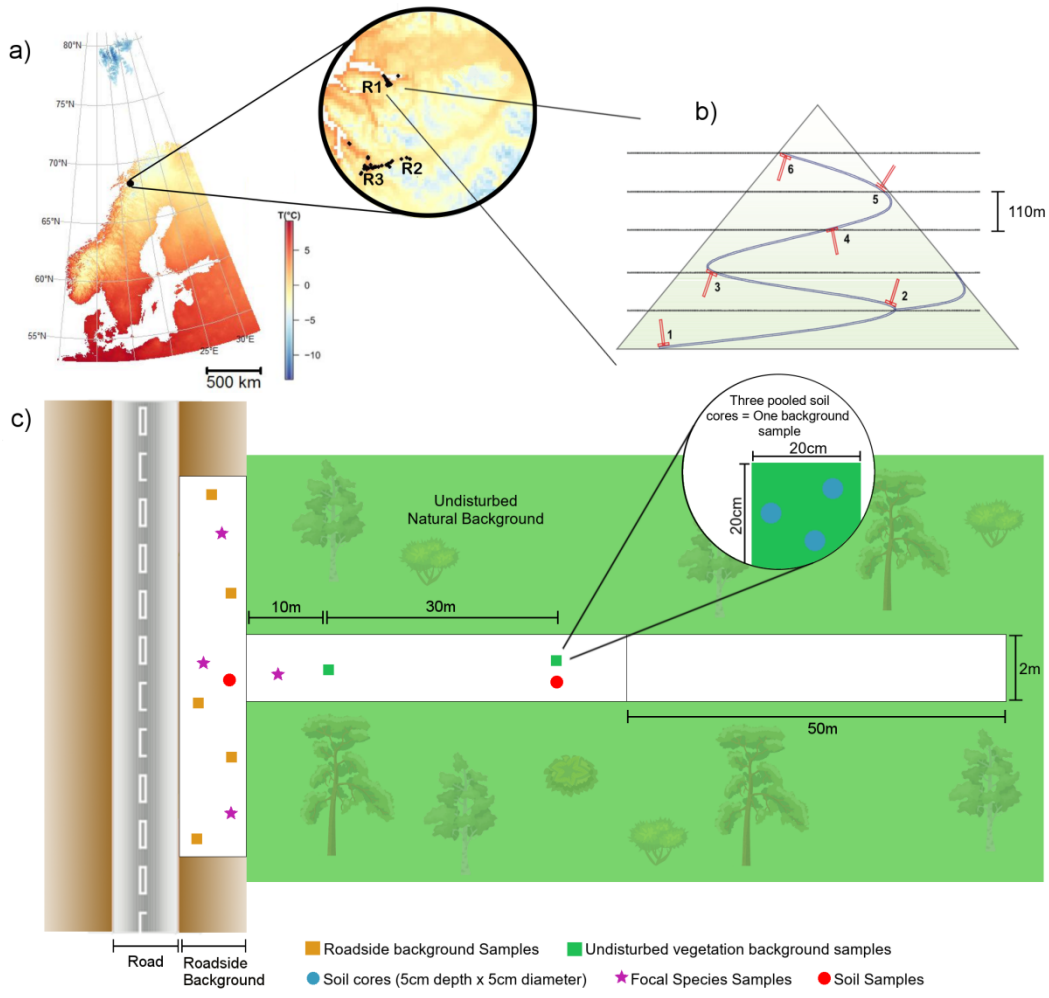
Model	Intercept	pH	N	P	AICc	$\Delta$ AICc
1	-0.209*** ( $P < 0.001$ )	0.052** ( $P = 0.003$ )			-90.2	0
2	-0.238*** ( $P < 0.001$ )	0.057** ( $P = 0.002$ )	0.002 ( $P = 0.250$ )		-89.6	0.6
3	-0.215*** ( $P < 0.001$ )	0.053** ( $P = 0.005$ )		0.0001 ( $P = 0.877$ )	-88.3	1.9

d) Non-native plant species

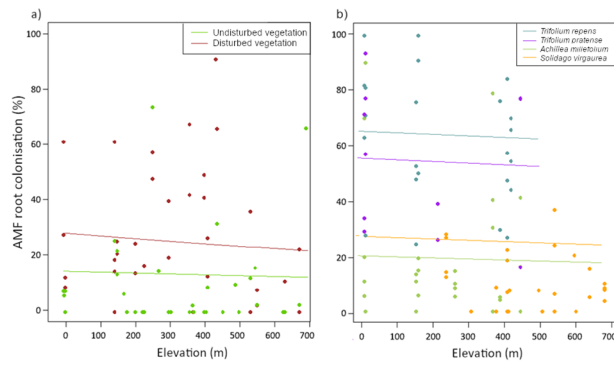
Model	Intercept (Absence of non-natives)	pH	Presence of non-natives	pH x Presence of non-natives	AICc	Δ AICc
1	-5.450*** (P<0.001)	0.600** (P=0.001)	1.320*** (P<0.001)		-83.9	0
2	-4.503*** (P<0.001)	0.454** (P=0.002)	1.116*** (P<0.001)		-82.5	1.4
3	-5.663*** (P<0.001)	0.645** (P=0.001)	2.397 (P=0.242)	-0.223 (P=0.595)	-82.2	1.7

Model selection was performed by selecting all models with a delta AICc < 2 from the best model (i.e. Model 1).

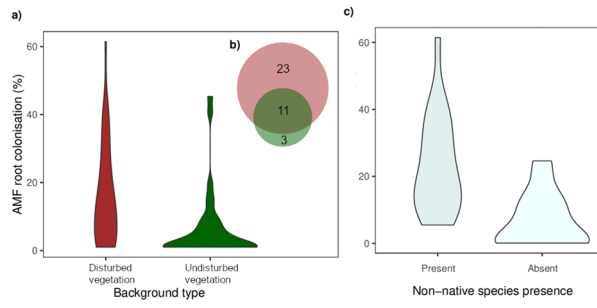
Blank spaces represent explanatory variables that were not retained in a given model. The factor level that serves as intercept is alphabetically assigned; other factor levels are compared to this baseline effect. The symbol “x” in between factors denotes an interaction.



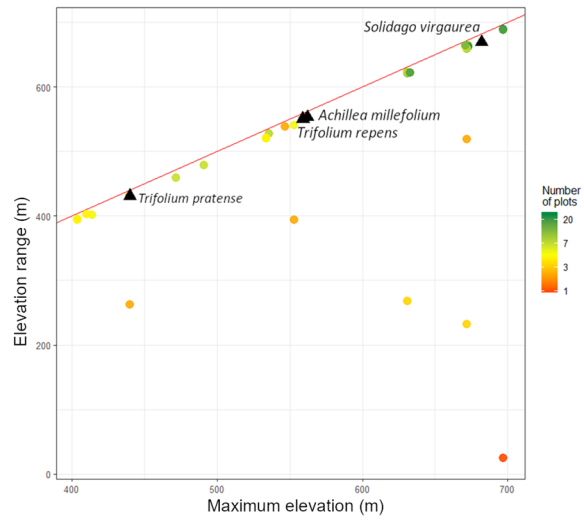
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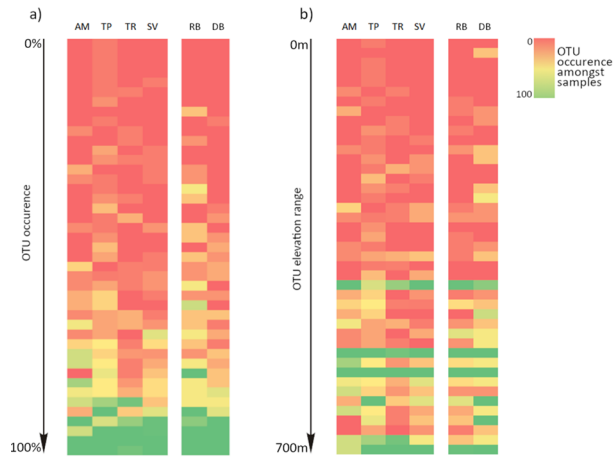


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