Plant invasion shifts soil microbiome and nutrient pools along altitudinal gradients

Khalid Hussain[1], Rameez Ahmad[1], Martin Nuñez[2], Tanvir Ul Dar[3], Irfan Rashid[1], and Anzar Khuroo[1]

[1]University of Kashmir
[2]University of Houston
[3]Baba Ghulam Shah Badshah University

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Abstract

Soil microbial communities, being at the interface of plant-soil feedback systems, can play a pivotal role in facilitating ecosystem response to the drivers of global environmental change, including invasive alien species (IAS). Studies evaluating the effect of plant invasion on soil microbial communities along altitudinal gradients can provide novel insights regarding the spread and impact of IAS and elevational range shifts in response to ongoing climate warming in mountains. In this study, we used metagenomic tools to investigate the impact of invasive Leucanthemum vulgare on taxonomic composition, relative abundance, alpha and beta diversity of soil microbial (bacterial and fungal) community, physicochemical properties and their interaction between invaded and uninvaded plots selected across multiple sites along an altitudinal gradient in Kashmir Himalaya. We found that the invasion by L. vulgare shifted composition, relative abundance, alpha and beta diversity of soil microbiome significantly in invaded plots. The relative abundance of soil microbial communities interestingly showed an increasing trend along the altitudinal gradient. The soil physicochemical properties were significantly correlated with microbial community abundance with temperature, K, pH, EC and Fe being the dominant determinants. Also, we found a significant effect of soil physicochemical properties on the microbial community abundance along the altitudinal gradient. Our findings unravel the plant invasion-induced shifts in the underground soil microbiome and physicochemical properties, which improve our understanding of plant invasion impacts on belowground biotic and abiotic components and can contribute in guiding integrated management of invaded mountain landscapes globally.

Introduction

The impacts of invasive alien plants (IAPs) are generally considered context-specific and vary across species, regions, ecosystems and habitat types (Pyšek et al., 2020). In recent times, several studies have investigated the ecological impacts of IAPs in order to unravel the invasion patterns and the underlying processes (Vilà et al., 2011; Tekiela and Barney 2017; Uddin & Robinson 2017; Pyšek et al., 2020). Nonetheless, majority of these studies have focused on the impact of plant invasions on aboveground community characteristics and ecosystem processes (Ahmad et al., 2019b), while as the impacts of IAPs on belowground soil biotic and abiotic components are relatively less investigated, although known to play a pivotal role in the success of IAPs in non-native regions (Bell et al., 2020). Our limited understanding of microbial community response to IAPs in belowground soil system stems from the fact that soil microbial diversity and abundance is dependent upon a number of edaphic factors as well as the management history of the invaded landscapes, which has impeded our ability to generalize impacts of plant invasion on soil microbial communities and as such remains an important research gap in invasion biology (Custer & van Diepen 2020).

The soil microbial communities, being situated at the interface between plant-soil-atmosphere systems, are

likely to play a crucial role in facilitating ecosystem response to the drivers of global environmental change, including invasive species (Bell et al., 2020). Invasive species, coupled with ongoing climate change, can disrupt the essential plant-microbe interactions from local to global scales (Cavicchioli et al., 2019, Rudgers et al., 2020). Therefore, a better understanding of climate-driven plant-microbe interactions over space, from individual plots to the entire landscapes is urgently required (Rudgers et al., 2020). Such studies, while considering plant-microbe interactions across broad spatial gradients (e.g. altitude), can aid in detecting potential nonlinear climate sensitivity functions and has the potential to provide a robust tool to predict the effects of increasing climate variability on belowground microbial communities (Cavicchioli et al., 2019; Rudgers et al., 2020). In an era of global environmental change, the belowground response to plant invasion is crucial because these soil microorganisms perform fundamental role in regulating key ecosystem processes, including the decomposition and nutrient cycling. In addition to these critical ecological processes, soil physicochemical properties determine the establishment and spread of IAPs (Nuñez et al., 2009; Dawson & Schrama 2016; Ricciardi et al., 2017). Given the crucial role that the unseen soil microbial communities play in regulating the ecosystem functioning and thereby facilitating and/or impeding the process of plant invasion, (Dawson & Schrama 2016; Ricciardi et al., 2017; Custer & van Diepen 2020) exploring the response of soil microbial communities to plant invasion merits adequate research attention.

Although, some research attention has been directed in the recent past to study the impacts of IAPs on soil biotic and abiotic communities and their potential feedbacks to the process of invasion (Dawson & Schrama 2016; Ricciardi et al., 2017; Custer & van Diepen 2020; Duchesneau et al., 2021), however the direction and magnitude of the associated changes are highly variable (Vilà et al., 2011; Zhang et al., 2019; Custer & van Diepen 2020). The differences in the response of soil biotic and abiotic communities to plant invasion may be an outcome of local environmental conditions, traits of the plant invader itself, and/or the interaction of both (Vilà et al., 2011; Hulme et al., 2013). Other than the vegetation type itself, the local micro-climatic conditions (e.g. temperature, precipitation) are considered to be the prime determinants of soil biotic and abiotic components (Massaccesi et al., 2020; Tang et al., 2020). Several environmental gradients like latitude and altitude (Cardelli et al., 2019) have been used to investigate the regional and global patterns in plant invasion impacts. Altitude has been often used as a useful proxy to unravel the effect of micro-climatic changes along environmental gradients on belowground soil biotic and abiotic components (Massaccesi et al., 2020; Tang et al., 2020). As invasion success of IAPs at high altitudinal ranges is attributed to broad climatic tolerance combined with enough residence time in a given region, mountains are more prone to invasions with rapid spread of these species under ongoing climate change (Pauchard et al., 2009; Pyšek et al., 2011). The altitudinal gradients in mountainous landscapes provide the natural experimental systems to investigate the patterns and processes of IAPs across multiple sites within a shortest spatial distance (Ahmad et al., 2019a, b). Such type of studies along increasing altitudinal gradient can provide novel insights regarding the expected up-climbing of IAPs in response to ongoing climate warming in mountains (Lamsal et al., 2018; Thapa et al., 2018). Towards this end, the recently emerging metagenomic tools offer opportunities to characterise soil microbiome at an unprecedented scale and taxonomic resolution (Fricker et al., 2019). Such an approach can be an important step forward in understanding the structural, functional and interaction aspects of this complex belowground soil system, and has huge implications in developing scientifically well-designed and integrated management strategies to restore the invaded landscapes (Ricciardi et al,. 2017; Custer & van Diepen 2020; Tang et al., 2020; Rudgers et al., 2020).

In recent times, rapid land-use and climate changes in mountains have made them highly vulnerable to the risks of plant invasions (Lamsal et al., 2018; Thapa et al., 2018; Pathak et al., 2019). The Himalaya – one of global biodiversity hotspot – is increasingly experiencing the introduction and invasion of alien plants with severe ecological and economic impacts (Ahmad et al., 2019a, b; Lamsal et al., 2018; Thapa et al., 2018; Pathak et al., 2019). Kashmir Himalaya –an important region of the Himalayan biodiversity hotspot – is recently experiencing a rising risk from the spread of IAPs (Khuroo et al., 2010; Ahmad et al., 2019a,b). One such invasive plant is *Leucanthemum vulgare* Lam. (Ox-eye daisy), which is fast invading the high-altitude landscapes of this Himalayan region and has reported to impact the native biodiversity, community diversity and soil system (Khuroo et al., 2010; Ahmad et al., 2019a, b, 2021).

The present study, employing metagenomic tools, investigated the composition and abundance patterns of soil microbiome (bacterial and fungal communities) between invaded and uninvaded plots at multiple sites selected along an altitudinal gradient in the Kashmir Himalaya using *L. vulgare* as a model invasive plant species. Simultaneously, we also studied soil nutrient pools between the invaded and uninvaded plots to explore the possible role of nutrients in shaping bacterial, fungal and *L.vulgare* invasion patterns along the altitudinal gradient. More specifically, we aimed to address the following research questions: (i) Does the species composition and relative abundance of soil microbiome (bacterial and fungal communities) differ consistently between the invaded and uninvaded plots across the multiple sites along the altitudinal gradient in Kashmir Himalaya? (ii) Do the patterns of alpha and beta diversity in soil microbial communities differ between invaded and uninvaded plots across the sites along the altitudinal gradient? (iii) Whether invasive *L. vulgare*exerts a consistent effect on key soil nutrient pools along the altitudinal gradient? (iv)Which environmental and soil physicochemical factors best explain the abundance patterns of soil microbiome between invaded and uninvaded plots along the altitudinal gradient? Considering plant invasion as one of the major drivers of global environmental change, and with soil microorganisms occupying a pivotal position in the terrestrial habitats, the present study while addressing the afore-mentioned research questions will be helpful in devising efficient management policies on plant invasions and effective restoration of invaded landscapes.

Materials and Methods

Site selection and sampling design

The present study was conducted in the Kashmir Himalayan region which is recognized as a biogeographical province situated along the north-western side of the Himalaya (Dar & Khuroo 2020). The region is surrounded by Zanskar Range of the Greater Himalaya in the north and northeast and PirPanjal Range of the Lesser Himalaya in the south and south-west. The study region lies between 32° 20' to 34deg 54' N latitude and 73deg55' to 75deg35' E longitude and covers an area of about 15,948 km^2 with 64% of the region being mountainous (Husain 2002; Khuroo et al., 2007). Due to relatively dry and hot summers followed by wet and cold winters, temperature usually fluctuates between 15degC - 31degC during summer and -4degC - 4degC in winter. The average annual precipitation received by the region, most of which comes in the form of snow during winter, is *ca.* 1055 mm. Coniferous evergreen forests and the alpine meadows are the characteristic natural vegetation types of the region (Dar & Khuroo 2013).

Field surveys were conducted across the study region for selection of appropriate sampling sites. In the region, the model invasive plant species (i.e., *Leucanthemum vulgare*) occurs along an altitudinal gradient ranging from *c.* 1700 to 2900 metres above sea level (m.a.s.l) (Ahmad et al., 2019a, b). Therefore, for the present study, we adopted the space-for-time substitution approach (Diekmann et al., 2016; Kiełtyk & Delimat 2019) by following coordinated distribution experiment methodology developed by the Global Invader Impact Network (Barney et al., 2015). This approach adopts the within-site comparison, wherein invaded and uninvaded plots that are located as close as possible are compared (Ahmad et al., 2019 a, b). Being the most preferred methodological design adopted globally for standardized evaluation of ecological impacts of invasive species, this method allows direct attribution of any ecological impacts to a specific invasive species (Barney et al., 2015; Tekiela & Barney 2017; Ahmad et al., 2021; Brooks et al., 2021). A total of four sampling sites namely Kunzar (KZ), Tangmarg (TM), Kashmir University–Gulmarg Research Station (KU) and Kongdoori (KD) were selected along the altitudinal gradient, where Kunzar is the low-altitude and Kongdoori the high-altitude site (Fig. 1). For soil microbiome and nutrient analysis, at each sampling site, a suite of two different types of plots (i) invaded (with *L. vulgare* present and well colonized i.e., $>$ 50% cover), and spatially separated (ii) uninvaded (*L. vulgare* absent) plots were selected. At each site, the two types of plots were selected in such a manner that they were environmentally similar (i.e., almost similar slope, aspect, vegetation, land-use history).

Soil sampling

For soil microbiome and nutrient pool studies, we collected soil samples from the four sampling sites along

the altitudinal gradient during the peak vegetation season (June-July 2019). At each site, we collected soil samples in triplicates from the invaded (IN) plot and one sample from uninvaded (UN) plot which served as a control. To maintain the viability of the soil microbiota sampled at each site, we employed proper soil collection and storage protocols (McPherson et al., 2018). For each sample, we collected *ca.* 1000g of soil using sterilized gloves and placed them in sampling bags. Digging equipment was also sterilized with bleach and ethanol after each soil sample collection to avoid any possible cross-contamination of soil biota. The collected samples were immediately transported to the laboratory and processed in less than 6 hours after collection from the field. All the visible macrobiota and roots were removed from the soil and sieved using a mesh of 2mm pore size. The sieved soil samples for the microbiome studies were stored in 50 ml tubes and kept inside deep freezer at -80^{o}C, while as, for nutrient studies all the soil samples were air dried and stored at -4^{o}C till further analysis.

DNA extraction, PCR amplification and high throughput sequencing

Total genomic DNA was extracted from 250 mg of soil per sample using the PowerSoil? DNA Isolation Minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA integrity was checked on 1.0% (w/v) agarose gel using 1X TAE (Tris acetate EDTA) as running buffer. The quantification of isolated DNA was carried out by Qubit^{TM} dsDNA BR assay kit (Thermo Fisher Scientific MA, USA).

Identification of bacterial and fungal microbiota was accomplished by amplicon sequencing of the 16S rRNA and ITS2 genes, respectively. 20 nanograms (ng) of DNA was used to amplify hyper variable (V3-V4) region of the prokaryotic small-subunit (16S) rRNA and the internal transcribed spacer region 2 (ITS2) of the fungal organisms using KAPA HiFi HotStart Ready Mix PCR Kit (Kapa Biosystems, Wilmington, MA, USA). The reaction mixture included KAPA HiFi Hot Start Ready Mix (2X), 100 nm concentration of primers and 20 ng of DNA. The PCR involved an initial denaturation of 95degC for 5 min followed by 25 cycles of 95degC for 30s, 55degC for 45s and 72degC for 30s and a final extension at 72degC for 7 min and holding at 4degC. After amplification, the PCR products were cleaned up, using 0.8X AMPure XP beads (Beckmann-Coulter, CA, USA) to remove unused primers and eluted in 15 µls of 0.1X TE buffer. 5 µLs of the purified PCR product from each sample was taken for performing P7 and P5 barcoding by additional 8 cycles of PCR. The final PCR products were again purified using 1X AMPure XP beads and the final library was eluted in 20 µLs of 0.1X TE buffer and the sequencing was performed on Illumina MiSeq, using MiSeq Reagent Kit v3 (600-cycle) according to the manufacturer's instructions (Illumina, Inc., San Diego, CA, United States).

OTU clustering and taxonomic assignments

The bacterial and fungal sequencing data were analysed using Quantitative Insights into Microbial Ecology (QIIME v 1.9.1, http://qiime.org/) and pipeline for analyses of fungal internal transcribed spacer (PIPITS v2.3, https://anaconda.org/bioconda/pipits) softwares, respectively. Paired end reads of approximately 300bp were obtained which were analysed for quality using FastQC tool v 0.11.7. (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). A wide range of information related to the quality profile of the reads: basic statistics, GC content, over-abundance of adaptors and over-represented sequence was determined by the FastQC report. Raw sequencing reads containing adaptors and primer sequences were quality trimmed with the help of Cutadapt tool (http://code.google.com/p/cutadapt/)

Fastq-Join tool (https://anaconda.org/bioconda/fastq-join) was used to convert paired-end reads into longer contigs of V3-V4 consensus sequence region. This tool operates by finding the overlap for each pair of reads and combines them into a single read. These reads were de-replicated to combine identical tags into unique sequences for the construction of consensus quality profiles and identified sequencing errors from the samples removed. Singletons and Chimeric sequences, caused by the hybridization of DNA fragments from various species, were filtered out using the parameter reference_chimera_detection default implemented in the QIIME.

High quality bacterial and fungal sequences were binned/clustered into operational taxonomic units (OTUs) by UCLUST and RDP classifier method with reference to latest Green gene database (http://greengenes.lbl.gov) and UNITE database (https://unite.ut.ee) in QIIME and PIPITS respectively using a similarity threshold of 97%. Finally, non-redundant and representative OTUs obtained were classified

up to species level (wherever possible) followed by individual sample quantification.

Nutrient pool analyses

The dried soil samples were analysed at Department of Environmental Sciences and University Science Instrumentation Centre (USIC) University of Kashmir, Srinagar. Water content (%) was measured using the fresh weight to the dry weight ratio. Soil pH, Electrical conductivity (EC), Total dissolved solids (TDS) and Salinity (SAL) were determined in distilled water (1:2.5 Soil: Distilled water) (Allen 1989). Total soil organic Carbon % (TOC) was determined by Walkley-Black chromic acid wet oxidation method (Walkley & Black 1934). Total soil Nitrogen (N) content (%) was assessed using the standard method of Kjeldahl (Bremner 1965). The soil available phosphorous (P) was estimated by Olsen's sodium bicarbonate method (Olsen 1954). In addition, potassium (K) and micronutrients such as iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn) were also estimated using the double-beam atomic absorption spectrophotometer (AAnalyst 600, Perkin-Elmer, USA)

Data analyses

Before analysis, data was subjected to Shapiro-Wilk test for checking its normal distribution. Two-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) post hoc tests with p-value adjusted using "BH" method were applied to analyze the difference in microbial communities and soil physicochemical properties between the plots and among sites. Data was also subjected to principal component analysis (PCA) using the FactoMineR (https://CRAN.R-project.org/package= FactoMineR) and factoextra (https://CRAN.R-project.org/package= factoextra) packages, to determine whether the overall physicochemical properties of the invaded plots were different from those of uninvaded plots across different sites along the altitudinal gradient.

We also used the community ecology R package'vegan_2.5.6' (https://github.com/vegandevs/vegan) for plotting the rarefaction curves for assessing the sequencing depth of individual samples both in bacteria and fungi at species level. We measured the α-diversity of bacterial and fungal communities within the samples. Both richness (i.e. number of different species present in a sample) and evenness (i.e. how common in number different species in a sample are) measures were computed for the invaded and uninvaded plots across all the sampling sites along an altitudinal gradient using QIIME v1.9.1. Total observed OTUs and Chao-1 estimator were employed to measure the observed and real richness (i.e. observed plus less frequent species) of the samples. For measurement of species diversity in each sample, we used Shannon index and Simpson index – the two commonly employed diversity indices in community ecology.

We determined the β-diversity (the differences of bacterial and fungal diversity between the samples) using non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity index in vegan_2.5.6. This index measures the relatedness of the species composition of soil microbial communities across different sites and is a distance-based method that maximizes rank dependent correlation between the original distances and the distances between samples from multidimensional space into new reduced 2D ordination space. Analysis of similarities (ANOSIM), a non-parametric statistical test, was carried out to determine the variation in the community composition.

Similarly, the most significant soil nutrient parameters governing the composition of microbial communities at different sites were determined by Canonical Correspondence Analysis (CCA). Forward selection method of explanatory variables was carried out to find out the model that includes only those parameters which contributed significantly to the overall microbial diversity at different sites using 'vegan_2.5.6'. Chart.Correlation() function from the "PerformanceAnalytics" package was used to estimate Spearman's *rho* (ρ) statistic, which is a rank-based measure of association between environmental variables (https://github.com/braverock/PerformanceAnalytics). For data visualization, all plots were created using the package ggplot 2_3.3.2 (https://ggplot2.tidyverse.org) in R software (R Core Team 2020).

Results

Composition and abundance of soil microbiome

The Illumina analysis generated a total of 39,52,344 bacterial and 21,89,062 fungal sequences with satisfactory average GCQ20 values of 55.42% and 98.96% respectively (Appendix S3: TableS1). These sequences were classified into 1959 and 2475 operational taxonomic units (OTUs) respectively with a similarity threshold of [?] 97%. Individual rarefaction curves of all the samples reached saturation showing a typical plateau formation for the OTUs detected, thereby indicating sufficient sampling effort (Appendix S2: Fig. S1). Taxonomically, the 1959 identified bacterial OTUs belong to 23 phyla classified under 73 classes, 105 orders, 126 families, 128 genera and 152 species. Similarly, the 2475 identified fungal OTUs represent 13 phyla, 49 classes, 116 orders, 210 families, 401 genera and 589 species.

The bacterial community was dominated by Proteobacteria which ranged from 22.19% in KD_UN to 27.75% in KZ_IN plots and differed significantly between the plots ($F_{1,\ 16} = 854.649, p < 0.0001$) and among the sites ($F_{3,\ 16} = 326.205$, $p < 0.0001$) (Appendix S3: Table S2). Across the sites, phylum Actinobacteria ranged from 12.90% in KZ_IN to 19.96% in KU_UN plots and showed slightly higher abundance in uninvaded (16.3%) as compared to invaded plots (15.3%). Similarly, other phyla that showed different ranges of abundance with significant p -values between the plots and among the sites along the altitudinal gradient are given in Appendix S3: Table S2. The relative abundance in different phyla ranged as follows: Planctomycetes: 13.64% in KU_UN to 20.22% in KZ_UN plots, Verrucomicrobia: 6.77% in KZ_UN to 19.34% in KU_UN plots, Bacteroidetes: 8.08% in TM_UN to 13.46% in KD_IN plots, Acidobacteria: 7.48% in KZ_UN to 11.15% in TM_UN plots, and Chloroflexi: 3.21% in KU_UN to 5.67% in KD_IN plots (Fig. 2a). Fungal community was dominated by Ascomycota forming 53% and 60% in invaded and uninvaded samples respectively. The relative abundances differed significantly between the plots ($F_{1,\ 16} = 52.373$, $p < 0.0001$) and among the sites ($F_{3,\ 16} = 48.838$, $p < 0.0001$) along the altitudinal gradient (Appendix S3: Table S2). The abundance of other fungal phyla with significant p -values between plots and among sites along the altitudinal gradient is given in Appendix S3: Table S2. The abundance of Basidiomycota ranged from 6.0% in KZ_UN to 36% in KU_IN plots. Similarly, other phyla such as Mortierellomycota, Mucoromycota, Chytridiomycota, Glomeromycota and Rozellomycota showed different abundance patterns between the invaded and uninvaded plots across the sites (Fig. 2b). The detailed results of abundance profiles of both bacteria and fungi at other taxonomic levels (classes, orders, families, genera and species) are given in Appendix S1: S1 to S5 and Appendix S1: Figures S1-S6.

Diversity of soil microbial communities

Αλπηα (α) διερσιτψ

Irrespective of the plot type, significantly (p <0.05) higher values of total observed OTUs and Chao1 estimator of bacterial communities were obtained in the high-altitude sites as compared to the low-altitude sites, though the pattern was inconsistent (Fig.3a, b and Appendix S3: Table S3). The Shannon index followed similar pattern, as observed above in case of observed OTUs in bacteria, reaching its peak at KU site followed by decline at KD site (Fig. 3c and Appendix S3: Table S3), however the Simpson index did not follow a monotonic trend along the altitudinal gradient but differed significantly between the plots and among the sites along the altitudinal gradient (Fig. 3d and Appendix S3: Table S3).

The observed OTUs and Chao1 estimator in fungal communities also followed a monotonic increase along the altitudinal gradient but dropped sharply at KD site – the highest altitudinal site (Fig. 3e, f and Appendix S3: Table S3). Overall, the alpha diversity of soil microbial communities increased from KZ to KU and differed significantly (p<0.05) between the invaded and uninvaded plots (Appendix S3: Table S3). The invaded plots at all sites showed higher diversity except KD, where opposite trend was observed. On the other hand, both Shannon and Simpson indices in fungal communities were significantly influenced by both the plot and site type (Fig. 3g, h and Appendix S3: Table S3). In particular, both indices were significantly higher in the invaded as compared to the uninvaded plots except KD site.

Βετα (β) διερσιτψ

For bacterial β-diversity, the results of ANOSIM revealed significant differences among the sampling sites with stress value = 0.063, R = 0.6997 and $p < 0.05$(Fig.4a) , thereby indicating that altitude has a significant

role in shaping the structure of the soil bacterial community. For soil fungal communities, more distinct clustering in terms of sites was observed and ANOSIM once again showed significant differences among the sites with stress value of 0.073, R = 0.5955 and $p < 0.05$ (Fig.4b) . Therefore, the results clearly demonstrate that both the soil bacterial and fungal communities are highly influenced by altitude.

Impact of plant invasion on soil nutrient parameters

Analysis of soil physicochemical properties revealed that pH, EC, TDS and WC differed significantly (p <0.001) between plots (Table 1). Soil pH, TDS, SAL and WC values were higher in the invaded plots as compared to uninvaded, whereas EC showed the opposite trend. Furthermore, pH, EC, TDS and SAL were highest at KZ site and showed a gradual decrease along the increasing altitude, while WC revealed the opposite trend (Table1). Significant differences were found in TOC, N and P between the plots at all the sites except KD site where P did not show any significant difference ($p = 0.9$). TOC and N were higher in the invaded plots at all sites and P showed an opposite trend. Between the plots, K differed significantly and showed progressively increasing trend from KZ to KU followed by decline at KD site. Furthermore, micronutrients were lower in concentration in the invaded plots as compared to uninvaded plots and increased gradually along the altitude (Table 1).

PCA analysis illustrated that first two axes (PC1, 52.4% and PC2, 32.4%) explained approximately 85% of total variance (Fig. 5). There was a striking pattern for soil physicochemical properties observed at different sites with invaded and uninvaded plots separated distinctly. WC, TOC, K, N and Cu were positively related with invaded plots at the higher-altitudinal KU and KD sites whereas Fe, Zn, Mn and P were strongly associated with uninvaded plots at these two sites. Similarly, pH, TDS and SAL were positively related with invaded plots at the lower-altitudinal KZ and TM sites but negatively associated with invaded plots at KU and KD sites. EC was strongly associated with uninvaded plot at KZ site, but negatively related to uninvaded plots at KU and KD sites (Fig. 5).

Relationship between soil microbial communities and nutrient parameters

The results of the Mantel test based on Spearman's rank correlation revealed that the diversity of bacterial communities in invaded plots along the altitudinal gradient were influenced by all the environmental variables (p <0.05) except SAL, Zn and Temp (p >0.05), whereas in uninvaded plots only three parameters viz. SAL, Zn and Temp determine the bacterial community diversity (p <0.05) (Table 2). The most influential variable explaining community composition is TOC (rho =0.97,$p < 0.001$) in invaded plots and SAL in uninvaded plots (rho = - 0.81, $p < 0.001$) (Table 2). Similarly, the fungal community composition in invaded plots was influenced by all the environmental variables except SAL and Zn ($p > 0.05$), whereas in uninvaded plots EC, K, Zn, Temp and Alt did not show any significant correlation (p> 0.05). WC in invaded plots (rho = 0.92, p< 0.001) and Mn in uninvaded plots (rho =0.93, p< 0.001) were most significantly correlated with fungal community composition (Table 2).

Further, Canonical Correspondence analysis (CCA) was used to analyze the relationship between microbial community structure and environmental variables along the elevation gradient (Fig. 6). Based on Variance Inflation Factors (VIF) values obtained, it was observed that among the 15 environmental variables only five (pH, EC, K, Fe and temperature) were more significant (p <0.001) in determining the bacterial and fungal diversity among the plots along the altitudinal gradient. All the five environmental variables were found to be statistically significant based on Monte Carlo permutation F test (Appendix S3: Table S4). Constrained inertia or variance explained by the environmental variables in bacteria and fungi was found to be 1.039 and 1.605 respectively.

In bacteria, first two axes explained 70.9% of the variation (Axis 1˜ 41.5% and Axis 2˜ 29.4%). Axis 1 was positively related with pH and negatively associated with Fe. Axis 2 was positively correlated with EC and Temp and negatively correlated with K. The relative abundance of bacteria at the KZ_IN and KD_IN were related with pH and negatively associated with Fe. However, the bacterial communities of uninvaded plots at KZ and TM showed a positive relation with EC and Temp, whereas KD_UN were correlated with K. TM and KU sites were positively related with Fe (Fig. 6a). In fungi, first two axes explained 93.33%of

variation (Axis 1˜ 49.98% and Axis 2˜ 43.35%). Axis 1 was positively correlated with EC and Temp and negatively associated with K. Axis 2 showed positive relation with pH and negatively related to Fe. The relative abundance of fungi in both plots at KZ site was correlated with EC and Temp, whereas both plots of KD site along with KU_IN were associated with K. Both plots of TM and KU_UN plot were positively related with Fe and negatively associated with pH (Fig. 6b). Our results revealed that there is a significant effect of soil physicochemical properties on the bacterial and fungal community abundance along the altitudinal gradient.

Discussion

Our results show that invasive plant species can cause significant shifts in the taxonomic composition and relative abundance of microbial communities across multiple sites along the altitudinal gradient. Also, the plant invasions can induce shifts in soil nutrient pools in the invaded plots and these shifts vary among the sites along the altitudinal gradient. Therefore, the present study provides new insights in elucidating the patterns of plant invasion-induced responses in soil microbial communities that operate underground along altitudinal gradient in mountainous landscapes.

Plant invasion-induced shifts in the soil microbial community along altitudinal gradient

To understand the underlying mechanisms of interactions between plant invasion and soil microbes, the facilitation of nutrient pools by soil microbial communities for plant performance has recently attracted research attention (Dawson & Schrama 2016; Dawkins & Esiobu 2018). In this regard, the role of soil microbial community in determining invasion success along the altitudinal gradient in mountain landscapes has been little explored yet. Towards this end, our study examines the effect of invasive *L.vulgare* on soil microbial communities' composition, richness and diversity by disentangling the differences between invaded and uninvaded plots across multiple sites along an altitudinal gradient in Kashmir Himalaya.

The drivers of global environmental change have the potential to disrupt ecological interactions between plants and microbes, thereby influencing the community structure and ecosystem functioning from local to global scales (Cavicchioli et al., 2019; Rudgers et al., 2020). The importance of microbial responses to drivers of change creates the context-dependent plant-microbe pairs in terms of temporal or spatial mismatches that accompany species'invasions and climate change-driven range shifts (Rudgers et al., 2020). In this regard, the microbes may respond differently to the drivers of change varying across spatial scales. In the present study, the abundance patterns of soil microbial communities in plots as well as among sites were significantly altered along the altitudinal gradient. Generally, an increase in altitude changes the environmental conditions in mountains, and a decrease in microbial abundance along altitudinal gradients is expected (Slater et al., 2008). However, our results have shown that microbial community composition shows an increasing trend with increase in altitude, which is similar to results of some recent studies (Siles et al., 2016, Siles & Margesin 2016). Higher nutrient availability and less immobilization rates at higher altitudes may lead to enhanced microbial growth, which in turn could explain the higher bacterial and fungal abundance at high altitudes (Doolittle et al., 2006). We also observed increased microbial abundance in all the invaded plots as compared to uninvaded ones at all altitudes except the highest altitude KD site. Some recent studies (Yang et al. 2020, Wang et al.2020) have also shown that invasion by*Spartina alterniflora* and *Bidens alba* greatly enhanced the abundance and diversity of soil bacterial communities. Likewise, an increase in soil fungal diversity has been reported in case of invasion by *Erigeron annuus* , *Solidago canadensis* and *Wedelia trilobata* (Schroeder et al., 2013, Wang et al., 2018).

We observed an increased abundance of several bacterial and fungal phyla in the *L.vulgare* invaded plots (Fig. 2a, b). Abundance of specific bacterial and fungal taxa belonging to the Acidobacteria and Ascomycota have been reported to increase with the increasing invasion levels (Rodrigues et al., 2015; Kong et al., 2017), while as opposite trend for Actinobacteria. The higher abundance of Chloroflexi has been found to contribute to soil fertilization and promote plant growth (Hug et al., 2013). The phylum Glomeromycota, which includes many arbuscular mycorrhizal (AM) fungi forming symbiotic association with plants (Rodriguez et al., 2004), were found at relatively higher abundance in the plots invaded by *L.vulgare* . This indicates that the symbiotic

interaction possibly facilitates invasion by *L.vulgare* , a finding which has been previously reported for the *Conyza canadensis* and *Thismia* sp. as well (Zhang et al., 2020; Merckx et al., 2017). Our results clearly indicate that the increasing relative abundance of various taxa in the invaded plots facilitated *L. vulgare* growth compared to uninvaded plots along the altitudinal gradient.

Patterns of diversity in soil microbial community along altitudinal gradient

In the present study, the highest species richness and diversity of bacterial and fungal communities were observed in the invaded plots at all the four sampling sites with highest values reaching at KU site (Fig.3). Yang et al., (2020) observed a similar pattern of species richness and diversity for soil bacterial communities invaded by *Spartina alterniflora* and reported a unique bacterial community composition with highest relative abundance associated with the invasive plant. The potential of plant invasion to shift the abundance and diversity of soil microbial communities has been demonstrated by few recent studies as well (Rodríguez-Caballero et al., 2017; Xiang et al., 2018). For instance, invasion of *Alliaria petiolata* significantly increased fungal richness under field conditions with shifts in soil microbial community composition (Duchesneau et al., 2021). Similarly, a distinct microbial community structure was observed among the rhizospheres of invasive *Mikania micrantha* , (Yin et al., 2020). Therefore, our study provides more empirical evidence to generalize the fact that the rhizosphere of invasive plants generally contains a higher diversity of microbial communities than uninvaded plots. This could be possibly attributed to secretion of numerous root exudates and signalling molecules produced by the invasive plants (Lagos et al., 2015) as well as the mycorrhizal advantages, root predation shield benefits and increased attachment niches (Dawkins & Esiobu 2018). In terms of microbial community relative abundance, the results of NMDS using the Bray-Curtis similarity index revealed that different sites were clustered together and formed distinct groups along the altitudinal gradient (Fig.4). This further substantiates that bacterial and fungal communities significantly differ in relative abundance at the invaded and uninvaded plots (Yannarell et al., 2011).

Impacts of plant invasion on soil nutrient parameters along altitudinal gradient

Our study clearly indicate that invasion by *L.vulgare* altered the soil physicochemical properties consistently between the plots and among the sites along the altitudinal gradient. More specifically soil pH, TDS, SAL, WC, TOC, N and K values were higher in the invaded plots as compared to the uninvaded plots (Table 1). The results provide further empirical support to a recent study carried on *L. vulgare*invasion, which reported that invasion significantly altered the overall soil properties to influence nutrient cycling and expedite its establishment and spread (Ahmad et al., 2019b). Particularly, the higher values of soil pH and WC in the invaded plots are in agreement with the findings of few other studies where invasion has led to the enhancement of the soil physicochemical properties (Manzoni et al., 2012; Simba et al., 2017; Ruwanza & Shackleton 2016). The possible reason for increased pH at invaded plots can be due to rapid uptake of nitrate ions from the rhizosphere by invasive plants (Ehrenfeld et al., 2001).The decrease in soil pH with increasing altitude can result from the fact that conifers tend to make soil pH acidic in character (FitzPatrick 1983). The increased WC in the *L.vulgare* invaded plots can be due to the excessive utilization of water for its growth and development. Similarly, the lower soil EC value in the invaded plots is in agreements with the results from several other studies (Osunkoya & Perrett 2011; Ahmad et al., 2019b). Our results revealed that TDS, TOC, N and K differed significantly between plots and among sites, which are in agreement with the findings of other studies where invasion has been reported to increase various nutrients such as carbon (Schaeffer et al., 2003; Pollierer et al., 2007), N and K (Niu et al., 2007) and C and N (Uddin & Robinson 2017). The increase in P at invaded plots could be due to microbial phosphatase activities and secretion of certain root exudates that displace P from humic metal complexes and enhance P concentration (Hinsinger 2001; Li et al., 2009). The low concentration of iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn)in the invaded plots, owing to rapid uptake and utilization by vigorously growing invasive plant species, could be the plausible explanation for their depletion in the invaded soils (Osunkoya & Perrett 2011; Ahmad et al., 2019b).

Relationship between soil microbial communities and nutrient parameters

In the present study, the most important variables explaining bacterial community composition was TOC in invaded plots and SAL in uninvaded plots (Table 2). It has been reported that soil nutrients provide ample substrates to stimulate the growth of bacteria and serve as drivers of bacterial diversity and abundance (Santonja et al., 2017; Yu et al., 2019). The significantly increased soil bacterial abundance and diversity following *L.vulgare* invasion can be possibly a result of the TOC levels, which provides enriched substrates for the proliferation of bacteria (Yu et al., 2019). Higher values of soil salinity were observed in the invaded plots (Table 1), but showed a significantly negative correlation with uninvaded plots (Table 2). By altering the osmotic potential of soils, high salinity can lead to alterations in microbial community structure with reduced microbial biomass and can restrict the growth of heterotrophic bacteria (Kamble et al., 2014; Xi et al., 2014). Among other variable examined, WC in plots were most significantly and closely correlated with fungal community structures (Table **2**). Canini et al., (2019) has reported that water content and pH has a strong effect on fungal community composition and found a positive relationship between the fungal richness and the vegetation cover. Similarly, invasion of *Impatiens glandulifera*has been reported to effectively change soil characteristics particularly soil moisture and soil pH in invaded areas (Ruckli et al., 2014; Gaggini et al., 2018). Therefore, fungal communities can be influenced by soil moisture and soil pH and thus can be viewed as an indirect effect of the plant invasion.

Additionally, according to Canonical Correspondence analysis, significant correlation were observed between soil physicochemical properties and altitude, which indicates that sites with distinct soil properties possess different microbial abundances and the plot-wise distribution of both soil physicochemical properties and microbial abundances are strongly influenced by temperature, K, pH, EC and Fe (Fig.6). The present study, therefore, enhances our understanding of the altitudinal patterns of variation in soil physicochemical properties and the impact of these factors on belowground soil microbial communities to drive plant invasions in the study region.

Conclusions

Our study highlights that invasion by *L.vulgare* shifts the composition, relative abundance and diversity of soil microbial communities in the invaded plots across multiple sites along the altitudinal gradient. The results further suggest that the plant invasion-induced shifts in soil microbiome and nutrient pools may be a belowground self-reinforced invasion mechanism used by *L.vulgare*in new habitats along the altitudinal gradient to facilitate its successful invasion and possibly suppress native biodiversity. The triggering of such shifts may also represent hitherto least explored key belowground functional traits for species invasiveness by promoting positive feedbacks that support the microbial community dynamics. Therefore, our study helps understanding the shifting patterns of soil microbial communities and their drivers following plant invasion along an altitudinal gradient. Our study also provides crucial insights in elucidating the patterns and processes of plant invasion that operate underground in the soil system along altitudinal gradient in mountain landscapes in Kashmir Himalaya. These mountains are relatively understudied areas in comparison to areas in the global north, but our scientific findings and management implications are for mountainous landscapes elsewhere in the world.

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Data Accessibility

Illumina next-generation DNA sequencing data were submitted to National Centre for Biotechnology Sequence Read Archive under Accession no. PRJNA730654.

Author Contribution

AAK conceptualized the research idea, managed fund acquisition and project supervision. KH planned and designed the research experiment with inputs from AAK, RA and THD. KH conducted the field work with help from AAK and RA. KH carried out experiment, analyzed data and prepared figures and tables. KH and AAK led the manuscript writing with inputs from RA, MN, THD and IR. All the authors agreed to the final submitted version.

Table 1 . Soil physicochemical properties of *L. vulgare*invaded and uninvaded plots at four sampling sites along an elevation gradient in Kashmir Himalaya.

Sites	KZ	KZ	TM	TM	KU	KU	KI
	KZ_IN	KZ_UN	TM_IN	TM_UN	KU_IN	KU_UN	KI
pH	7.776±0.189	7.033±0.015***	6.936±0.045	6.410±0.020***	6.820±0.029	6.146±0.015***	6.8
EC	126.200±1.126	149.900±3.534***	95.600±5.501	106.966±4.932*	97.000±7.463	143.700±9.100***	89
TDS	106.133±2.589	91.133±2.871***	96.733±1.234	70.833±1.305***	87.466±0.814	68.000±4.313***	88
SAL	74.033±2.218	57.166±2.990***	70.400±2.022	51.400±2.007***	69.033±3.023	45.433±1.823***	74
WC	19.103±0.711	16.326±0.090***	24.816±0.254	19.026±0.712***	29.936±0.064	23.480±0.902***	26
TOC	1.630±0.009	0.910±0.026***	2.473±0.047	2.353±0.051**	3.616±0.100	2.093±0.037***	2.3
N	0.160±0.020	0.053±0.015***	0.410±0.020	0.360±0.036*	0.646±0.015	0.430±0.039***	0.5
P	17.544±0.449	16.497±0.048**	20.147±0.894	19.134±0.027**	23.217±0.301	21.982±0.038***	19
K	104.856±3.728	97.856±0.310**	114.550±2.489	106.113±1.852***	129.173±3.088	117.850±1.549***	12
Mn	0.800±0.079	0.926±0.056*	1.113±0.020	1.240±0.070*	1.263±0.047	1.453±0.058***	1.1

Sites	KZ	KZ	TM	TM	KU	KU	KI
Fe	11.800±0.793	15.933±0.251***	12.266±0.971	17.666±0.208***	14.633±0.757	19.933±1.266***	13
Cu	0.510±0.020	0.580±0.036*	0.576±0.032	0.603±0.070^{ns}	0.956±0.037	1.039±0.017**	0.6
Zn	0.413±0.060	1.293±0.085**	0.486±0.050	2.426±0.056***	1.910±0.091	2.723±1.097*	2.9

Data were shown as mean±standard deviation, n=3. EC: Soil electrical conductivity; TDS: total dissolved solids; SAL: salinity; TOC: total soil organic Carbon; N: total soil nitrogen content; P: soil available phosphorous; K: potassium; Fe: iron; Cu: copper; Mn: manganese; Zn: zincSignificance levels: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.^{ns} stands for not significant with$p > 0.05$.

Full forms of the sample codes are given in Fig. **2** .

Table 2. Mantel statistic based on Spearman's rank correlation rho (ϱ) considering the bacterial and fungal community structures in invaded and uninvaded plots as well as the different environmental and physicochemical parameters analyzed.

Environmental Variables	**Bacteria**	**Bacteria**	**Fungi**	**Fungi**
	Invaded	Uninvaded	Invaded	Uninvaded
pH	**-0.79****	-0.19	**-0.88*****	**-0.77****
EC	**-0.66***	-0.16	**-0.80****	-0.39
TDS	**-0.84*****	-0.10	**-0.89*****	**-0.63***
SAL	-0.50	**-0.81****	-0.38	**-0.89*****
WC	**0.94*****	0.27	**0.92*****	**0.73****
TOC	**0.97*****	0.12	**0.82****	**0.73****
N	**0.91*****	0.23	**0.90*****	**0.80****
P	**0.94*****	0.57	**0.77****	**0.92*****
K	**0.82****	-0.10	**0.85*****	0.41
Mn	**0.90*****	0.57	**0.90*****	**0.93*****
Fe	**0.76****	**0.70***	**0.67***	**0.89*****
Cu	**0.93*****	**0.59***	**0.73****	**0.72****
Zn	0.49	0.10	0.53	0.47
Temp	-0.45	0.46	**-0.58***	-0.17
Alt	**0.75****	0.10	**0.83*****	0.56

Values in bold indicate statistical significance. Significance levels are shown at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

Full forms of environmental variables are given in Table **1** .

Fig. 1. Map of the study area showing the location of selected sampling sites in Kashmir Himalaya at different altitudes. These sites are Kunzar, Tangmarg, Kashmir University Gulmarg Research Station and Kongdoori.

Fig. 2. Stacked bar chart of the relative abundance of top nine (a) bacterial and (b) fungal phyla in the *Leucanthemum vulgare* invaded and uninvaded plotsalong an altitudinal gradient in the Kashmir Himalaya. (Full forms of the sample codes are KZ_IN: Kunzar invaded; KZ_UN: Kunzar uninvaded; TM_IN: Tangmarg invaded; TM_UN: Tangmarg uninvaded; KU_IN: Kashmir University Gulmarg Research Station- invaded; KU_UN: Kashmir University Gulmarg Research Station-uninvaded; KD_IN: Kongdoori invaded; KD_UN: Kongdoori uninvaded).

Fig. 3. Patterns of α-diversity for bacterial (a-d) and fungal (e-h) communities in *L. vulgare* invaded and uninvaded plots along an altitudinal gradient in the Kashmir Himalaya, (Shown are bacterial and fungal

observed OTUs (a, e) Chao1 estimator (b, f) Shannon index (c, g) and Simpson index (d, h). Sites are grouped by different colours with increasing altitude (left–right). (Full forms of the sample codes are given in Fig. **2**).

Fig. 4. Non-metric multidimensional scaling (NMDS) plot displaying β-diversity (Bray-Curtis dissimilarity) for bacterial (a) and fungal (b) communities. The samples are coloured according to the sites along the altitudinal gradient: bright cyan (Kunzar), light yellow (Tangmarg,), green (Kashmir University-Gulmarg Research Station) and bright pink (Kongdoori).

Fig.5 . Principal component analysis (PCA) biplot of *L. vulgare* invaded and uninvaded plots across the sampling sites along an altitudinal gradient in the Kashmir Himalaya. (Directions and magnitudes of the soil variables driving each axis are also indicated.Full forms of the sample codes are given in Fig. **2**).

Fig. 6. Canonical Correspondence analysis (CCA) biplot based on the microbial sequencing data and environmental variables for bacteria (a) and fungi (b). (Arrows indicate the direction and magnitude of environmental variables associated with microbial communities. The length of an arrow-line indicates the strength of the relationship between the environmental variable and microbial community. Each green dot represents individual microbial species and red text shows plots of different altitudes.Full forms of the sample codes are given in Fig.**2**).

Fig. 1.

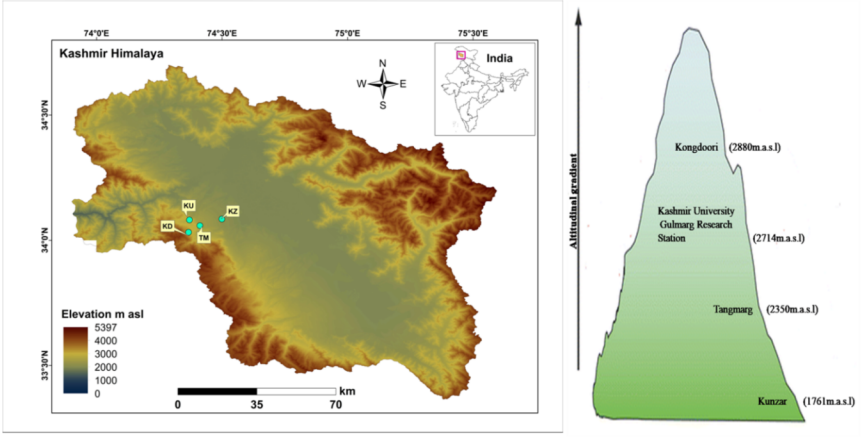
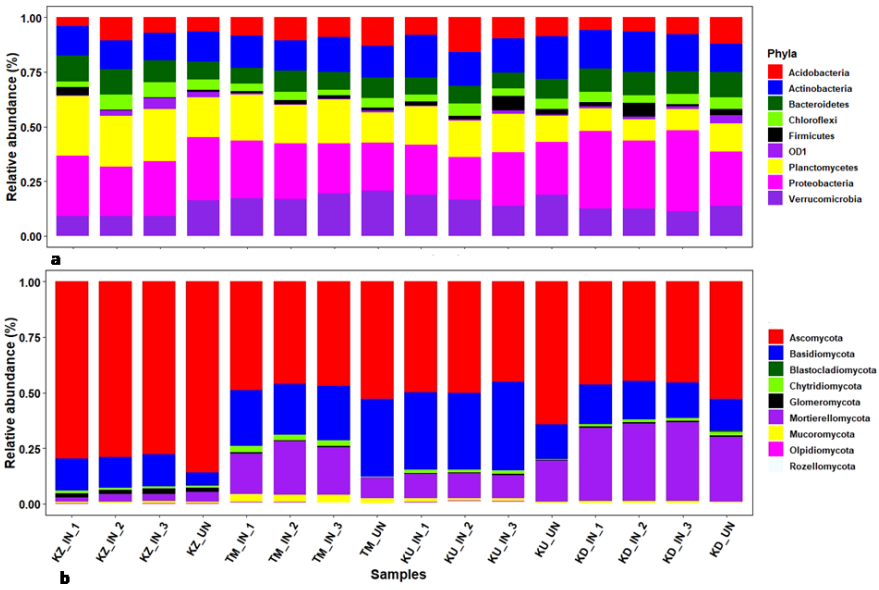


Fig. 2.



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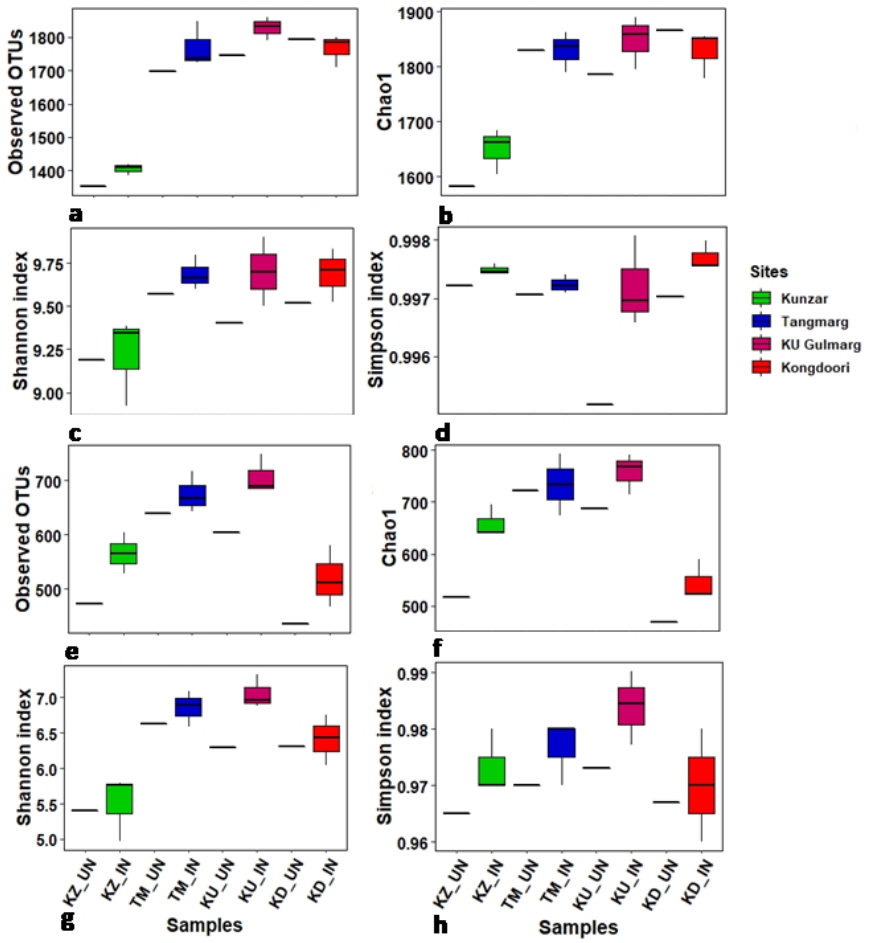

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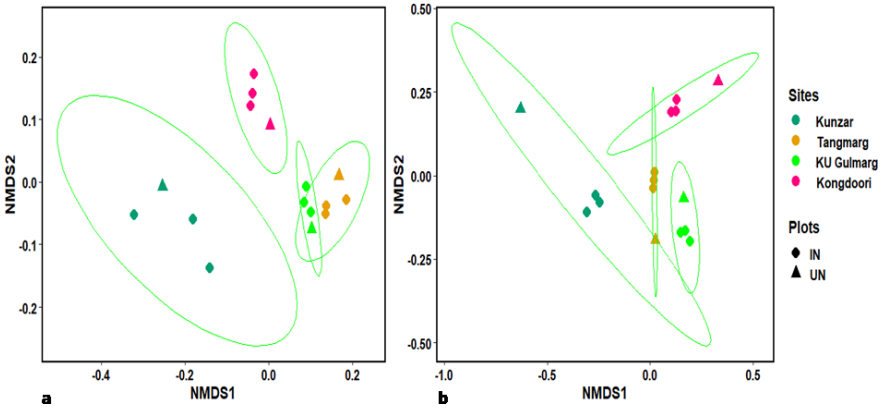

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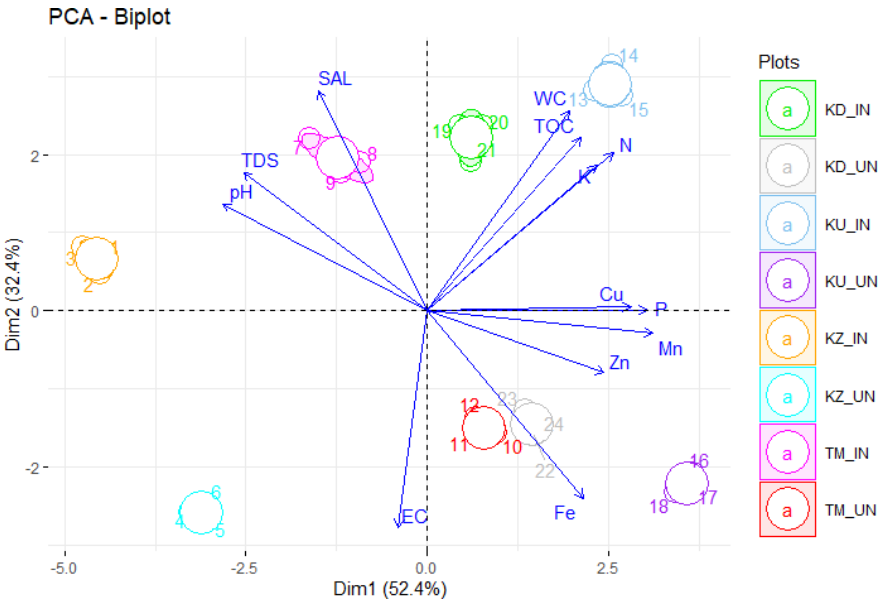

Fig. 6.