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Investigation of diazotrophic bacteria association with plants

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, which are referred in the text by their Roman numerals:

I. Tedersoo, L., Laanisto, L., **Rahimlou, S.,** Toussaint, A., Hallikma, T., & Pärtel, M. (2018). Global database of plants with root-symbiotic nitrogen fixation: Nod DB. Journal of Vegetation Science, 29, 560–568.

II. **Rahimlou, S.,** Bahram, M., & Tedersoo, L. (2021). Phylogenomics reveals the evolution of root nodulating alpha- and beta-Proteobacteria (rhizobia). Microbiological Research, 250, 126788.

III. **Rahimlou, S.,** Delaux, P.M., Karlsen-Ayala E., Gazis R., Hosseyni Moghadam M., Bahram M., & Tedersoo, L. (2022). Is the nitrogen-fixing root nodule symbiosis polyphyletic? Manuscript.

IV. Kariman, K., Moreira-Grez, B., Scanlan, C., **Rahimlou, S.,** Boitt, G., & Rengel, Z. (2022). Synergism between feremycorrhizal symbiosis and free-living diazotrophs leads to improved growth and nutrition of wheat under nitrogen deficiency conditions. Biology and Fertility of Soils, 58, 121–133

Author's contributions to the publications:

Was responsible for ***, contributed substantially **, contributed *

	Designing the study	Carrying out the experiment	Analyzing the data	Preparing the manuscript
I	–	*	–	**
II	***	***	***	***
III	***	***	***	***
IV	–	–	*	**

INTRODUCTION

Nitrogen (N) is an integral component of key biomolecules (e.g., chlorophyll, amino acids, and nucleic acids), and its supply is crucial for plant growth and productivity in agroecosystems (Berman-Frank et al. 2003). To meet the increasing N demand in farming systems, synthetic N fertilizers are intensively used; in 2022, the global consumption worldwide is estimated to exceed 110 Mt of N (FAOSTAT 2019). Nitrogen fertilizers are costly and energy-intensive to produce, and may lead to severe or even irreversible damages to the environment, including nitrate (NO_3^-) pollution of surface- and groundwater, formation of coastal dead zones, and elevated nitrous oxide and carbon dioxide emissions (Eickhout et al. 2006).

Root symbiotic associations with diazotrophic bacteria and mycorrhizal fungi are important evolutionary adaptations of plants to compete for nutrients. Nitrogen-fixing plant-bacterial associations are widely distributed across all terrestrial biomes and continents apart from Antarctica. Nodulated plants form important components of plant communities, especially in N-limited early-successional ecosystems, riparian habitats, and tropical savanna and shrubland biomes (Cleveland et al. 1999). In early successional habitats, N-fixing plants and their root symbiotic microbes contribute to soil development and facilitate the recruitment of other plant species and consumers (Walker et al. 2003). The global symbiotic biological N fixation amounts roughly to 45 Mt annually, which is the main contributor to natural terrestrial N sources (Vitousek et al. 2013). It is crucial to boost biological N_2 fixation in farming systems to reduce the use of synthetic N fertilizers. Root fungal symbionts such as AM and EcM fungi have been shown to synergistically interact with both free-living and symbiotic diazotrophs in soil, providing additional N benefits to host plants (Paul et al. 2007, Sabannavar and Lakshman 2008). To ensure environmental sustainability while maintaining the economic viability of farming systems, enhancing biological N fixation is essential.

Several bacterial groups fix atmospheric nitrogen in root symbiosis with plants. Frankiaceae (Actinobacteria) form actinorhizal root nodules with genera from multiple eurosid plant families (Chaia et al. 2010). Nostocaceae (Cyanobacteria) form specific root nodules in all examined members of Cycadophyta and inhabit leaves of the angiosperm genus *Gunnera* (Gunneraceae), aquatic fern genus *Azolla* (Salviniaceae, Rai et al. 2000), and shoots of bryophytes (DeLuca et al. 2007). Rhizobiaceae (α-Proteobacteria) and Burkholderiaceae (β-Proteobacteria) are the most well-known N-fixing bacterial groups that form root nodule symbiotic associations with legumes (Sprent et al. 2017). A small genus *Parasponia* (Cannabaceae) has evolved independently symbiotic associations with Rhizobiaceae (Trinick 1973). There have been a few reports of γ-Proteobacteria inducing nodules on legumes, however, they have not yet been confirmed (Shiraishi et al. 2010, Gyaneshwar et al. 2011, Moulin et al. 2015). In addition, rhizobial root nodules have been reported in three zygophyllaceous genera, *Tribulus*, *Fagonia*, and *Zygophyllum* (Mostafa and Mahmoud 1951), but these plant taxa have received limited attention in the recent treatments despite

the mono-dominance of some species in desert habitats around the world (Sheahan 2007). Moreover, rhizobial root nodule structures were observed in monocot species *Roystonea regia* L. (Arecaceae) for the first time in India (Basu et al. 1997). Further evidence of N fixation was provided by the presence of nitrogenase. In addition to evidence of N fixation, the nodules were also found to be producing indole acetic acid, an important plant hormone (Basu and Ghosh 1998, Basu and Ghosh 2001). Proteobacterial leaf nodules occur in some species belonging to several genera of Rubiaceae, Myrsinaceae, and Dioscoreaceae (Miller 1990).

In symbiotic and free-living microbes, N fixation is catalyzed by nitrogenase, the structural subunits of which are encoded by the *nif*H, *nif*D, and *nif*K genes (Igarashi and Seefeldt 2003). Along with the core *nif*HDK genes, there are several other types of *nif* genes, which contribute to nitrogenase synthesis that are found in all diazotrophs irrespective of their lifestyle (Cooper 2004). Rhizobia symbiosis is based on the specific recognition of signal molecules, which are produced by both bacterial and plant partners. Flavonoids and isoflavonoids – polyphenolic plant secondary metabolites – are secreted by the host plants to induce the expression of nodulation (*nod*) genes in the cognate rhizobial bacteria (Cooper 2004, Spaink et al. 2012). The products of *nod* genes constitute enzymes involved in the biosynthesis of species-specific, substituted lipo-chitooligosaccharides (LCOs) termed Nod factors (Roche et al. 1992, Dénarié and Cullimore 1993). Nod factors are the most important elements for bacteria-legume communication during the first steps of nodule formation by acting as key components for host plant recognition (Lerouge et al. 1990). The ubiquitous presence of Nod factors in all rhizobia has led to the development of a universal "lock-and-key" hypothesis, which assumes that all symbiotic legumes and rhizobia encode host nodulation determinants and homologs of the known nodulation genes, respectively (Giraud et al. 2007). In response to plant signaling molecules, bacterial symbionts produce Nod factors, encoded by more than 60 different bacterial *nod*, *nol*, and *noe* genes (John et al. 1993). The *nod*A, *nod*B, and *nod*C proteins play a pivotal role in the synthesis of the LCO-backbone structure of Nod factors, by functioning as acyltransferase, chitin oligosaccharide deacetylase, and chitin oligosaccharide synthase, respectively (John et al. 1993, Atkinson et al. 1994, Röhrig et al. 1994, Spaink et al. 1994, Barnett et al. 1998). All other *nod*, *nol*, and *noe* genes encode enzymes that add a variety of substituents to the core structure (Röhrig et al. 1994). Thus, synthesis of the Nod factor chitin oligomer backbone requires the activity of three specific enzymes, encoded by the *nod*A, *nod*B, and *nod*C genes, which are present in all rhizobia (except a few photosynthetic *Bradyrhizobium* strains) characterized so far (Giraud et al. 2007).

Genes associated with symbiosis signaling are invariably conserved in all land plant species possessing intracellular endosymbionts including arbuscular mycorrhizal, ericoid mycorrhizal, orchid mycorrhizal fungi, and root nodulating bacteria (Radhakrishnan et al. 2020). The perception of LCOs is not specific to the N-fixing clade (rosid I, angiosperms) but appears to be a phylogenetically more widespread phenomenon as suggested by the overall growth-promoting effect of

LCOs in a variety of higher plants (Tanaka et al. 2015, Sun et al. 2015). LysM receptor-based LCO perception system involved in AM fungus detection is present in legumes, tomatoes, and monocots (Buendia et al. 2016, Carotenuto et al. 2017). It can therefore be safely concluded that LCO perception by LysM receptors *per se* is not a novel invention of the N-fixing clade, implying that the symbiosis signaling pathway has been repeatedly recruited by all land plants forming intracellular symbiosis after its emergence in arbuscular mycorrhizal plants around 450 million years ago (Mya). Thus, species forming exclusively extracellular symbioses, such as ectomycorrhizae, and those forming associations with cyanobacteria, have lost this signaling pathway.

Nitrogen-fixing plants are paraphyletic, distributed across four plant orders including Fabales, Fagales, Cucurbitales, and Rosales sharing a common ancestor in the rosid I clade of angiosperms. In addition to the scattered distribution, a further mystery that shrouds the evolution of N-fixing symbiosis is its diversity at multiple levels: Legumes (Fabales) and the non-legume *Parasponia* (Rosales) form nodules with rhizobia, whereas species of actinorhizal plants from eight plant families associate with the actinobacterial genus *Frankia* (Pawlowski and Demchenko 2012). Furthermore, basal legume subfamilies Detarioideae, Cercidioideae, Duparquetioideae, and Dialioideae, as well as the early-branching members of the main legume subfamilies Caesalpinioideae and Papilionoideae, are not associated with the symbiotic N-fixing bacteria (Dilworth et al. 2008, Sprent 2009, Sprent et al. 2017, Tedersoo et al. 2018, Ardley and Sprent 2021). A recent phylogenomic analysis of plants suggests that symbiosis involving angiosperms and N-fixing rhizobia/*Frankia* evolved 92–110 Mya from the most recent common ancestor of the N-fixing clade, followed by multiple independent losses of the symbiotic capacity (Griesmann et al. 2018). Considering the paraphyletic and scattered distribution of nodulating taxa, it remains challenging to study the evolutionary origins of symbiotic N fixation in plants, especially if nodulating taxa such as Zygophyllaceae and *R. regia* are considered.

So far, two hypotheses proposed to explain the paraphyletic distribution of nodulating taxa. First, nodulation originated independently multiple times, preceded by a single hypothetical predisposition event in the common ancestor of the N-fixing clade (Doyle 1998, Doyle 2011, Werner et al. 2014, Li et al. 2015, Doyle 2016, Martin et al. 2017). Using plant genome comparison analysis, Griesmann et al. (2018) found no evidence for parallel gene expansion in nodulating taxa, therefore rejecting the multiple gain hypothesis. They proposed that nodulation originated at the root of the currently known N-fixing clade, followed by massive independent losses. It has been shown that the *NIN* and *RPG* genes are partially conserved in nodulating taxa with known functions in symbiosis (Griesmann et al. 2018). While it is obscure why these symbiosis genes were maintained over an extended period in non-nodulating plant species and were subsequently independently lost in some nodulating taxa. Loss of those symbiotic genes in non-nodulating plant species is not absolute, as functional copies of those genes are observed in several non-symbiotic taxa within and outside the N-fixing clade. There are still ambiguities regarding the evolution of N-fixing symbiosis in plants.

My thesis addresses the following research gaps and hypotheses:

I. Available information about plants that are capable of establishing nodulation is fragmented and somewhat outdated. Hence, we present a freely accessible database of plant genera with nodulated roots. This represents a consensus on the nodulation and N-fixing status based on several reviews and accounts for phylogenetic information, which will allow interpretation of the nodulation trait in unstudied groups and detect potential erroneous reports.

II. Nodulation emerged for the first time in major rhizobial genera after legume divergence at around 60 Mya (Sprent 2007). This hypothesis was tested by molecular clock calibration analysis and reconstructing the ancestral states of structural *nod*ABC genes using a phylogenomic approach.

III. Nodulation is not a phenomenon confined to the rosid I clade of angiosperms. For this purpose, we investigated the nodulation occurrence in the unconventional plant species *T. terrestris* (Zygophyllaceae) and *R. regia* (Arecaceae). We sampled *T. terrestris* and *R. regia* roots from the middle east and the Caribbean respectively and applied molecular genomic and metagenomic techniques to study the biodiversity of microbial endophytes inhabiting the root nodules of the sampled plant species.

IV. We assumed that *A. occidentalis* – a plant-associated fungus that improves growth and nutrition without the development of interface structures – enhances the activity of indigenous soil-borne N_2-fixing microbes, which might thereby account for the significant N nutritional benefits in the fungal treatments. Thus, we explored the potential of the fungus *A. occidentalis* in combination with free-living diazotrophs consortium, to improve the growth and N nutrition of wheat plants grown under N deficiency conditions, characterized soil chemical/microbial factors linked with symbiotic N nutritional benefits to host plants via assessing soil N forms (NO_3^--N, NH_4^+-N, and total N) and changes in soil microbial composition.

MATERIALS AND METHODS

Data collection, sampling sites, and study design

For developing the nodDB database (I), we examined five meta-studies/reviews (Rai et al. 2000, Sprent 2009, Chaia et al. 2010, Werner et al. 2014, Li et al. 2015) and three databases – GRIN (last updated Feb 2009, no longer publicly available; species level for Fabaceae); Nodulation_clade (Afkhami et al. 2018; accessed 25 Jan 2018; genus level for Fabaceae); and TRY (Kattge et al. 2011; accessed 25 Jan 2018; species level for streptophytes) to obtain the majority of records about nodulation and non-nodulation in terrestrial plant roots. Genera with no reports or conflicting reports in the above data sets were thoroughly searched for nodulation status in the literature. In addition, genera whose nodulation reports did not match expectations based on phylogeny were further searched for additional support. We used a Boolean search combining each genus name AND 'nodulation OR fixation' in Google Scholar (as of 01 Jun 2017) and studied all hits with relevant matches in the whole text. All plant genera were considered associated or not associated with rhizobia (incl. Rhizobiaceae, Burkholderiaceae), Frankiaceae, or Nostocaceae. We also generated extra categories 'likely nodulated' and 'unlikely nodulated' for plant genera that possessed only phylogenetic implications or reports from unreliable sources. Notably, all other plant genera absent from this data set are expected to lack the nodulation capacity, although these may form undifferentiated interactions with free-living N-fixing bacteria.

For phylogenomic and ancestral state reconstruction analysis of rhizobia (II), the NCBI taxonomy browser was used to search for the genome sequences available for published rhizobial and non-rhizobial nodule-associated Proteobacteria (Sprent et al. 2017, Martínez-Hidalgo and Hirsch 2017). We selected 800 high-quality genome assemblies (including plasmid sequences) according to their genome coverage value from 12 families of Proteobacteria, where each species whether symbiotic, pathogenic or free-living represented by a single assembly of an isolate. Reference genomes or the genomes with the highest assembly level were preferentially chosen. To avoid any potential phylogenetic bias, a comparable number of representatives from both putatively symbiotic and non-symbiotic strains were selected. Nine species from *Acidobacteria* (families: Acidobacteriaceae, Bryobacteraceae, and Pyrinomonadaceae) were taken as outgroups according to the ARB living tree (Yarza et al. 2008), and the iTOL tree of life (Letunic and Bork 2019).

To investigate nodulation occurrence in Zygophyllaceae and Arecaceae (III), root samples were collected from *T. terrestris* growing in semi-arid lands of Iran, Saudi Arabia, Western Australia, South Africa, and Benin and *R. regia* growing in the USA (Florida), Mexico (Yucatán), Dominican Republic, and Cuba between the years 2018–2021 (Fig. 1). The samples were stored in fixation buffer, RNA*later*, and 1% PBS solution for microscopy, DNA extraction for metagenomics, and culturing bacteria respectively.

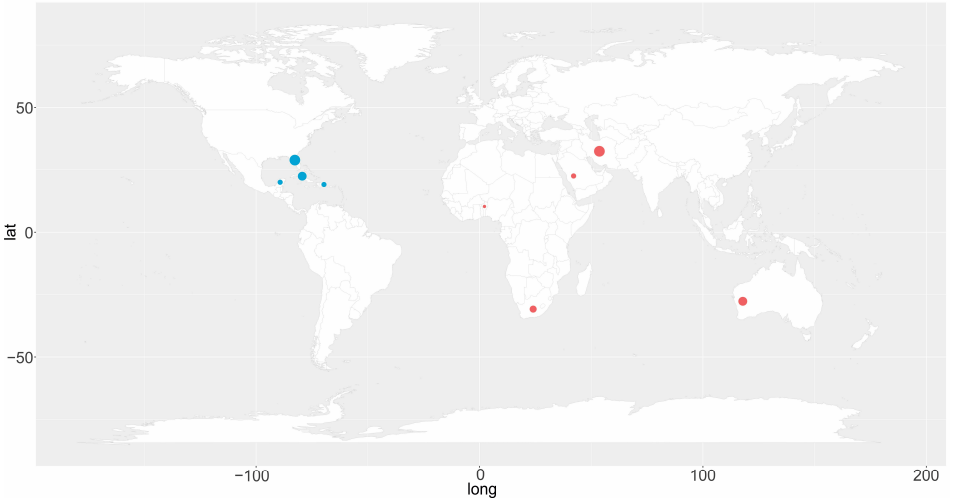

Figure 1. Location map of *Tribulus terrestris* (red) and *Roystonea regia* (blue) root nodule sampling areas.

To isolate bacterial endophytes from nodules (III), nodules were excised from the roots and surface sterilized in 70% ethanol for 1 min and 2.5% Na hypochlorite for 1–3 min and washed with sterile water 3 times. Then nodules were homogenized in a 1.5 ml tube using sterile plastic pestles and the solution was dispersed across the YMA plates (mannitol: 10 g/L, KH_2PO_4: 0.5 g/L, K_2HPO_4: 0.5 g/L, $MgSO_4$*$7H_2O$: 0.2 g/L, yeast extract: 0.5 g/L, NaCl: 0.2 g/L, Agar: 15 g/L, pH: 6.8). Plates were incubated upside down overnight at 28 °C. Individual colonies were selected and streaked onto fresh YMA plates. Once re-grown, a single colony from a pure isolate on the YMA plate was taken and used to inoculate a sterile 5 ml TY broth (tryptone 5 g/L, yeast extract 3 g/L, $CaCl_2$*xH_2O: 0.89 g/L, pH: 6.8). After 24 h (at log phase), 0.9 ml of culture was combined with 0.9 ml of sterile 50% glycerol. The stock was mixed and frozen in liquid-N_2 and later the solution is used for DNA extractions and PCR.

To determine the effects of free-living diazotrophs in association with *Austroboletus occidentalis* on wheat growth (IV), two soil samples were used to isolate the diazotrophic bacteria: the soil used for our controlled-environment trial, and a soil sample collected from Jarrahdale, WA (–32.318397, 116.042577). A Jarrahdale soil subsample (10g) was added to 500-mL Erlenmeyer flasks containing 100 mL of sterile deionized (DI) water and shaken at 150 rpm for 30 min. Then, serial dilutions of 10^{-3} to 10^{-5} were prepared in sterile DI water, and 0.1 mL aliquots of each serial dilution were inoculated onto plates containing 25 mL of Burk's N-free medium (sucrose: 20 g L^{-1}; K_2HPO_4: 0.8 g L^{-1}; KH_2PO_4: 0.2 g L^{-1}; $MgSO_4 \cdot 7H_2O$:0.2 g L^{-1}; $CaCl_2 \cdot 2H_2O$: 90 mg L^{-1}; $FeCl_3$:1.45 mg L^{-1}; $Na_2MoO_4 \cdot 2H_2O$: 0.25 mg L^{-1}; agar: 15 g L^{-1}; pH: 7.0). The inoculated plates were incubated at 25 °C for 3–5 days. The growing colonies were considered diazotrophs and were re-inoculated onto Burk's N-free medium plates to confirm their diazotrophic activity. After 3–5 days of growth at 25 °C, morphologically different

isolates were selected, named, and maintained on nutrient agar (NA) plates for short-term storage at 4 °C, or in nutrient broth (NB; same composition as NA, but without agar) medium amended with 20% (v/v) glycerol for long-term storage (−80 °C). The controlled-environment trial was carried out in a completely randomized design with four replications (IV). Four inoculation treatments included control (no added microbes: only containing soil indigenous microbes), diazotrophs (a consortium of four free-living diazotroph isolates in 20 mM $MgSO_4$), FM (hyphal inoculum of *A. occidentalis*), and dual (co-inoculation with *A. occidentalis* and diazotrophs consortium). To equalize the amount of nutrients/organic matter across treatments, heat-sterilized fungal inoculum (for control and diazotrophs treatments; added before sowing) or heat-sterilized diazotrophs inoculum (for control and FM treatments; added during sowing) was also added to the soil in respective treatments. To prepare the inoculation treatments, the living or sterilized hyphal inocula were thoroughly mixed with soil (1:10 v/v, equivalent to 70 mL of inoculum per kg soil) within clean plastic bags, and 2 kg of the prepared mixtures were placed into the pots. Before sowing, an aqueous KNO_3 solution was added to the soil (33 mg N kg^{-1}) to assist with the early establishment of plants; there was no additional N input during the growth period in order to have N deficiency conditions. Wheat seeds were surface-sterilized and submerged in DI water for 3 h to imbibe. The imbibed seeds were drained and incubated in the dark at 4 °C overnight to break any possible dormancy and achieve uniform germination. Ten seeds were sown per pot (at 2 cm depth), and each seed received 1 mL of the diazotrophs consortium (in diazotrophs and dual treatments) or autoclaved diazotrophs consortium (in control and FM treatments). All pots were covered with sterile plastic beads (3–4 mm in diameter, 35 g per pot) to minimize cross-contamination and reduce evaporation. Seedlings were thinned to six seedlings/pot 1 week after sowing. To assure an effective diazotroph inoculation, seedlings were re-inoculated with 1 mL of the living or sterilized diazotrophs consortium (added to the soil around each seedling) 2 weeks after planting. Plants were grown in controlled-environment growth chambers at 12/12 h light/dark and 20/15 °C day/night temperature. During the growth period, pots were watered to field capacity (14% volumetric water content).

Plants were harvested after 7 weeks of growth. Shoots were cut 1 cm above the soil surface and were dried in an oven (70 °C for 72 h). Roots were separated from the bulk soil and washed over a 2-mm sieve to remove debris and the adhering soil particles. The root total fresh weight was measured for all samples, and each root system was subsequently split into two subsamples, one of which was weighed and oven-dried (70 °C for 72 h) to be used for dry weight calculations, and the other subsample was stored in 50% ethanol (v/v) for root AM colonization measurements. Soil from each pot was homogenized manually, and two subsamples (about 50 g each) were subsequently placed in zip-lock bags, one of which was immediately placed in a foam-insulated container containing dry ice (−78 °C) and stored at −20 °C to be used for microbial analysis. The other soil sample was air-dried and used for mineral and total N analyses. Oven-dried shoot samples were ground to a fine powder, and a measured amount (195–205 mg, the

exact weight recorded for nutrient content calculations) was digested in a mixture of nitric and perchloric acid (4:1 v/v). The sample digests were used to determine the concentration of P, K, Ca, Mg, S, Zn, Fe, and Mn using inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 5300 DV; PerkinElmer). Shoot N content was determined using a combustion analyzer (Elementar Vario Macro, Hanau, Germany). For soil extractable NH_4^+ and NO_3^- content determination, soil samples were extracted using 0.5 M K_2SO_4, and the mineral N fractions were quantified spectrophotometrically (Joergensen and Brookes 1990, Rayment and Lyons 2011). To determine soil total N, the air-dried soil samples were ground to a fine powder, and total N was measured using the combustion analyzer.

Molecular analysis

Total DNA from root nodules was extracted using the CTAB protocol (dx.doi.org/10.17504/protocols.io.bhx8j7rw) and DNeasy Plant mini kit (Qiagen) according to the manual. To amplify the structural *nod*ABC and *nif*HDK genes we used primer sets designed for the amplification of the corresponding genes in γ-Proteobacteria (Shiraishi et al. 2010) and custom-designed primer pairs. Individual PCR reactions were carried out using 5 µl of HOT FIREPol® Blend Master Mix (Solis Biodyne) with 2 mM $MgCl_2$ final concentration, 1 µl of each primer pair, and 2 µl of DNA sample diluted to a final volume of 25 µl. The PCR conditions were adjusted to initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94°C for 1 min, suitable annealing temperature for each primer pair for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min using Eppendorf 5345 Mastercycler Ep Gradient S PCR Thermal Cycler (Applied BiosystemsTM). PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega) and Sanger sequenced in Eurofins-Germany.

To prepare libraries for metagenomics we used Nextera XT DNA Library Preparation Kit (Illumina Inc.) according to the manual. Metagenome sequences were generated for ten root nodule samples of *T. terrestris* and four samples of *R. regia* using Illumina NovaSeq 2×150 at Novogen and Genewiz companies, respectively. Five bacterial isolates were prepared for genome sequencing on the PacBio Sequel II instrument in the Norwegian Sequencing Center. Metagenome and genome assemblies are freely accessible through MG-RAST (https://www.mg-rast.org/mgmain.html?mgpage=search) and NCBI genome database under Bioproject number PRJNA818535 respectively.

Soil subsamples (0.3 g) were used to extract DNA from four biological replicates per treatment (n = 16) using a Qiagen PowerSoil Kit; the manufacturer's protocol was followed throughout the process with the sole modification of reloading the final elution buffer onto a filter column in order to maximize the DNA recovery yield. The V4 region of the bacterial 16S rRNA gene was targeted for amplicon sequencing using the 505F/806R primer pair (Liu et al. 2007), which was carried out at the Australian Genome Research Facility (Melbourne, Australia) using an Illumina MiSeq v2 platform (250 PE chemistry).

Data analysis

The UBCG (Up-to-date Bacterial Core Gene) pipeline v.3 was used for Proteobacteria species tree construction (Na et al. 2018) (II). Multiple sequence alignments were generated using MAFFT v.7.271 (Katoh et al. 2005) applying the 50% cut-off value for gap-containing positions and the 'codon' method to account for amino acid sequences. Finally, a concatenated alignment of 88,989 nucleotide character states was subjected to tree estimation. RAxML v.8.2 implemented in UBCG was applied for phylogenetic tree construction with default settings (Stamatakis 2014). The number of single-gene trees supporting a branch in a UBCG tree is calculated as Gene Support Index (GSI) at a 95% confidence threshold. The GSI value of 92 indicates that a branch is supported by all single-core gene trees used for phylogeny reconstruction (Na et al. 2018). Phylogenetic trees were visualized and annotated using the online tool iTOL v.4.3.3 and released under the username 'Saleh' (https://itol.embl.de/shared_projects.cgi). Clock calibration analysis was performed using the 'chronopl' function with $\lambda = 0$ from the ape package using R v.3.6. This function estimates the node ages of a tree using a semi-parametric method based on penalized likelihood (Sanderson 2002). We constrained our genome-based phylogeny at the origin of α-Proteobacteria around 1900 (1786–2012) Mya (Wang and Luo 2021). Ancestral states for nodulation genes were reconstructed using the 'ace' function by setting an equal rate (ER) model from the ape package. The results were mapped onto the time-calibrated species tree using R v.3.6.

For metagenomic analysis (III) raw reads were trimmed and quality filtered using Trimmomatic v.0.39 (Bolger et al. 2014) with ILLUMINACLIP: NexteraPE.fa:2:30:10:2:keepBothReads LEADING:3 TRAILING:3 MINLEN:36 parameter values. MegaHit assembler v.1.1.3 (Li et al. 2016) was applied to assemble the filtered sequences with kmer sizes ranging from min=15 to max=255 with increment=2 after each iteration using the university of Tartu computer cluster system. The assemblies were annotated using EggNOG-mapper pipeline v.2.0.6 (Cantalapiedra et al. 2021). For taxonomic and functional sequence annotation purposes the assemblies were subjected to MG-RAST pipeline v.4.0.3 (www.mg-rast.org). Using Blobtools v.2 (Challis et al. 2020) we parsed the assembled contigs into taxonomic groups. For checking the presence/absence of conserved *NIN*, *RPG*, and *NFP2* genes, the nucleotide sequences of the corresponding genes from the nodulating plant species *Medicago truncatula* and *Parasponia rugosa* were mapped to contigs classified in Streptophyta using nhmmer v.3.3.2 (Wheeler and Eddy, 2013). Genome assembling with PacBio raw reads was performed using Canu v.2.2 (Koren et al. 2017) with default parameter settings. Genome completeness was measured using BUSCO v.5.1.12 (Manni et al. 2021). The assemblies were annotated using EggNOG-mapper pipeline v.2.0.6 (Cantalapiedra et al. 2021). The GEnView pipeline was used to characterize the structural N-fixing *nif*HDK genes and their 7000 nucleotide gene-environment with 50% identity and 60% coverage (Ebmeyer et al., 2022). Average nucleotide identity analysis (ANIb) was performed with 14 *Kosakonia* genome assemblies

retrieved from GenBank including our *Kosakonia sacchari* genome (R1.Fl) with the Pyani v.0.2 program (Pritchard et al. 2016).

Amplicon sequence bioinformatics analysis was performed using the QIIME2 pipeline (Bolyen et al. 2019) (IV). In total, 2.88 million reads were generated for the 16S rDNA region, out of which about 580,000 reads (~ 20%) were retained after quality filtering steps such as read overlap detection, de-noising, and chimera filtering. Amplicon sequence variants (ASVs) were selected using the DADA2 plugin after trimming the low-quality nucleotides of each sequence. Taxonomic assignment was carried out using the Scikit-learn algorithm within the QIIME2 pipeline. Chloroplast- and mitochondria-like sequences as well as low-abundance ASVs were discarded (representing 15% of the dataset). Due to the unbalanced read count among samples, a rarefaction step (set at 19,276) was performed prior to the statistical analyses. The Bray–Curtis dissimilarity matrix was employed to generate the non-metric multidimensional scaling (NMDS) plot based on the relative microbial abundance using the 'metaMDS' function of the 'vegan' package in R (Oksanen et al. 2019). Similarity percentage analysis (SIMPER, implemented in the 'vegan' R package) was also performed to quantify dissimilarity between groups and better explain the observed clustering (Clarke 1993). Shannon diversity was calculated using the function 'Diversity' in the 'vegan' package and visualized using the ggplot2 package (Wickham et al. 2020). Due to the short length of the targeted amplicons, a fine taxonomic resolution is rarely achieved for soil microorganisms. Two diazotrophs (SP5 and SP12) were taxonomically identified as the same *Arthrobacter* species, despite presenting different morphology. A phylogenetic approach was taken to differentiate these two isolates. The 16S rDNA region sequences obtained from the pure cultures of these two diazotroph isolates were analyzed separately following the same methodology as described above. A total of 331,000 quality reads were binned into 10 ASVs, all of which were identified as an unknown *Arthrobacter* species. The ASV sequences were extracted and placed into a comprehensive phylogenetic reference tree (Hug et al. 2016). Reference sequences were aligned using the INFERNAL, and tree construction was performed using the RAxML algorithm using the GTRGAMMA substitution model. The ASV sequences of SP5 and SP12 isolates were concatenated with the reference sequences and aligned as described above. The reference package and the alignment were then fed into the "pplacer" (Matsen et al. 2010) with the flag "-keep-at-most" set to one. Phylogenetic placements were then visualized and annotated in iToL v.5 (Letunic and Bork 2019). All 16S rRNA gene sequences were deposited in the European Nucleotide Archive (accession number: PRJEB46901).

RESULTS AND DISCUSSION

Diversity of nitrogen-fixing plants

We propose a consensus reference database NodDB on nodulation in plant genera that account for critically revised records and plant phylogeny (I). To date, the existing databases cover highly variable amounts of information about plants associated with N-fixing bacteria. Sprent (2009), Werner et al. (2014), Li et al. (2015), the Nodulation_clade, GRIN and TRY databases provided information about nodulation for 391, 360, 469, 505, 490, and 1,800 (220 genera in the target group) currently recognized genera, respectively. More specific reviews by Rai et al. (2000) and Chaia et al. (2010) supplemented information about ten *Cyanobacteria*-associated genera and 25 actinorhizal plant genera, respectively. Combining these basic sources, as well as neglected older and more recent studies increased the available information about plants with or without N-fixing root nodules to 590 genera based on 9,446 records. The likelihood for nodulation was assigned to a further 234 genera (mainly Fabaceae and Zygophyllaceae) based on their phylogenetic relationships. These data can be further used to address evolutionary ecological hypotheses regarding nodulation. Information about the potential N fixers may greatly improve estimates of N cycling in plant communities from fine to landscape scales and planning of further research on coevolution and host shifts. Knowledge about N-fixing capacity facilitates the development of agroforestry planning and selection of plant species for bio fertilization and reclamation of soil. Our data support the hypothesis that rhizobial symbiosis has evolved more than twice and up to six times in Fabaceae, once in Cannabaceae, and probably once in Zygophyllaceae. Actinorhizal associations evolved on nine independent occasions in angiosperms, whereas cyanobacterial nodules are known only from all three extant families of cycads.

The origin of rhizobia–legume N-fixing symbiosis

To estimate the origin of rhizobia-legume N-fixing symbiosis, we calibrated our genome-based phylogeny of Proteobacteria using the origin of α-Proteobacteria at ~1900 (1786–2012) Mya, as the only reliable and host-independent calibration available for Proteobacteria (Wang and Luo 2021) (II). Applying this calibration, major root-nodulating genera diverged relatively recently (51 Mya), with *Rhizobium* as the oldest genus among major nodulating genera of Proteobacteria (Tab. 1). It is suggesting that most probably horizontal gene transfer played a pivotal role in the transition of symbiotic genes from Rhizobiaceae to several other root-nodulating Proteobacteria. Interestingly, our estimated time for the emergence of nodulation in rhizobia is matching the early Eocene climatic optimum (~51 Mya), which was characterized by high CO_2 levels (Zhao et al. 2021).

Table 1. Main symbiotic nodule inducing proteobacterial genera.

Genus	Family	Relative Divergence Time (Mya)*	Host Plant (Genus)
Rhizobium s.str.	Rhizobiaceae	~50.86	*Phaseolus, Trifolium, Mimosa, Lathyrus, Pisum, Vicia, Lens, Onobrychis, Albizia, Arachis, Astragalus, Caragana, Desmanthus, Kummerowia, Oxytropis, Clitoria, Dalea, Leucaena, Calliandra, Macroptilium, Vigna, Sophora, Hedysarum, Trigonella, Acacia, Faidherbia, Lotus, Neptunia*
Sinorhizobium (*Ensifer*)	Rhizobiaceae	~19.07	*Sesbania, Acacia, Phaseolus, Tephrosia, Astragalus, Glycine, Prosopis, Medicago, Leucaena, Neptunia, Vachellia*
Mesorhizobium	Phyllo-bacteriaceae	~3.71	*Alhagi, Amorpha, Cicer, Robinia, Biserrula, Lotus, Sesbania, Astragalus, Sophora, Anthyllis*
Bradyrhizobium	Bradyr-hizobiaceae	~32.04	*Arachis, Chamaecytisus, Lupinus, Teline, Aeschynomene, Glycine, Macroptilium, Vigna, Phaseolus, Pachyrhizus, Lablab, Centrolobium, Retama, Erythrina, Neonotonia, Centrosema, Lespedeza*
Paraburk-holderia	Burk-holderiaceae	~37.12	*Mimosa, Phaseolus, Rhynchosia, Piptadenia, Lebeckia, Cyclopia, Macroptilium*

* The divergence time estimates are calculated with ±3 My confidence interval.

It is noted that nodulation genes of *Paraburkholderia* branched earlier compared to α-proteobacterial genes and thus suggested that nodulation may be ancestral in β-Proteobacteria (Bontemps et al. 2010). Using a broader set of isolates and phylogenomic approaches, we demonstrated that phylogenies of *nod* genes are topologically incongruent with the species tree constructed based on genome sequences, which most probably results from horizontal gene transfer. Our analyses indicate that nodulation genes emerged earlier (< 51 Mya) and are more diverse in α-Proteobacteria. We are suggesting most probably the ancestor of the four legume subfamilies that are emerged before this time including Cercidoideae, Detarioideae, Dialioideae, Duparquetioideae, and the early branching Caesalpinioideae and Papilionoideae lineages were non-symbiotic due to the absence of nodulating symbionts in nature (Lavin et al. 2005, Azani et al. 2017, Koenen et al. 2020, Ardley and Sprent 2021).

Nodulation occurrence outside the conventional N-fixing clade

Nodule-like structures in *T. terrestris* L. (Zygophyllaceae) root systems were observed in samples collected from the Middle East (III) (Fig. 2). The structures averaged 1.5 to 2 mm in diameter and were loosely attached to the root system and are white to yellowish, similar to the color of the roots. Field observations demonstrated that maximum nodulation occurred in sandy soils and sandy gravels with low moisture content. Root hair deformation was observed in the field which is indicative of early physiological responses initiated by the Nod factor perception. The Indeterminate type nodules with an apical meristem and a central vascular system resemble those of actinorhizal plants. No common nodulating genera were detected in metagenome assemblies of *T. terrestris*. In addition, we found no evidence of cyanobacterial infection in *T. terrestris* nodules in contrast to Mahmood and Athar (1998) who reported heavy colonization of nodules by a new species described as *Newmania karachiensis*. However, we were able to amplify *nod*ABC and *nif*HDK genes of *Sinorhizobium meliloti* from *T. terrestris* nodular tissues. On the other hand, we detected the conserved *NIN*, *RPG*, and *NFP2* genes in *T. terrestris* metagenome assemblies suggesting potential physiological responses to Nod factor perception. However, despite many attempts, *S. meliloti* could not be isolated from surface-sterilized nodules of *T. terrestris*.

Nodule-like structures also formed on the young roots of *R. regia* (III). Mature nodule-like structures are creamy in color, oval to cylindrical, 2 to 2.5 mm in diameter with an apical meristem (Fig. 3). The structures resemble indeterminate type actinorhizal nodules with a central vascular system. Tubular structures were observed inside the nodule cortex cells that are similar to fungal hyphae in terms of thickness. Nodules are less common in rocky and dry soils, and young plants also nodulate in natural conditions. Nodules were found in every sample collected from the USA (Florida), Mexico (Yucatan), Dominican

Republic, and Cuba. *Kosakonia sacchari* was the single dominant species isolated from *R. regia* root nodules. *K. sacchari* is fast-growing, gummy white mucilaginous, convex, and has many peritrichous flagella which are matching the description of the strain isolated by Basu et al. (1997) from *R. regia* nodules. This species is known to be able to colonize and fix atmospheric N_2 in association with sugarcane plants (Chen et al., 2014). The *nif*HDK operon coding for α and β subunits of nitrogenase was identified in the chromosome of *K. sacchari* R1.Fl strain but the *nod* genes coding for Nod factor backbone structure synthesis are missing.

Two possible hypotheses explain the occurrence of nodulation in *T. terrestris*: nodulation occurs via infection by *S. meliloti*; or *S. meliloti* is present on the nodule surface but cannot penetrate the nodule – so inducing bacteria-free nodule-like structures. It has been demonstrated that there are plant mutations that result in spontaneous root nodule formation in the absence of rhizobia. Tirichine et al. (2006) indicated that mutation of the autophosphorylation residue (T265I) of *Lotus japonicus* CCaMK leads to spontaneous nodulation in the absence of rhizobia when expressed from the native promoter. Furthermore, it has been shown that removal of the autoinhibition domain in *DMI3* (encoding for CCaMK in *Medicago truncatula*) leads to the autoactivation of the nodulation signaling pathway in the plant, resulting in the induction of nodules and the nodulation gene expression in the absence of bacterial elicitation (Gleason et al. 2006). Mutation of *Lotus japonicus* cytokinin receptor gene (*Lhk1*) also resulted in the spontaneous development of root nodules in the absence of rhizobia or rhizobial signal molecules (Tirichine et al. 2007). The second hypothesis appears more likely in both *T. terrestris* and *R. regia*, in which nodulation may be caused by certain rhizobia that cannot penetrate the nodules. Since after surface sterilization we could not isolate any rhizobia from root nodules and the other isolated bacteria are lacking the structural *nod* genes. In addition, data from metagenomics also could not reveal any dominant rhizobia living in nodules, and no structural *nod* genes were found in the assemblies. By sectioning and microscopically observing nodule-like structures, we found no signs of the bacteroid colony, symbiosomes, and infection threads formation, which are characteristic of legumes and actinorhizal nodules. The demonstrated biological N fixation of *T. terrestris* (Athar & Mahmood 1985) and *R. regia* (Basu et al. 1997) can be attributed to *S. meliloti* and *K. sacchari* respectively, which occupy the nodule-like structures as epi- or endophytes. These two plants that produce rhizobia-free nodules naturally provide a great opportunity to treat them as intermediate taxa between nodulating and other plant species in comparative genomics and transcriptomics analyses.

The presence of root nodules in *T. terrestris* and *R. regia* raises an important and still open question: Why does nodulation in plants exhibit such a polyphyletic pattern? Two major hypotheses have been proposed for explaining the scattered evolution of root nodule symbiosis in plants. First, nodulation may have been independently gained multiple times with a predisposition at the common ancestor of the currently known N-fixing clade (Soltis et al. 1995, Swensen 1996,

Doyle 2011, Werner et al. 2014). Based on comparative genomics analysis of plants, Griesmann et al. (2018) did not detect parallel gene expansion in nodulating plant species, therefore rejecting the multiple gains hypothesis. They proposed an alternative, single gain and massive loss hypothesis suggesting that nodulation has been acquired at the common ancestor of the conventional N-fixing clade around 92–110 Mya and subsequently lost multiple times. Genes *NIN* and *RPG* were found to be conserved in nodulating taxa supporting this hypothesis. However, functional copies of these genes were observed in non-nodulating plant taxa within the conventional N-fixing clade as well as other non-symbiotic outgroup plant species. Additionally, *NIN*, *RPG*, and *NFP2* were found in our metagenome data generated for *T. terrestris,* indicating that these genes are distributed taxonomically more broadly, in both nodulated and non-nodulated plants of the N-fixing clade (Griesmann et al. 2018) as well as earlier diverging lineages. This casts doubt on their exclusive functional association with nodulation, leaving the evolution of N-fixing symbiosis still ambiguous. Considering atypical, rhizobial-free nodulation in *T. terrestris* and *R. regia* and the probable occurrence of similar nodules in some other members of Zygophyllaceae, it is highly improbable that nodulation was gained at a single point at the common ancestor of the currently known N-fixing clade. Based on the phylogenomics study of rhizobia, it has shown that nodulation emerged around 51 Mya in Rhizobiaceae suggesting the legume subfamilies that emerged before this period remained non-symbiotic probably due to the absence of symbiotic bacteria in nature (Rahimlou et al. 2021). It seems more likely that angiosperms acquired nodulation more than once, which supports the first hypothesis.

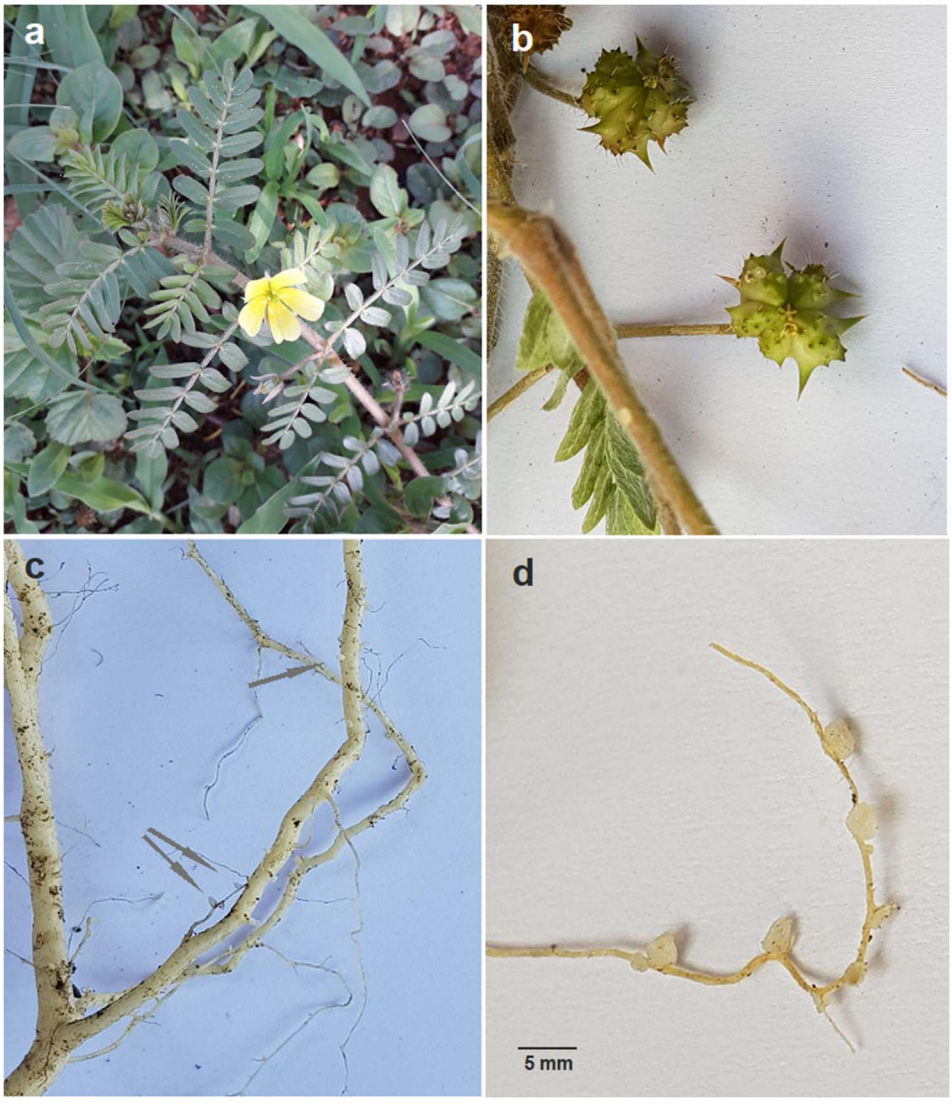

Figure 2. *Tribulus terrestris* compound leaves and yellow five-petal flower (a), spiky fruits (b), and root nodule-like structures (c,d).

Figure 3. *Roystonea regia* tree (a), fruit bunch (b), ripe and unripe fruits (c, d), and root nodule-like structures (e, f).

The role of diazotrophic bacteria in plant growth promotion

***Austroboletus occidentalis* and free-living soil diazotrophs enhanced wheat plant growth synergistically (IV)**, as reflected in higher shoot N content and shoot/root biomass of plants in the fungal and dual treatments compared to the control and the diazotrophs treatments. The dually inoculated plants had significantly higher extractable NH_4^+-N compared to the control and diazotrophs treatments (Fig. 4), as well as the highest shoot N content and soil total N across the treatments (Fig. 4). Although plants in the dual treatment had higher shoot nutrient content than in the fungus-only treatment, their shoot biomass was numerically higher than in the fungal treatment, a possible indication of higher C allocation to the fungus and diazotrophs in the dual treatment. Soils in all inoculated treatments had significantly lower NO_3^--N content compared to the control (Fig. 4), likely due to the greater root biomass of inoculated plants, which used the soil available NO_3^--N. This phenomenon was more pronounced in the fungal and dual treatments, which had the lowest NO_3^--N content across treatments, because of their larger root and shoot biomass. The improved N nutrition of plants in the fungal treatment compared to both control and diazotrophs treatments can be associated with the enhanced N_2-fixing activities of the indigenous diazotroph populations that were detected in the field soil. We assume that *A. occidentalis* enhanced the activity of indigenous soil-borne N_2-fixing microbes, which might thereby account for the significant N nutritional benefits in the fungal treatment. This was supported by the significant fungus-driven modification of the soil microbial community uncovered by NMDS ordination of the bacterial 16S rRNA gene amplicon data (Fig. 5).

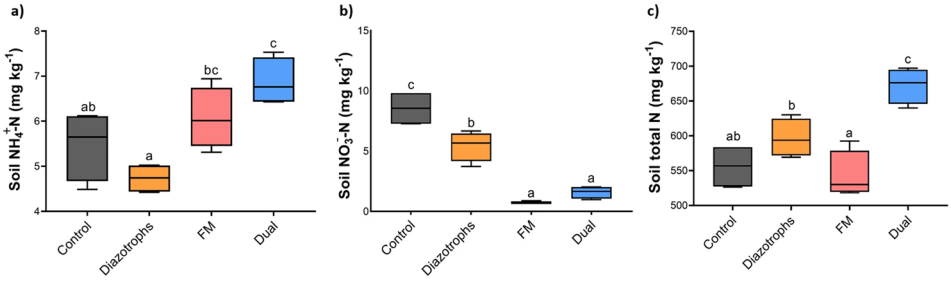

Figure 4. Soil content of extractable NH_4^+-N (a) and NO_3^--N (b) and total N (c) at the end of the controlled-environment study (7 weeks). Control, no added microbes, only containing soil indigenous microbes; diazotrophs, inoculated with a consortium containing four free-living diazotroph isolates; FM, inoculated with the hyphal inoculum of *Austroboletus occidentalis*; and dual, inoculated with both diazotrophs and *A. occidentalis* inoculums. For each parameter, bars with different letters are significantly different according to Fisher's protected least significant difference test ($p \leq 0.05$). Error bars indicate standard errors ($n = 4$).

The dual treatment had the highest soil total N content, which can be attributed mainly to the organic N pool accumulated in biomass of the introduced fungus/diazotrophs because the soil total extractable mineral N (NH_4^+-N + NO_3^--N) content of the dual treatment only made a small proportion (~ 1.2%) of the total N (Fig. 4). A proportion of the nutritional improvement in the dual treatment can also be attributed to possible plant growth-promoting activities of the added diazotrophs (e.g., P solubilization, production of siderophores, and phytohormones such as auxin) (Backer et al. 2018), which might have been stimulated by the fungus, leading to greater shoot biomass and nutrient content in the dual treatment compared to the diazotrophs treatment. These are important findings within the agricultural context, as this fungus as well as effective diazotrophs can be propagated on synthetic media containing hexoses (e.g., glucose), and be applied to enhance biological N_2 fixation to benefit both AM and non-mycorrhizal crops.

Synergism between mycorrhizal fungi and diazotrophs has been attributed to diverse factors, including the role of fungal partners in increasing the mobilization/solubilization of soil nutrients, particularly those involved in the biosynthesis of nitrogenase and the oxygen-carrying compound leghemoglobin (e.g., Fe, P, Mo) and/or direct provision of C source for diazotrophs as organic acid anions (Olsson and Wallander 1998, Abd-Alla et al. 2014, Püschel et al. 2017). Based on the proximity of the bacterial symbionts and plant roots, the diazotrophs are classified into three main groups, namely free-living (e.g., *Azotobacter* and *Azospirillum*), endophytic (e.g., *Azoarcus* and *Herbaspirillum*), or endosymbiotic (e.g., *Rhizobium* and *Frankia*) (Mus et al. 2016). Endophytic and endosymbiotic diazotrophs rely directly on the C source provided by their associated host plants inside their roots, whereas the free-living (rhizosphere-associative) diazotrophs typically gain their energy by oxidizing organic molecules – exudates – released by roots, other organisms, or from the decomposition of organic matter (Mus et al. 2016, Dellagi et al. 2020). *Austroboletus occidentalis* was previously shown to solubilize water-insoluble P compounds via exudation of organic acid anions such as oxalate and citrate (Kariman et al. 2020), which may also act as a food source for free-living diazotrophs in soil, thus representing one of the possible mechanisms underlying the synergism we observed in our study between *A. occidentalis* and free-living diazotrophs.

Nitrogen fixation by soil microbes is a gradual process that provides plants with a steady N supply (James 2000); microbe-mediated N_2 fixation is accompanied by other nutritional (e.g., P, K, Mg, Fe, Zn) benefits as we observed in the present study and previous studies (Abd-Alla et al. 2014, Püschel et al. 2017). This may result in the nutritional biofortification of food/feed crops in comparison with the application of synthetic N fertilizers. The diazotroph isolates used in this study were identified as *Arthrobacter* sp. (SP5/SP12), *Bacillus flexus* (SP15), and *Paraburkholderia bryophila* (J7), all of which have been documented as free-living diazotrophic bacteria (Mongodin et al. 2006, Eberl and Vandamme 2016, Yousuf et al. 2017). Out of the four added diazotrophs, only two of them (SP5/SP12; *Arthrobacter* isolates) had higher relative abundance in the corre-

sponding treatments (i.e., diazotrophs and dual treatments) (Fig. 5), suggesting an effective establishment of these two isolates compared to the other two isolates (SP15 and J7) that had the same relative abundance across treatments. The enhanced soil total N in the dual treatment (Fig. 4) could also be partially attributed to the enhanced relative abundances of the SP5/SP12 isolates. Nonetheless, we should bear in mind that the relative abundance does not necessarily reflect the magnitude of N_2-fixing activities of diazotrophs. Future research, therefore, should focus on the activity of *nif* genes to further elucidate the interactions between *A. occidentalis* and diazotrophs.

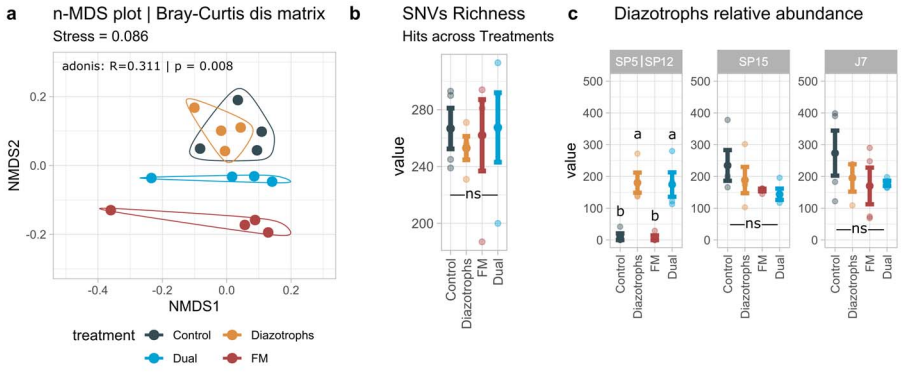

Figure 5. Soil microbiome analysis based on the bacterial 16S rRNA gene sequences at the end of the controlled-environment study (7 weeks). a Non-metric multidimensional scaling (NMDS) analysis using the Bray–Curtis dissimilarity matrix. b Species richness in different treatments based on the amplicon sequence variants (ASVs). C Relative abundance of the four added diazotroph isolates in soil. Control, no added microbes, only containing soil indigenous microbes; diazotrophs, inoculated with a consortium containing four free-living diazotroph isolates; FM, inoculated with the hyphal inoculum of *Austroboletus occidentalis*; and dual, inoculated with both diazotrophs and *A. occidentalis* inoculums. Bars with different letters are significantly different according to the Fisher's protected least significant difference test ($p \leq 0.05$). ns, not significant. Error bars indicate standard errors (n = 4).

In the present study, the addition of microbial inocula (diazotrophs and *A. occidentalis*, alone or in combination) resulted in a significant reduction in AM colonization of wheat roots under severe N deficiency conditions. Previously, Root AM colonization of maize plants was shown to be negatively affected by the N_2-fixing bacterium (*Bacillus subtilis*) due to the exudation of unknown volatile antifungal compounds (Xiao et al. 2008); hence, the decline in AM colonization of wheat plants in the present study could be due to antifungal properties of the added diazotrophic isolates. The decline in root AM colonization of plants inoculated only with *A. occidentalis* (compared to the control) can be possibly due to partial C allocation to the introduced fungus, leading to reduced C allocation to the indigenous AM fungi accompanied by decreased root colonization.

CONCLUSIONS

The following conclusions and further hypotheses can be inferred from my thesis:

- Understanding the distribution of N fixation among plants is far from complete. Resolving the multiple potential gains and losses in Fabaceae certainly requires further confirmation of nodulation or the lack of it in critical taxa.
- Although N-fixation of plants *T. terrestris* and *R. regia* was discovered in the 1970s, some rare N-fixing taxa may remain to be discovered within poorly studied habitats.
- Our analysis indicated that nodulation capacity evolved for the first time in Rhizobiaceae around 51 Mya (Eocene epoch) and further spread to multiple nodulating lineages via horizontal gene transfer. This suggests that the ancestor of the legume lineages that evolved over this time remained non-symbiotic most probably owing to the absence of nodulating symbionts in nature.
- Nodule-like structures were observed in *T. terrestris* and *R. regia* sampled from the Middle East and the Caribbean, respectively. Although genomic evidence for biological N fixation has been acquired, their N symbiotic association with rhizobia seems unlikely. Our study suggests that there is a possibility of similar spontaneous nodulation in other members of the Zygophyllaceae and, perhaps, Arecaceae, for which further investigation is required. Although rhizobia may have stimulated nodule formation in *T. terrestris* and *R. regia*, these bacteria or *Frankia* are not among the dominant members colonizing the nodules.
- Nodulating plant taxa may have independently lost/downregulated certain resistant genes against rhizobial infection. Parallel gene contractions in nodulating taxa warrant further investigation based on genome comparison analyses of symbiotic and non-symbiotic species.
- The fungus *A. occidentalis*, alone or in combination with diazotrophs, significantly improved the shoot and root biomass and nutrition (e.g., N, P, K, Zn, Fe) of wheat plants grown under N deficiency, which can be attributed to the synergistic interactions between *A. occidentalis* and indigenous or added diazotrophs. The presence of *A. occidentalis* led to the modification of the soil microbial composition while preserving the microbial species richness.

SUMMARY

Nitrogen is essential to life because it is required for the biosynthesis of all N-containing organic compounds, such as proteins. N fixation is carried out naturally in soil by microorganisms termed diazotrophs including various groups of bacteria and archaea. Three main groups of bacteria – Nostocaceae (Cyanobacteria), rhizobia (Proteobacteria), and *Frankia* (Actinobacteria) fix atmospheric N both in a free-living form and in root symbiosis with plants. Here, we introduce the NodDB database of N-fixing plants based on morphological and phylogenetic evidence (available at https://doi.org/10.15156/bio/587469) and discuss plant groups with conflicting reports and interpretations, such as the certain legume clades and the Zygophyllaceae and Arecaceae family. This compiled and re-interpreted information about N-fixing plants enables accurate analyses of biogeography and community ecology of biological N fixation.

Root nodulating Proteobacteria produce nodulation (Nod) factors during the initiation of rhizobial nodule organogenesis on the roots of legumes. We screened the Nod factor production capacity of the previously reported nodule-inducing Proteobacteria genera using their genome sequences and assessed the evolutionary history of symbiosis based on phylogenomics. Based on molecular clock analysis, we estimate that rhizobial N-fixing symbiosis appeared for the first time in about 51 Mya (Eocene epoch) in Rhizobiaceae, and it was laterally transferred to multiple symbiotic taxa in the α- and β-Proteobacteria.

Root nodule symbiosis with rhizobia and actinobacteria is considered to be limited to four plant orders Fabales, Fagales, Cucurbitales, and Rosales, which are nested in the rosid I clade of angiosperms. However, several articles have reported root nodule symbiosis in Zygophyllaceae and *Roystonea regia* Cook (Arecaceae), but have remained unnoticed or ignored for decades. We collected root samples of *Tribulus terrestris* L. (Zygophyllaceae) and *R. regia* Cook (Arecaceae) from the Middle East and the Caribbean, respectively. Nodule-like structures were observed on the root systems of both plant species. Genomes of five bacterial strains isolated from surface-sterilized root nodules were sequenced and metagenome data were generated for 14 nodule samples from *T. terrestris* and *R. regia*. Phylogenomic analysis indicates that the dominant bacterial species isolated from *T. terrestris* and *R. regia* are clustered with *Klebsiella* and *Kosakonia* (γ-Proteobacteria), respectively. structural *nod/nif* genes identified matching *Sinorhizobium meliloti* could be amplified from *T. terrestris* root nodule samples. In contrast to previous reports, cyanobacterial infection in root nodules of *T. terrestris* was not detected based on metagenome sequence data analysis. Conserved *NIN*, *RPG*, and *NFP2* genes in N-fixing plant species were detected in *T. terrestris* metagenome assemblies. We conclude that *T. terrestris* and *R. regia* evolved to produce natural 'empty nodules' without intracellular rhizobial infection. The demonstrated biological N fixation of *T. terrestris* and *R. regia* can be attributed to *S. meliloti* and *K. sacchari* respectively, which colonize the nodule-like structures as epi- or endophytes.

A controlled-environment study was conducted to explore possible synergistic interactions between the fungus *Austroboletus occidentalis* and soil free-living N-fixing bacteria (diazotrophs). Wheat (*Triticum aestivum*) plants were grown under N deficiency conditions in a field soil without adding microbial inoculum (control: only containing soil indigenous microbes) or inoculated with a consortium containing four free-living diazotroph isolates (diazotrophs treatment), *A. occidentalis* inoculum (FM treatment), or both diazotrophs and *A. occidentalis* inoculums (dual treatment). After 7 weeks of growth, significantly greater shoot biomass was observed in plants inoculated with diazotrophs (by 25%), *A. occidentalis* (by 101%), and combined inoculums (by 106%), compared to the non-inoculated control treatment. All inoculated plants also had higher shoot nutrient contents (including N, P, K, Mg, Zn, Cu, and Mn) than the control treatment. Compared to the control and diazotrophs treatments, significantly greater shoot N content was observed in the FM treatment (i.e., synergism between the FM fungus and soil indigenous diazotrophs). Dually inoculated plants had the highest content of nutrients in shoots (e.g., N, P, K, S, Mg, Zn, Cu, and Mn) and soil total N (13–24% higher than the other treatments), *i.e.,* synergism between the FM fungus and added diazotrophs. Root colonization by soil indigenous arbuscular mycorrhizal fungi declined in all inoculated plants compared to control. Non-metric multidimensional scaling (NMDS) analysis of the bacterial 16S rRNA gene amplicons revealed that the FM fungus modified the soil microbiome. Our in vitro study indicated that *A. occidentalis* could not grow on substrates containing lignocellulosic materials or sucrose, but grew on media supplemented with hexoses such as glucose and fructose, indicating that the FM fungus has limited saprotrophic capacity similar to ectomycorrhizal fungi. The results revealed synergistic interactions between *A. occidentalis* and soil free-living diazotrophs, indicating a potential to boost microbial N_2 fixation for non-legume crops.

SUMMARY IN ESTONIAN

Lämmastikku fikseerivate bakterite sümbioos taimejuurtega

Lämmastik on eluks hädavajalik keemiline element, sest seda on tarvis kõigi lämmastikku sisaldavate orgaaniliste ühendite, näiteks valkude, biosünteesiks. Lämmastikku seovad mikroorganismid, keda nimetatakse diasotroofideks – näiteks erinevad bakterid ja arhed. Kolm peamist bakterirühma – *Nostocaceae* (tsüanobakterid), mügarbakterid (proteobakterid sugukondadest *Rhizobiaceae* ja *Burkholderiaceae*) ja *Frankia* „kiirikseened" (aktinobakterid) – seovad õhulämmastikku nii iseseisvalt kui ka taimejuurtega sümbioosis olles. Käesolevas doktoritöös tutvustan morfoloogilistel ja evolutsioonilise ajaloo tõenditel põhinevat NodDB andmebaasi (kättesaadav aadressil https://doi.org/10.15156/bio/587469) ning arutlen vastuoluliste kirjelduste ja tõlgendustega taimerühmade üle, näiteks teatud liblikõieliste (*Fabaceae*) rühmad ning eksootilised sugukonnad seiglehelised (*Zygophyllaceae*) ja palmilised (*Arecaceae*). Koondatud ja ümbertõlgendatud teave lämmastikku siduvate taimede kohta võimaldas läbi viia analüüsi bioloogilise lämmastiku sidumise ökoloogia ja leviku kohta.

Juuremügaraid moodustavad proteobakterid toodavad liblikõieliste juurte noodulite arengu algfaasis valke, mida nimetatakse nodulatsioonifaktoriteks (nod-faktorid). Geenijärjestusi kasutades uurisin varem kirjeldatud juuremügaraid moodustavate proteobakterite perekondade nod-faktoreid ja hindasin sümbioosi evolutsioonilist ajalugu fülogenoomika analüüside põhjal. Molekulaarse kella analüüsile tuginedes prognoosin, et mügarbakterite lämmastikku siduv sümbioos ilmnes *Rhizobiaceae* sugukonnas esimest korda umbes 51 miljonit aastat tagasi (eotseeni ajastu) ja kandus hiljem horisontaalselt edasi mitmele sümbiootilisele alfa- ja beeta-proteobakteri taksonile.

Juuremügara sümbioosi aktinobakterite ja mügarbakteritega peetakse esinevaks neljal seltsil taimedel – pöögilaadsed *(Fagales), oalaadsed (Fabales), kõrvitsalaadsed (Cucurbitales)* ja roosilaadsed (*Rosales)* – mis kuuluvad katteseemnetaimede pärisrosiidide I klaadi. Mitmed teadusartiklid on sugukonna seiglehelised ja palmiliigi *Roystonea regia* Cook sümbioosi kirjeldanud, kuid need on aastakümneteks tähelepanuta jäänud. Kogusin Lähis-Idast juureproove seigleheliste liigilt *Tribulus terrestris* L. ja Kariibi mere saartelt palmiliste liigilt *R. regia* ning täheldasin mõlema liigi juurestikul noodulilaadseid struktuure. Pindsteriliseeritud juurenoodulitest eraldasin ja sekveneerisin (järjendasin) viie bakteritüve genoomid. Lisaks sekveneerisin metagenoomi 14 *T. terrestris* ja *R. regia* juurenooduli proovile. Fülogenoomiline analüüs näitab, et *T. terrestris* ja *R. regia* proovidest eraldatud valdavad bakteriliigid on vastavalt *Klebsiella* ja *Kosakonia* perekondade esindajad (gammaproteobakterid). Seigleheliste liigi *T. terrestris* juurenoodulite proovidest leidsin amplifitseerimise (PCR) ja sekveneerimise tulemusel bakteriliigi *Sinorhizobium meliloti* struktuurseid *nod* ja *nif* geene. Bakterid kasutavad *nif* geene lämmastikku fikseerivate valkude sünteesiks. Erinevalt varasematest teadustöödest ei tuvastatud metagenoomi andmete ana-

lüüsi põhjal taimeliigi *T. terrestris* juurenoodulites tsüanobakteriaalset infektsiooni. *T. terrestris* metagenoomi kooslustes tuvastati lämmastikku siduvate taimeliikide *NIN*, *RPG* ja *NFP2* geene. Järeldan, et taimeliikidel *T. terrestris* ja *R. regia* arenes loomulike "tühjade noodulite" teke ilma rakusisese mügarbakterite infektsioonita. Liikidel *T. terrestris* ja *R. regia* esineva bioloogilise lämmastiku sidumist võib seostada vastavalt *S. meliloti* ja *K. sacchari* bakteritega, mis hõivavad noodulilaadseid struktuure epi- või endofüütidena, elades vastavalt taimekudede pinnal või sees.

Kontrollitud keskkonnas läbiviidud uuringus vaadeldi võimalikke samasuunalisi koostoimeid fakultatiivselt ektomükoriisse seeneliigi *Austroboletus occidentalis* ja mullas vabalt elavate lämmastikku siduvate bakterite vahel. Nisu (*Triticum aestivum*) kasvatati põllumullas lämmastikupuuduse tingimustes mikroobset inokulaati lisamata (muld sisaldas ainult looduslikke mikroobe) või inokuleeriti nelja vabalt elava diasotroofi isolaadi seguga (töötlemine diasotroofidega), seeneliigi *A. occidentalis* inokulaadiga või nii diasotroofide kui ka *A. occidentalis* inokulaadiga (topelttöötlemine). Pärast seitset kasvunädalat täheldati diasotroofide (25%), seeneliigiga *A. occidentalis* inokulaadi (101%) ja kombineeritud inokulaatidega (106%) nakatatud taimedes oluliselt suuremat biomassi kui inokuleerimata kontrollrühma taimedes. Ka oli inokuleeritud taimede võrsete toitainete sisaldus (sealhulgas N, P, K, Mg, Zn, Cu ja Mn elemendid) suurem kui kontrollrühma taimedel. Võrreldes kontrollrühma ja diasotroofide inokulaadiga inokuleeritud taimedega täheldati seemeliigiga *A. occidentalis* töödeldud taimede võrsetes oluliselt suuremat lämmastikusisaldust (st koostoime seene ja mulla looduslike diasotroofide vahel). Topeltinokuleeritud taimede võrsed sisaldasid kõige rohkem toitaineid (nt N, P, K, S, Mg, Zn, Cu ja Mn) ja nende mulla üldlämmastiku tase oli kõrgeim (13–24% kõrgem kui teiste töötluste puhul), mis viitab tugevale koostoimele seenetüve ja lisatud diasotroofide vahel. Kontrollrühma taimedega võrreldes vähenes juurte kolonisatsioon mulla looduslike arbuskulaar-mükoriissete seente poolt kõigis inokuleeritud taimedes. Bakterite 16S rRNA geeni põhjal tuvastatud koosluse mittemeetriline mitmemõõtmeline skaleerimise (NMDS) analüüs näitas, et seeneliik *A. occidentalis* mõjutas mulla bakterite koosseisu. Paralleelselt läbi viidud *in vitro* uuring näitas, et seeneliik *A. occidentalis* ei kasvanud lignotselluloosi või sahharoosi sisaldavatel substraatidel, kuid kasvas söötmetel, mida oli täiendatud heksoosidega, nagu glükoos ja fruktoos. See näitab, et seeneliik *A. occidentalis* sarnaneb toitumiselt teistele ektomükoriissetele seentele, kellel esineb piiratud saprotroofne võime. Uurimuse tulemused näitasid positiivseid koostoimeid seeneliigi *A. occidentalis* ja mullas vabalt elavate diasotroofide vahel taimede kasvule, mis näitab potentsiaali võimendada mikroobse N_2 sidumist mitteliblikõieliste põllukultuuride puhul.

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PUBLICATIONS

CURRICULUM VITAE

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Education

2009–2012	Plant Pathology, Urmia University, B.Sc.
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Scientific Publications

Soudzilovskaia, N. A., He, J., **Rahimlou, S.**, Abarenkov K., Brundrett, M. C., Tedersoo L. (2022). FungalRoot version 2.0. – an empirical database of plant mycorrhizal traits: reply to Bueno et al. New Phytologist, In Press.

Kariman, K., Moreira-Grez, B., Scanlan, C., **Rahimlou, S.**, Boitt, G., & Rengel, Z. (2022). Synergism between feremycorrhizal symbiosis and free-living diazotrophs leads to improved growth and nutrition of wheat under nitrogen deficiency conditions. Biology and Fertility of Soils, 1–13.

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Hosseyni Moghadam, M. S., Safaie, N., **Rahimlou, S.**, & Hagh-Doust, N. Inducing tolerance to abiotic stresses in Hordeum vulgare L. by halotolerant endophytic fungi associated with Salt Lake plants. Frontiers in Microbiology, 1827.

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Sayari, M., van der Nest, M. A., Steenkamp, E. T., **Rahimlou, S.**, Hammerbacher, A., & Wingfield, B. D. (2021). Characterization of the Ergosterol Biosynthesis Pathway in Ceratocystidaceae. Journal of Fungi, 7(3), 237.

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Rahimlou, S., Bose, T., Babaeizad, V., Sayari, M., & Tajick, M. A. (2015). Molecular data confirm the mitosporic state of Hyphodermella rosae (Phanerochaetaceae) as the pathogen of rosaceous fruits in northern Iran.

Mirhosseini, H. A., Babaeizad, V., & **Rahimlou, S.** (2014). Neofusicoccum parvum, agent of leaf spot on the new host Ginkgo biloba in Iran. New Disease Reports, 30.

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Grants and scholarships

2022 Dora Plus Action 1.1 short term mobility
2022 Grant for short-term mobility provided by the supervisor
2021 Dora Plus Action 1.1 short term mobility
2020 Dora Plus Action 1 – Study Mobility of Doctoral Degree Students
2020 Proposal (WIP) ID: 506772. Bahram M. (PI), Cubeta M. (Co-PI), Tedersoo L., Ryberg M., Põldmaa K., Yagame T. **Rahimlou S.** Evolution of nutritional modes of Ceratobasidiaceae (Basidiomycota, Fungi). Joint Genome Institute (JGI).
2017 Dora Plus doctoral studies scholarship, Faculty of Science and Technology (2017–2022), Botany and Ecology. University of Tartu, Estonia.

Participation in international courses and workshops

PNGOO9S Introduction to Meta-analysis in ecology (Royal Holloway University of London, UK)

PK.1680 Applied Biostatistics in Biological Sciences Using R (Estonian University of Life Sciences)

LT2017/2018-O308 Community Assembly Rules in Fungal and Microbial Ecology: State of the Art and Up-to-date Tools (University of Copenhagen, Denmark)

LT2017/2018-O308 Sample Preparation for High-throughput Sequencing of Fungal Communities (Swedish University of Agricultural Sciences, Sweden).

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Publikatsioonid

Soudzilovskaia, N. A., He, J., **Rahimlou, S.**, Abarenkov K., Brundrett, M. C., Tedersoo L. (2022). FungalRoot version 2.0. – an empirical database of plant mycorrhizal traits: reply to Bueno et al. New Phytologist, In Press.

Kariman, K., Moreira-Grez, B., Scanlan, C., **Rahimlou, S.**, Boitt, G., & Rengel, Z. (2022). Synergism between feremycorrhizal symbiosis and free-living diazotrophs leads to improved growth and nutrition of wheat under nitrogen deficiency conditions. Biology and Fertility of Soils, 1–13.

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Toetused ja stipendiumid

2022 Dora Pluss Tegevus 1.1 lühiajalise õpirände stipendium
2022 Juhendaja poolt antud lühiajalise mobiilsuse toetus
2021 Dora Pluss Tegevus 1.1 lühiajalise õpirände stipendium
2020 Dora Pluss Tegevus 1 – doktorantide õpirände stipendium
2020 Proposal (WIP) ID: 506772. Bahram M. (PI), Cubeta M. (Co-PI), Tedersoo L., Ryberg M., Põldmaa K., Yagame T. **Rahimlou S.** Evolution of nutritional modes of Ceratobasidiaceae (Basidiomycota, Fungi). Joint Genome Institute (USA energiaministeeriumi ühendatud genoomiinstituut).
2017 Dora Pluss doktorantide stipendium, loodus- ja täppisteaduste valdkond (2017–2022), ökoloogia ja maateaduste instituut. Tartu Ülikool, Eesti.

Osalemine rahvusvahelistel kursustel ja töötubades

PNGOO9S "Sissejuhatus metaanalüüsi ökoloogia valdkonnas" (Royal Holloway, Londoni Ülikool, Ühendkuningriik)

PK.1680 "Rakenduslik biostatistika bioloogiateaduses kasutades R-I" (Eesti Maaülikool, Eesti)

LT2017/2018-O308 "Koosluste kujunemise reeglipärad seente ja mikroobide ökoloogias: Parimad ja uusimad tööriistad" (Kopenhaageni Ülikool, Taani)

LT2017/2018-O308 "Proovide ettevalmistamine suure läbilaskevõimega sekveneerimiseks tuvastamaks seenekooslusi" (Rootsi Põllumajandusteaduste Ülikool, Rootsi)

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