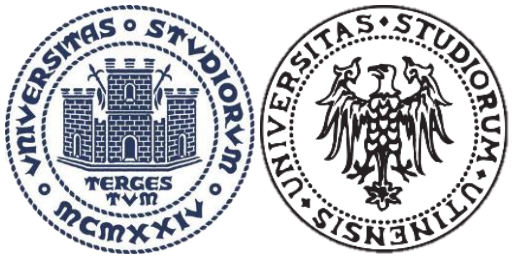

# UNIVERSITÀ DEGLI STUDI DI TRIESTE
# UNIVERSITÀ DEGLI STUDI DI UDINE

## XXXIII CICLO DEL DOTTORATO DI RICERCA IN AMBIENTE E VITA

# EXTRACELLULAR DNA IN BEECH-SPRUCE FOREST SOILS: DISTRIBUTION, EFFECTS AND PRODUCTION

Settore scientifico-disciplinare: **BIO/07 ECOLOGIA**


Dottorando/Ph.D. Student

**Dott. Alessandro Foscari**

Coordinatore/Ph.D. Program Coordinator

**Prof. Giorgio Alberti**

Supervisore di Tesi/Thesis Supervisor

**Prof. Guido Incerti**


**ANNO ACCADEMICO 2019/2020**

# Summary

# Chapter 1

# General introduction

# and Synopsis

## 1.1. Extracellular DNA in soil: distribution and persistence

Environmental DNA (exDNA) is a complex mixture of genomic DNA from many organisms found in and environmental sample (Taberlet et al., 2012a). The location of these exDNA mixtures can be every matrix such as soil, sediment, water, air and even different organic materials (e.g. digestate, compost, manure). In the case of soil, the total DNA pool can be divided, and experimentally separated (Ascher et al., 2009), into an intracellular fraction, comprising a complex mixture of the genomic DNA located within cell membranes, and extracellular DNA, which comprises degraded and fragmented DNA molecules (Pietramellara et al., 2009). Although different acronyms have been used to indicate such DNA fractions, following Nagler et al. (2018), we use iDNA and exDNA to indicate intracellular and extracellular DNA, respectively. The biogeochemical cycle of exDNA in soil has been previously clarified and synthesized, at least qualitatively, with focus on the fate of plant exDNA (Levy-Booth et al., 2007). Plant DNA enters the soil in different ways, from the sloughing off of root cap cells (Hawes, 1990), as a result of pathogen colonization of below-ground biomass (Polverari et al., 2000), through pollen dispersal (de Vries et al., 2003), and during litter and crops residue decomposition (Ceccherini et al., 2003). The importance of the different mechanisms of DNA entry will differ among plant species, such as those with different life cycles and architecture (Levy-Booth et al., 2007). DNA is largely degraded *in planta* within 5 days of fresh plant tissue addition to soil at 21.5 °C (Pote´ et al., 2005). The decomposition of plant tissues by microbial enzymes facilitates the release of undegradated exDNA into the rhizosphere, which is accessible to decomposing microorganisms (Ceccherini et al., 2003). In general, exDNA release during plant residue decomposition is poorly characterized quantitatively. In temperate climates and agricultural systems, the entry of crop residue DNA into soil is believed to follow seasonal oscillations following patterns of plant growth and senescence, while in tropical systems the entry of DNA in soil may be continuous (Levy-Booth et al., 2007). Distribution and persistence of plant exDNA in the soil matrix

are two complex and poorly understood phenomena that can be influenced by different parameters (Levy-Booth et al., 2007; Nielsen et al., 2007). exDNA can diffuse in the soil vertically, with movement either directed towards the groundwater through leaching or towards the soil surface through advection in water capillaries (Nagler et al., 2018) or horizontally, movement follows the soil water flow direction (Agnelli et al., 2004; Ascher et al., 2009; Ceccherini et al., 2007; Poté et al., 2003). The persistence of exDNA depends on a number of factors such as its composition, methylation or conformation and the prevailing environmental conditions (Nagler et al., 2018). In that context, rapid desiccation, low temperatures, high salt concentrations, low pH and a high content of expandable clay minerals have all been found to slow down exDNA degradation (Crecchio et al., 2005; Pietramellara et al., 2009). Degradation of DNA in soil follows different phases: once free in the interstitial water the DNA is restricted and digested by extracellular DNases of microbial origin (Demaneche et al., 2001), which are ubiquitous in the soil environment and provide oligonucleotides and nutrients then used in metabolism by microorganisms and plants (Levy-Booth et al., 2007). Based on the overview reported above, it is clear that the processes and the environmental factors controlling plant exDNA distribution and persistence in soil are mostly understood at a qualitative level, while the quantification of plant exDNA at different soil depths, as well as under different plant cover conditions, are not yet fully clarified. Indeed, the analysis of soil DNA, more than aimed at quantifying its distribution, has been traditionally carried out with a biodiversity monitoring purpose (Taberlet et al., 2018) with different approaches.

## 1.2. Environmental DNA assessments: methodological issues

Different methodological approaches are available to study environmental DNA, according to different purposes. When the study aim is to identify or quantify the abundance of a single species or taxon, the assessment can be based on standard or quantitative PCR (Logan et al., 2009). These

techniques are already popular for different taxonomic groups (Hebert et al., 2003). Alternatively, if the aim is to identifying a large numbers of species can be use an approach based a target sequence (White et al., 1989), aiming to detect all taxa of a given group. These approach is commonly called DNA Metabarcoding (Riaz et al., 2011) and involve the analysis of a metabarcode sequences amplified from exDNA. These sequence consist in a short and taxonomically informative DNA region bordered by two conservative regions serving as primer anchors for the PCR. Differently, if the metabarcoding analysis is performed without the metabarcode amplification we have a shotgun analysis (Deininger et al., 1983). These last approach, despise can be used for metabarcoding studies (Taberlet et al., 2012b), is extensively employed for studying the functional characteristics of genomes, belonging to metagenomics analysis (review in Simon & Daniel, 2011). Environmental DNA is used in different ecology studies, like diet studies (review in Pompanon et al., 2012), food web interactions (Eveleigh et al., 2007), changes in species distributions and stability of the niche (Yoccoz, 2012) and for biodiversity studies (Ficetola et al., 2008, Yoccoz et al., 2012). While the use of exDNA as tools analyses have rapidly gained boost in different study on the freshwater community, first for single species detection and more recently for diversity surveys, their success among terrestrial ecosystem have been less immediate. In particular, DNA from soil samples might provide an efficient metric for components of above- and belowground ecosystem diversity. Study of soil microbiology has approached quickly at these tools (Hugenholtz et al., 1998), followed by earthworm's study (Pansu et al., 2015) or macro-organism traces in soil (Zinger et al., 2019). Differently, studies of the vascular plants diversity are less limited (Yoccoz et al., 2012). Classic methods for estimating contemporary plant diversity rely on time-consuming above-ground sampling, usually abundance or biomass measurements of individuals and their taxonomic identification (Magurran, 2005; Stohlgren, 2007). While these methods remain invaluable,

information contained in the soil could both complement above-ground data and be used to estimate components of plant diversity over longer temporal scales.

The use of soil-derived DNA to identify the presence and abundance of plants poses technical challenges. It has been shown that different DNA extraction protocols are not as equally efficient at removing PCR inhibitors depending on the sample type (Humic substances in soil samples) ad these can lead to different results (Martin-Laurent et al., 2001; Asher et al., 2009; Taberlet et al., 2012a) so, the choice to use a type of extraction protocol have to be previously test.

It's important also remember that one of the main characteristics of environmental DNA is the heterogeneity of the extracts obtained from environmental samples (Taberlet et al., 2018). These heterogeneity, identified as different fragmentation levels, poses technical challenges in the choice of the PCR metabarcode for analysis. Several elements should be carefully considered to make an informed choice, like first of all the taxonomic group of interest. Fortunately, the CBoL Plant Working Group (2009) described standardized DNA barcodes for plants; these barcodes target relatively long DNA fragments (~550 bp for *rbcLa*). But using such long DNA sequences for taxonomic identification in environmental samples is likely to result in few positive matches and many species being missed, as experienced in ancient DNA studies (Willerslev et al., 2003) where exDNA is strongly degraded. Different authors (Coissac et al., 2007; Yoccoz et al., 2012) proposed the use of shorter DNA markers for degraded soil DNA samples but more recent study (Fahner et al., 2016) showed how full length DNA barcode regions could outperform shorter markers for surveying plant diversity from soil samples. Overall, *rbcLa* is recommended for DNA metabarcoding of vascular plants from exDNA because can take advantage of existing resources such as the growing DNA barcode database (Bell et al., 2017).

Translating the metabarcode copy number to biomass or individual abundance represent another challenge, as the number of metabarcode copies varies across cells, tissues, individual,

species and probably time (Taberlet et al., 2018). These occurs because the DNA to accumulate in the soil over more than a year, so should provide a temporally integrated view of plant community composition. Study of estimation of biomass of plants in soil are low; Yoccoz et al., 2012 showed how exist a relationship between proportion in above-ground surveys and DNA survey for different groups of boreal plant and, more recently, Matesanz et al., 2019 focused on the correlation between the root biomass and the percentage of sequences assigned to each taxon. Environmental DNA metabarcoding can be effectively used to determine not only species presence but also their relative abundance in field samples, but these possibility requires additional experiments and implementations.

Metabarcoding could provide information about the temporal and spatial distribution of vegetation. In a study on alpine fields, Yoccoz et al. (2012) did not detect crop exDNA in the areas with no history of cultivation, even if crops DNA could be amplified at low levels in low cultivated fields 1 Km away. The less exDNA moves in soil, the less its signal could be shared between two adjacent sites. Strong small scales (<10 m) horizontal variations have been observed with either traditional observation (Ettema et al., 2002) and through exDNA metabarcoding (Zinger et al., 2019). Similarly, vertical community composition of Fungi, Bacteria and meiofauna show a clear distribution gradient between organic and mineral horizons in soil (Ascher et al., 2009; Yang et al., 2014) but we have a lack of similar study for the plant community vertical and horizontal distribution.

A further, interesting methodological approach to study DNA distribution and persistence in soil is the use of stable isotopes to track plant DNA during its biogeochemical cycle. As an example, microbial exDNA degradation dynamics have been elucidated primarily in microcosm studies in controlled environments, with the use of stable isotopes in the culture medium, then incorporated in bacterial DNA. Morrissey et al. (2015) performed a laboratory experiment wherein soils were

amended with $^{13}C$-labelled exDNA (extracted from bacterial cultures) observing that the amount of exDNA-carbon remaining in the soil declined exponentially over time, suggest that the fraction of exDNA-carbon that remained in the soil was incorporated into microbial biomass, firmly bound to soil constituents, or fragmented and no longer amenable to sequencing. Interestingly, the exDNA amendment affected the composition of the bacterial community, opening to the possibility that different exDNA type could select different types of microbiome. Along the same methodological path, it could be really innovative and certainly interesting, to produce labelled plant DNA and track its dynamic in soil to investigate the less understood steps of exDNA biogeochemical cycle, such as nucleotide salvage pathway (Ingraham et al., 1983; Katahira & Ashihara, 2002) or plant exDNA horizontal transfer to soil microbiome (Levy-Booth et al., 2007). By a methodological point of view, the promising use of stable isotopes could be based on two different approaches. First, labelling plant DNA with $^{13}C$, which however requires growing the plant in a costly, controlled atmosphere enriched in the heavy C isotope. Second, using fertilizers enriched in $^{15}N$ during plant growth, then extracting from the leaves DNA enriched in the heavy N isotope.

## 1.3. Functional effects of soil exDNA

The known functions of exDNA in soil are manifolds. As in other different environment, soil exDNA plays a crucial role in the formation of microbial biofilms, mainly with structural functions (Nagler et al., 2018) and its long persistence under favourable conditions could enhance the occurrence of horizontal gene transfer, as in the case antibiotic resistance genes passed from cell to cell (Poté et al., 2003). At plant root level, the presence of exDNA in the growth medium of plants enhances the growth of lateral roots and root hairs (Paungfoo-Lonhienne et al., 2010) and, according to Wen et al., (2009) could even enhance the natural mechanisms of root disease resistance. Indeed, the apical 1-2 mm root tip of *Pisum sativum*, housing apical and root cap meristems, is resistant to fungal

infection, when other root sites are invaded. The underlying mechanisms are unclear, but the phenomenon appears to involve root cap “slime”, a mucilaginous matrix composed of proteins, polysaccharides, and detached living cells called “border cells” (Iijima et al., 2008). Wen et al. (2009) observed that DNase added to root tips eliminates resistance to infection, lending support to the hypothesis that exDNA is present in the matrix and that its presence and structural integrity are required for root tip defence. In a further study, Wen et al. (2017) showed that soil bacteria and fungi are trapped by a matrix combining exDNA released by active secretion with other molecules and cells shed from the plant root. Wrapped up in strands of sticky exDNA, pathogens can’t infect the growing plant (Hawes et al., 2011; Hawes et al., 2012). Finally, Mazzoleni et al. (2015a) found a very interesting biological effect by fragmented extracellular self-DNA (i.e. DNA originating from conspecifics). In particular, DNA extracted and purified from leaves of several plant species, and fragmented by sonication to mimic its degradation in natural condition by plant debris decomposition, showed inhibitory effects on seed germination and root elongation on conspecific seedlings, in a concentration-dependent manner, without affecting heterospecifics, in bioassays carried out both *in vitro* and in greenhouse. Such evidence clearly indicated a causal role of plant exDNA in the frame of species-specific plant-soil negative feedbacks (see next section). Moreover, self exDNA inhibitory effect was found to be a generalized biological phenomenon by testing on several taxa including bacteria, protozoa, algae, fungi, and insects (Mazzoleni et al., 2015b), opening interesting perspectives on the use of the self-DNA inhibition principle for the control of weed, parasitic and even pathogenic species (Mazzoleni et al., 2014). More recently, Duran-Flores & Heil (2018) confirmed the self-DNA inhibition effects, arguing that exDNA could act in an analogy to other damage associated molecular patterns (DAMP) that cause the local development of resistance-related responses by the affected plant, with the reduced seedling growth being a side-effect of the energetic cost of the immunity response.

## 1.4. Ecological relevance of self-DNA inhibition

Tree species diversity is critical for the maintenance of ecosystem functions such as production, nutrient cycling and carbon storage (Nadrowski et al., 2010; Liang et al., 2016). Long before the term "ecology" was coined by Haeckel in 1866, naturalists and explorers observed that forest composition changes with climate at a broad scale even though it is still unclear what determines tree species composition and richness at a narrower scale. According to the Janzen–Connell (J-C) hypothesis (Janzen, 1970; Connell, 1971) a widely accepted explanation for the maintenance of tree species diversity, host-specific herbivores, pathogens, or other natural enemies exert a controlling role on the areas near a parent tree, making it inhospitable for seedling survival. Several studies in both temperate (Packer & Clay, 2000) and tropical (Mangan et al., 2010) forests provided empirical support to the J-C hypothesis, reporting a stronger reduction in growth and survival of recruitment near than away from conspecific adult trees, resulting in negative density-dependent (Bell et al., 2006) and distance-dependent (Swamy et al., 2010) seedling mortality. It has been postulated that plant-soil feedbacks may play an important role in driving tree species composition (Van der Putten et al., 2016): when the soil surrounding a given plant promotes the growth of conspecific plants, plant-soil feedback is positive, but when the soil discourages the growth of conspecific plants, the feedback is negative. Plant-soil negative feedback (NF) is the rise in soil of negative conditions for plant performance induced by the plants themselves (Klironomos, 2002). This means that a particular plant species changes abiotic and/or biotic soil conditions such that establishment and growth of individuals of that species are reduced, while other plant species that are less harmed by the specific soil conditions are favoured (Bever et al., 2012).

NF is recognized as an important factor shaping natural plant communities (Van der Putten et al., 1993), allowing species coexistence (Bever et al., 1997; 2003; Bonanomi et al., 2005) and contributing to plant community dynamics in natural vegetation (Van der Putten et al., 2003). NF

has been observed in a variety of environments, including grasslands (Reynolds et al., 2003) and temperate and tropical forests (Mangan et al., 2010). Species-specific NF (Kulmatisky et al., 2008) has been related to different, not mutually exclusive hypotheses of underlying mechanisms, such as soil nutrient depletion (Ehrenfeld et al., 2005) and both the build-up (Packer & Clay, 2000) and changing composition (Kardol et al., 2007) of soil-borne pathogen populations, with the latter being the most credited interpretations as explanatory NF mechanisms (Van der Putten et al., 2013).

Another hypothesis of possible NF mechanism (Singh et al., 1999) was based on the observation that, in addition to the well-recognized role of litter as a nutrient source (Vitousek & Sanford, 1986), leaf and root litter can also inhibit plant growth by immobilizing nitrogen (Hodge et al., 2000) or by releasing phytotoxic compounds during decomposition (An et al., 2001; Trifonova et al., 2008). Similar findings were done for natural ecosystems (Bonanomi et al., 2011). However, the NF putative toxins were known to be rapidly degraded by soil microbial activity. In most cases, the litter inhibitory effects have been found to be limited to short-term phases of early decomposition stages, usually lasting only a few weeks (Hodge, 2004; Bonanomi et al., 2011), and for this reason the toxicity hypothesis has been widely criticized (Harper, 1977; Fitter, 2003). Indeed, in traditional studies of litter allelopathy, hundreds of organic compounds, extracted from plant tissues, purified and identified (Rice, 1984; Reigosa et al., 2006), only showed a general toxicity without species-specific effects. It seems evident that such phytotoxic compounds, given their short persistence in the soil and lack of specificity, could hardly explain species-specific NF.

The fact that NF occurred mainly in terrestrial systems, while it was rarely observed in aquatic environments (Mazzoleni et al., 2007) supported the idea that the inhibiting factor could be a water-soluble compound. Secondly, the spatial scale and patterns of many observed negative density-dependence distributions (Wright, 2002) seemed consistent with diffusion processes of putative inhibitory substances related to litter accumulation, but less compatible with the

involvement of pathogens, whose mobility range is plausibly larger than the observed NF spatial scale. This was first investigated by a theoretical modelling approach showing that the occurrence of negative feedback could affect species coexistence (Mazzoleni et al., 2010) and the emergence of spatial patterns (Cartenì et al., 2012). However, these models remained speculative without a demonstration of the actual existence of the supposed chemical compounds.

The studies of self-DNA inhibition by Mazzoleni et al. (2015a, b) demonstrated that fragmented extracellular DNA (exDNA), accumulating in litter during the decomposition process, has a concentration dependent inhibitory effect on conspecifics, reducing conspecific root growth and seed germination without affecting heterospecifics (see previous section). Such findings, while representing a chemical basis of autotoxicity to be taken into account in mechanisms explaining NF, also suggest an unexpected new functional role of exDNA in intra- and interspecific plant interactions at ecosystem level (Cartenì et al., 2016). Within an ecological perspective, it was also suggested that exDNA is implicated in plant signaling and self-recognition (Duran-Flores & Heil, 2015; 2016), plant root defense mechanisms (Hawes et al., 2011) and damage-associated molecular patterns (Panstruga, 2016).

All in all, it is clear that the presented findings raise many issues deserving further investigation. In a recent commentary paper by Veresoglou et al. (2015) the authors state: “The ecological, physiological and molecular significance of the observations of Mazzoleni et al. is thought-provoking. A priority now is to start a discourse on the interpretation of the results of these studies, because this will help design focused experiments to further investigate the role of self-DNA on growth. …There are currently a lot of open questions with regards to the ecological significance of self-DNA effects, the relative magnitude of these effects on different organisms and whether there is room for improvement in competition models through considering self-DNA effects. We currently do not know why the growth effects could only be observed with partially

fragmented DNA and how sensitive organisms can be to differences in the level of self-DNA degradation. We thus expect that this topic will attract considerable scientific attention over the next couple of years."

In this regard, little is known about the cellular and molecular mechanisms underlying growth inhibition by extracellular self-DNA (Barbero et al., 2016), whose disclosure would certainly require a huge research effort in system biology disciplines. On the other hand, within an ecological perspective, it is particularly interesting to clarify the level of persistence and quantify the fate of extracellular self-DNA in natural plant-soil systems.

Most of the studies on NF have been carried out under controlled conditions, and very few of them have assessed its role in the field as far as forests are concerned (Bennett et al., 2017; Teste et al., 2017). Moreover, no study so far has investigated the fate and effects of exDNA in forest ecosystems as related to NF. Possible testing areas where to study NF and exDNA dynamics in the field exist both in the Alps and in the Apennines. In fact, where the optimum for two key species (i.e. beech, fir, spruce) partially overlaps and no disturbance is present (Schütz, 1999; Wilson, 1999), these species may co-exist alternating each other. For example, where a spruce or fir stand is in its regeneration phase (Oliver & Larson, 1990), there will be a widespread beech regeneration in the understory and vice versa (i.e. species alternation mechanism, see Del Favero, 1998; 2004). Some early studies related this switch in species composition with time to human management (Giacobbe, 1929), while others focused on plant-soil feedbacks, related to shifts in soil chemical-physical properties (Susmel et al., 1951). However, the persistence, relevance and effects of extracellular DNA from conspecific litter decomposition in forest ecosystem are still poorly understood.

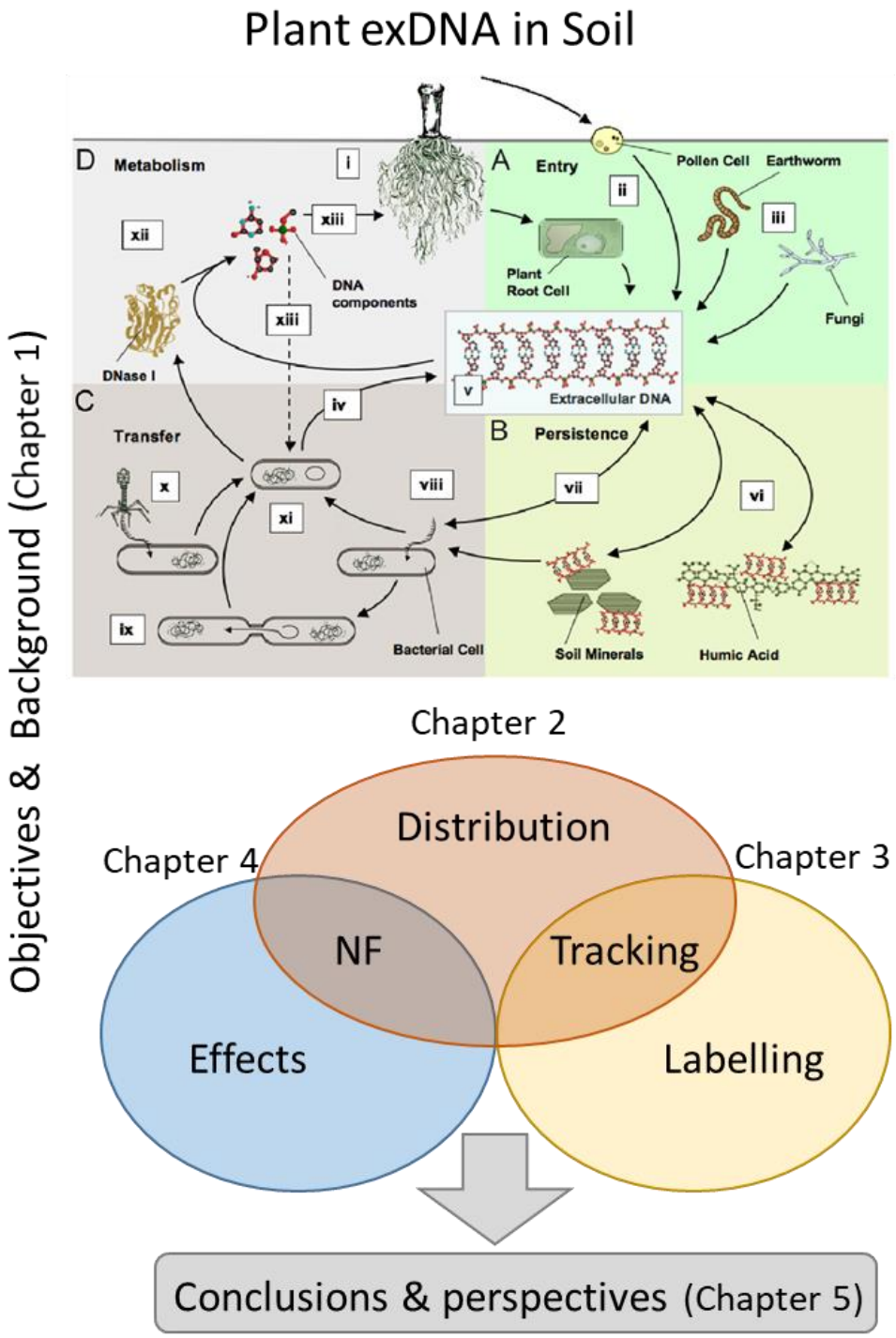


**Figure 1.1** – Background on exDNA biogeochemical cycle (ed. from Levy-Booth et al. 2007) and conceptual diagram of the inter-relationships between this PhD thesis topics (ellipses and intersection areas), with indication of the chapters dedicated to each topic.

## 1.5. Aims and scope

In this first chapter of my PhD thesis I presented a synopsis of the current knowledge on DNA biogeochemical cycle, as well as methodological issues and perspectives for quantitative assessments of plant exDNA distribution and persistence in soil, and its effects at individual and (possibly) community scale. These topics are inter-related, as showed in the conceptual scheme in Fig. 1.1, which is explained as follows, with keywords in bold type: on one hand, the reliability of an ecological role of plant exDNA in controlling **NF** and then plant biodiversity at community scale, must be challenged with different tests: a) quantifying plant exDNA **distribution** in the soil in natural conditions; b) testing its **effects** by more realistic experimental setup as compared to real filed conditions. On the other hand, in order to investigate the less understood steps of plant exDNA biogeochemical cycle, the possibility to **track** it by promising techniques based on stable isotopic **labelling**, in analogy to previous successful attempts carried out on the more easily manipulable microbial DNA, must be appropriately tested. In my thesis, a chapter is dedicated to each of those topics, whose specific aims are described in detail below.

This PhD thesis aims at deepening the knowledge of exDNA distribution and applications in natural environments, with particular focus on natural beech-spruce forest ecosystems. Under a basic research perspective, clarifying exDNA persistence and abundance along the soil profile and across different conditions of tree community structure is a primary objective of this work, which is specifically addressed in the second thesis chapter. It deals with a field study, carried out in the forest system of Fusine Lakes (UD). This study is based on soil DNA metabarcoding approach, specifically directed to characterize plant exDNA in forest soil at different depth and under different stand conditions.

Under a methodological perspective, besides the validation of using DNA metabarcoding for quantitative analysis of exDNA, a second aim of this thesis is to test the possibility to produce large

amounts of plant DNA labelled with stable isotopes in such a way to making possible to further experimental field work, such as e.g. to experimentally trace its fate in soil. Methodological aims are addressed in the third chapter of this PhD thesis. A first specific aim was to provide a simple protocol to label plant material with $^{15}N$ and extract isotopically labelled DNA, oriented to maximize the extract quality while minimizing the production costs. The possible isotopic fractionation effect during plant vegetative growth, eventually limiting the degree of labelling of plant DNA, was investigated with an experiment in controlled conditions. Then, in a controlled field study, an original automated system for labelled fertirrigation of beech juveniles was designed, set up, and used to two consecutive growth seasons, leading to massive litter production.

Linked to the recent development of basic research in the field of plant-soil negative feedback, and in particular to the proposed causal role of plant self-DNA in species-specific NF, a further aim of this thesis is to provide a first contribution specifically referred to beech forest systems. This is the content of the fourth chapter of this PhD thesis. In particular, the occurrence of NF on beech seedlings is tested in a bioassay in microcosms under controlled conditions, considering different species-specific substrate conditioning sources (natural forest soil as well as beech litter amendments). In order to disentangle possible mechanistic explanations of the observed pattern, substrate-dependent effects in the bioassays are factorially combined with substrate treatments intended to either release the possible causal factors of species-specific NF (i.e. substrate microbiome, low molecular weight phytotoxic compounds) or exacerbate the negative effects (i.e. self-DNA addition).

# Chapter 2

# DNA abundance and distribution in spruce-beech forest soil: a field study from NE-Italy

## 2.1. Introduction

Environmental DNA, first reported in a methodology for extracting microbial DNA from sediment (Ogram et al., 1987), can be described as the DNA that can be extracted from environmental samples, without first isolating any target organism (Taberlet et al., 2012a). It is widespread in different environments, including water bodies, sediments and soils (e.g. Collins et al., 2018; Nagler et al., 2018; Pathan et al., 2020) and can be found in any kind of environmental samples from many different organisms and at any possible degradation level (reviews in Taberlet et al., 2012b and Nagler et al., 2018). The total environmental DNA pool can be divided, and experimentally separated (Ascher et al., 2009), into an intracellular fraction comprising a complex mixture of the genomic DNA located within cell membranes, and extracellular DNA, which comprises degraded and fragmented DNA molecules (Pietramellara et al., 2009). Although different acronyms have been used to indicate such DNA fractions, in this paper, following Nagler et al. (2018), we use iDNA and exDNA to indicate intracellular and extracellular DNA, respectively.

In forest soil, the cycling of exDNA has been extensively reviewed (Pietramellara et al., 2009; Nagler et al., 2018). Most exDNA enters the soil mainly after the lysis of microbial cells, subject to degradation by nucleases, nuclease degradation by proteases, and DNA protection by impurities associated to the released DNA molecule. Plant DNA enters the soil from above-ground through pollen dispersal (Uribelarrea et al., 2002; de Vries et al., 2003), during litter decomposition (Widmer et al., 1997; Ceccherini et al., 2003) and below-ground through death of root cap cells (Hawes, 1990; de Vries et al., 2003) or as a result of pathogen colonization of below-ground organs (Polverari et al., 2000; Kay et al., 2002). The resulting exDNA pool in the interstitial soil solution can be bound to soil minerals (Crecchio & Stotzky, 1998; Morrissey et al., 2015) and humic substances (Lorenz & Wackernagel, 1987; Crecchio & Stotzky, 1998), and under specific environmental conditions can persist for years (Agnelli et al. 2007; Nielsen et al. 2007; Pietramellara et al. 2009).

Once unbound and released in the soil solution, exDNA can diffuse vertically (Potè et al., 2007), both downwards, through leaching, and upwards, through advection by water capillarity and horizontally, following the soil water flow (Ceccherini et al. 2007; Ascher et al. 2009). While diffusing, exDNA may be used as a nutrient source for plant and microbial growth (Macfadyen et al., 2001; Ceccherini et al., 2003; Morrissey et al. 2015). Partial DNA breakdown produces nucleotides, nucleosides, ribose, and bases that can be re-assimilated into nucleic acids without further degradation entering a living cell (Levy-Booth et al., 2007). Complete DNA degradation provides elemental nutrients, which in the case of P can became a relevant part of the soil pool (Baker, 1977), especially in soils with low nutrient input (Levy-Booth et al. 2007; Nielsen et al. 2007).

Additionally, soil exDNA can be incorporated into competent bacteria by uptake (de Vries & Wackernagel, 2005; Thomas & Nielsen, 2005) and integration in the prokaryote genome (Carlson et al., 1983, 1984; de Vries & Wackernagel, 2005). Then, transduction and conjugation may spread exDNA genetic information through the horizontal gene transfer (HGT) pathways (England & Trevors, 2003; de Vries & Wackernagel, 2005; Levy-Booth et al. 2007). HGT frequency in soil has been considered low (Nielsen et al. 1998; Pietramellara et al. 2007; Thomas & Nielsen 2005), or underestimated (Poté et al. 2003; Pietramellara et al., 2009).

More recently, it has been shown that not only HGT occurs frequently within the soil microbiome, but also that the genetic information can be transferred from the soil microbiome (Boto et al., 2019), as in the case of the antibiotic resistome (Forsberg et al., 2012). Moreover, exDNA was recently discovered to have a species-specific inhibitory effect, reducing germination and growth of conspecifics in plants (Mazzoleni et al., 2015a) and other organisms (Mazzoleni et al., 2015b). While the underlying mechanisms are not yet fully clarified (but see Veresoglou et al., 2015, Bhat & Ryu, 2016), assessing the relevance of exDNA functional roles at plant community and ecosystem scales (Cartenì et al., 2016) requires additional research (Nagler et al., 2018).

Preliminarily, a reliable quantification of specie-specific plant exDNA abundance and horizontal and vertical distribution in soil is a main issue still awaiting to be addressed.

Among the possible methodological approaches to quantify species-specific DNA in complex matrices such as soil samples, the use of advanced molecular techniques such as DNA metabarcoding, which allows the simultaneous identification of different species in a complex environmental sample through high-throughput sequencing methods (Bell et al. 2017), is increasingly promising. Indeed, DNA metabarcoding has been used to assess the occurrence of specie-specific plant DNA in environmental samples aiming at reconstructing past flora (Jørgensen et al. 2012; Epp et al., 2012), studying plant-pollinator interactions (Keller et al., 2015; Richardson et al., 2015) and tracing invasive species or genetically modified plants (Folloni et al., 2012). Moreover, the recent availability and progressive update of reference libraries for different genetic markers (e.g. CBOL Plant Working Group, 2009; Yu et al., 2011; Cheng et al., 2016; Bell et al., 2017) allowed to compile plant diversity inventories and assess community composition complementing above-ground investigation (Yoccoz et al., 2012).

However, when metabarcoding application is directed to assessing species-specific exDNA abundance, rather than occurrence, possible environmental drawbacks and technical issues must be considered (Bohmann et al., 2014). On one hand, species-specific persistence of DNA in soil, its interaction with the environmental conditions, as well as amplification conditions, different specificity and sensitivity of different markers, and possible sequencing biases should be taken into account (Hollingsworth, 2011; Dong et al. 2013; Lamb et al., 2019). On the other hand, recent observations showed significant relationships between the root biomass of a species in a community and the proportions of a that species reads in the mixed exDNA pool resulting from the amplification and sequencing of the selected genetic marker (Matesanz et al., 2019). Therefore, DNA metabarcoding of pooled root samples are increasingly used as a promising tool to track below

ground abundance of actively growing plants (López-Angulo et al. 2020; Illuminati et al. 2021), while the same approach applied to soil samples could allow to assess the abundance of exDNA from both actively growing and long-dead individuals.

In this study, we studied the vertical distribution of soil iDNA and exDNA fractions, separated following Ascher et al. (2009) along a gradient of tree density in mixed beech-spruce forest subjected to beech-spruce alternation (Del Favero et al., 1998). Therefore, it provides an optimal condition to assess whether the relative abundance of beech and spruce exDNA along the explored gradients reflects the actual tree density, or the effect of past conditions can still be detected in relation to exDNA persistence. Based on soil DNA extraction from soil cores collected at 36 locations selected on the base of a stratified random sampling design, and exDNA metabarcoding, specific aims of this work were: (i) to provide a quantitative overview of iDNA and exDNA abundance and distribution along the explored gradient and the soil profile; (ii) To assess the relationships between soil DNA content and physico-chemical properties; (iii) To estimate the abundance of beech and spruce exDNA, as well as the whole plant community diversity below ground in the tested conditions.

## 2.2. Material and methods

### Study site and sampling

The study area is located at Fusine lakes (46°30’15” N, 13°38’26” E), in NE Alps near the Italian border with Slovenia and Austria. It falls into the Eastern Alpine bioclimatic sector (Rivas-Martiez et al., 2004), with high annual precipitation (mean 1520 mm), mostly during the growth period, and low annual temperature (mean 7.3° C, absolute minimum -27° C). The site is characterized by patches of pastures surrounded by forests of spruce (*Picea abies* Karst), often mixed with fir (*Abies*

*alba* Mill.) an beech (*Fagus sylvatica* L.), lying on a Rendzinic Leptosoil (IUSS, 2006), over a substrate of moraine or alluvial deposits.

Within the forest compartments belonging to the Regional Government, 12 circular plots (radius 13 m) were randomly selected, according to three different forest types. In particular, plots where the basal area of either *P. abies* or *F. sylvatica* was higher than 65% of the total, were considered as spruce or beech stands, respectively. Otherwise, they were treated as mixed forest (Alberti et al., 2013). At each plot, all standing trees belonging to the two target species were recognized and their diameters at breast height (DBH, 1.30 m) were measured. Trees with DBH < 10 cm were not considered for further analysis (INFC 2006). Trees were aggregated into four classes of DBH, as follows: (I) 10 - 19 cm; (II) 20 - 29 cm; (III) 30 - 39 cm; (IV) > 40 cm.

Four soil cores per plot were sampled down to a maximum depth of 60 cm using a percussion drilling set (Cobra TT, Eijkelkamp, the Netherlands) interiorly equipped with a pre-sterilized PE foil liner. Each soil core, still enveloped into the liner, was brought to the laboratory and subdivided into four homogeneous soil horizons, corresponding to different layers and depths (Table 2.1), using sterilized cutters. Considerable effort was maintained throughout sampling to ensure clean, uncontaminated samples, including use of gloves during sample collection and decontamination of equipment prior to and during sampling. Then, aliquots were collected from each horizon with a stainless steel sterilized spatula and stored in Falcon tubes at -80 °C for subsequent DNA extraction. From the remaining materials, bulk soil samples were made mixing the homogeneous soil horizons from the four cores collected at the same plot, for a total of 48 bulk samples (12 plots x 4 soil horizons). Samples were sieved at 2 mm, dried in stove (65 °C for 48 h) and stored in plastic bags at 4 °C for further analysis.

**Soil chemical-physical analysis**

Soil pH was measured potentiometrically with a sureflow combine glass-calomel electrode in $H_2O$ solution 1:5 solid: liquid ratio (McLean, 1982). Carbonate content was assessed following the volumetric method with the Scheibler apparatus (Williams, 1948). The determination of a humification index (HI), calculated as the ratio of non-humified (non-phenolic) to humified (phenolic) organic carbon after extraction with alkaline sodium pyrophosphate, was performed following the methodology described in De Nobili & Petrussi (1988).

Organic carbon (C) and total nitrogen (N) content and corresponding C to N ratio were measured using a using a Vario Micro Cube (Elementar GmbH, Langenselbold, Germany) elemental analyzer in triplicated aliquots of 10 ± 0.5 mg of each sample weighed in a silver capsule and treated with HCl to eliminate carbonates (Nieuwenhuize et al., 1994).

Particle size analysis (PSA) and the conversion into a recognized texture class was performed according to Bowman & Hutka (2002), by dispersion and pipette sub-sampling to particle separation into size groups.

**Sequential extraction of soil DNA**

DNA was directly extracted from fresh, frost fine fraction aliquots (5 g) of each soil sample. Intra- (iDNA) and extra-cellular (exDNA) fractions were sequentially purified using the protocol by Ascher et al. (2009), modified as follows: exDNA was extracted by gentle soil washings with 5 ml of 0.12 M $Na_2HPO_4$ at pH 8 in 50 mL falcon tubes horizontally shaken for 30 min (80 rpm). The tubes were centrifuged (4°C, 30 min, 7500 g) and the supernatant was collected. The same procedure was repeated twice and the resulting supernatants were pooled together to a final volume of 15 mL of unpurified exDNA. The exDNA solution was purified using a commercial extraction Kit (DNeasy® PowerMax® Soil Kit, Qiagen, USA) following the manufacturer instruction, but avoiding the method

step of sample incubation in cell lysis buffer. The soil pellet residual after alkaline washing was used for iDNA extraction. The pellet was transferred into a new 50 mL Falcon tube and processed in the extraction kit according to the manufacturer's instructions, including the method step of sample incubation in the cell lysis buffer. At the end of the purification all DNA samples were separately suspended in 5 mL of 10 mM Tris solution.

Purified DNA samples were quantified by fluorimeter Qubit 3.0 (Life Technology, Carlsbad, California, USA) and the quality was assessed by NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The fragment length distribution was assessed by 0.8% agarose gel electrophoresis.

**Amplification and sequencing**

Amplification of exDNA and iDNA was performed in a final volume of 25 µL for each sample, using 10 µL of DNA extract (concentration of 12 ± 5 ng $\mu L^{-1}$), 1X concentrated OneTaq Hot Start Quick-Load, 2X Master Mix with Standard Buffer (New England Biolabs inc.), and 0.5 µM of the forward and reverse rbcL primers. These primer set amplifies a 553-bp fragment of the rbcL gene and is recommended by the CBoL Plant Working Group (2009) for plant metabarcoding as compared to bacterial and fungal ones. Sequence selected is: rbcLa_f 5' ATGTCACCACAAACAGAGACTAAAGC-3' and rbcLa_rev 5'-GTAAAATCAAGTCCACCRCG-3' (Fahner et al., 2016). The PCR conditions were 94°C for 30 s, followed by 40 cycles of 94°C for 30 s, 64°C for 60 s, 68°C for 30 s, and a final step of 68°C for 5 min. A subsequent amplification run integrating relevant flow-cell binding domains and unique indices was performed with NexteraXT Index Kit (Illumina). The amplification products were sequenced on MiSeq instrument platform (Illumina, San Diego, CA) using 300-bp paired-end and following the manufacturer's instructions.

Taxonomic classification was performed using a database containing 181133 rbcL sequences downloaded from NCBI Nucleotide section on 9 September 2020, using the following key words in search: rubisco [all fields] OR ribulose-1,5-biphosphate carboxylase/oxygenase [all fields]) AND plants[filter] AND biomol_genomic [PROP] AND large subunit [All Fields]. In addition, consensus sequences of *Cyamopsis tetragonoloba* and *Vitis vinifera* were manually inserted.

Bioinformatics pipeline steps were as follows: reads were trimmed in order to eliminate primer sequences by *cutadapt* (Martin, 2011) with standard parameters (-anywhere, -overlap 5, -times 2, -minimum-length 35, -mask-adapter). Low-quality bases were removed from 3' with *erne-filter* (Del Fabbro et al., 2013) by applying default parameters, excluding reads < 60 bp from further analysis. Reads with an error rate > 1% were removed. Chimeric sequences were removed with the algorithm *uchime_denovo* (Edgar et al., 2011) implemented in *usearch* (Edgar, 2010). Reads were clustered to a minimum identity of 97% generating representative sequences with the algorithm *cluster_fast* implemented in *usearch* (Edgar RC, 2010). Blast against the rbcL database was performed without a minimum identity filter, using the lowest unambiguous taxonomic attribution among all the possible blast hits. If there were best hits with the same score indicating different lineage, most common part was reported. Sequences not taxonomically attributed to *Streptophyta* were discarded and not used for further data analysis.

**Statistical analysis**

Main and interactive effect of forest type (three levels: beech, mixed, spruce) and tree species (two levels: *Fagus sylvatica, Picea abies*) on specie-specific basal area ($m^2$/Ha) at the sampling plots were tested by two-ways ANOVA. Main and interactive effect of forest type (three levels: beech, spruce or mixed), soil horizon (three levels: Oe, A1, A2), and DNA source (two levels: exDNA, iDNA) on soil DNA content were tested using three-ways ANOVA. Main and interactive effect of forest type and

soil horizon on total reads numbers from sequenced rbcLa amplicons from soil exDNA samples were tested using two-ways ANOVA. After estimating species-specific exDNA relative abundance in soil as the reads proportion of a given species on the total reads attributed to Streptophyta in the sample, a further three-ways ANOVA model was fitted for the relative abundance of beech and spruce exDNA in soil including main and interactive effects of soil horizon, forest type and tree species (either *F. sylvatica* or *P. abies*). For all ANOVA models, pairwise comparisons between combinations of independent factors were tested by Duncan's post hoc test at $\alpha$=0.05, in all tested comparisons.

The relationships between soil physical-chemical properties and the abundance of species-specific exDNA was assessed separately for different plant species (*Fagus sylvatica*, *Picea abies*) and for all plants, as well as for all data pooled and for different forest types, by an extensive correlation analysis based on Pearson's *r*. Correlation scores were considered statistically significant at $\alpha$=0.05/*N*=0.0019 after application of the Bonferroni's correction, with *N* being the number of multiple comparisons (n=27).

Based on the data matrix containing the number of reads recorded for all plant species in all soil samples, alpha diversity metrics were calculated including the simple species richness and the Shannon's index of evenness, and significant shifts in their horizontal and vertical distribution across forest types and soil horizons were tested at $\alpha$ = 0.05 by a Monte Carlo test after a Permanova analysis based on 999 permutations. A heatmap was generated to assess the variation in eDNA community composition at species level. A resemblance matrix calculated on Bray–Curtis dissimilarity was used to perform nonmetric multidimensional scaling (nMDS) to assess variation in plant species composition (i.e. species abundance) in soil exDNA across forest types. In association with nMDS, the significance of changes in plant species composition of soil eDNA in the three forest types were tested through Permanova (999 permutations), using the soil horizon and forest types

as fixed factors and the replicated plot as random factor. All statistical analyses were performed using the softwares Statistica 7.0 (StatSoft inc, Tulsa, Oklahoma, USA) and Primer-e v. 7 (Primer Ltd, Plymouth, UK).

## 2.3. Results

**Soil and forest overview**

Table 2.1 shows soil horizons properties at the three forest types. In general, horizons are rather thin, developed over largely gravelly and calcareous material (C horizon), with a maximum thickness of A-to-B of 28 cm under mixed forests. At all forest types, pH ranges from acid (4.5 to 5.8) in Oe horizons to basic in C horizons (ranging from 8.5 to 8.7). Organic C content is expectedly decreasing with depth, with a maximum of 30.11 ± 6.80 % in the Oe layers, followed by 11.62 ± 7.50 % and 2.55 ± 1.42 % in the A1 and A2 horizons, respectively, and by a steep decrease in the C horizons, with values < 1% at all sites. Total N content followed the same pattern, corresponding substantially to the same values of C/N ratio in Oe and A1 horizons, then progressively decreasing with depth. On the other hand, carbonate content increased with depth, up to values higher than 700 g $kg^{-1}$ in the C horizon, while being more variable at the Oe horizons (8.6 to 49.8 g $kg^{-1}$).

**Table 2.1**. Soil horizons properties at the sampling plots, according to forest type (beech, mixed and spruce). Data refer to mean ± standard error of 4 replicates (i.e. sampling plots) for each soil property, with the exception of thickness, where 16 replicates corresponded to 4 cores per plot. For each soil property, different letters indicate pairwise significant differences among soil horizons within each forest type (Duncan test $P < 0.05$).

| Forest type Horizon | Thickness (cm) | pH | C (%) | N (%) | $C_{org}/N_{tot}$ | $CO_3^{2-}$ (g $kg^{-1}$) | HI | Sand (%) | Silt (%) | Clay (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| *Beech* | | | | | | | | | | |
| Oe | 10 ± 1 | 5.3 ± 0.3 *a* | 29.6 ± 1.8 *c* | 1.7 ± 0.1 *c* | 18.5 ± 0.7 *bc* | 49 ± 45 *a* | 0.3 ± 0.0 *a* | n.a. | n.a. | n.a. |
| A1 | 7 ± 2 | 6.7 ± 0.3 *b* | 13.5 ± 3.6 *b* | 0.7 ± 0.2 *a* | 18.6 ± 0.9 *c* | 137 ± 49 *ab* | 0.3 ± 0.1 *a* | 32.7 ± 10.6 *a* | 53.7 ± 6.5 *a* | 13.5 ± 4.3 *a* |
| A2 | 10 ± 3 | 7.7 ± 0.2 *c* | 2.9 ± 0.4 *ab* | 0.2 ± 0.0 *a* | 13.9 ± 0.7 *b* | 498 ± 181 *b* | 0.3 ± 0.1 *a* | 47.2 ± 14.1 *a* | 42.0 ± 10.1 *a* | 10.7 ± 4.5 *a* |
| C | 15 ± 7 | 8.5 ± 0.1 *d* | 0.6 ± 0.1 *a* | 0.1 ± 0.0 *a* | 7.7 ± 1.6 *a* | 688 ± 54 *c* | n.a. | n.a. | n.a. | n.a. |
| *Mixed* | | | | | | | | | | |
| Oe | 11 ± 2 | 4.8 ± 0.7 *a* | 28.6 ± 4.0 *b* | 1.4 ± 0.2 *c* | 20.7 ± 0.5 *c* | 9 ± 5 *a* | 0.3 ± 0.1 *a* | n.a. | n.a. | n.a. |
| A1 | 12 ± 3 | 6.8 ± 0.5 *b* | 7.0 ± 0.9 *ab* | 0.3 ± 0.0 *a* | 20.6 ± 2.7 *c* | 189 ± 109 *a* | 0.2 ± 0.0 *a* | 26.2 ± 7.3 *a* | 56.0 ± 3.5 *a* | 17.7 ± 3.9 *a* |
| A2 | 17 ± 3 | 7.6 ± 0.4 *bc* | 3.0 ± 1.1 *ab* | 0.2 ± 0.0 *a* | 17.1 ± 2.1 *b* | 448 ± 187 *b* | 0.5 ± 0.1 *b* | 42.2 ± 12.5 *a* | 45.5 ± 7.6 *a* | 12.2 ± 4.9 *a* |
| C | 19 ± 5 | 8.6 ± 0.1 *c* | 0.5 ± 0.1 *a* | 0.1 ± 0.0 *a* | 5.8 ± 1.1 *a* | 765 ± 34 *b* | n.a. | n.a. | n.a. | n.a. |
| *Spruce* | | | | | | | | | | |
| Oe | 10 ± 4 | 5.8 ± 0.3 *a* | 32.1 ± 4.6 *c* | 1.7 ± 0.3 *c* | 18.8 ± 0.8 *c* | 15 ± 4 *a* | 0.4 ± 0.1 *a* | n.a. | n.a. | n.a. |
| A1 | 7 ± 2 | 7.0 ± 0.4 *b* | 14.3 ± 5.2 *b* | 0.8 ± 0.3 *b* | 17.4 ± 0.4 *bc* | 269 ± 130 *ab* | 0.3 ± 0.1 *a* | 40.7 ± 9.6 *a* | 51.7 ± 7.0 *b* | 7.5 ± 3.2 *a* |
| A2 | 14 ± 3 | 8.0 ± 0.1 *c* | 1.8 ± 0.4 *a* | 0.1 ± 0.0 *a* | 13.7 ± 0.8 *b* | 511 ± 100 *b* | 0.5 ± 0.1 *a* | 67.0 ± 7.8 *a* | 29.5 ± 6.0 *a* | 3.5 ± 1.8 *a* |
| C | 16 ± 5 | 8.7 ± 0.1 *c* | 0.5 ± 0.0 *a* | 0.1 ± 0.0 *a* | 4.8 ± 1.2 *a* | 718 ± 71 *b* | n.a. | n.a. | n.a. | n.a. |

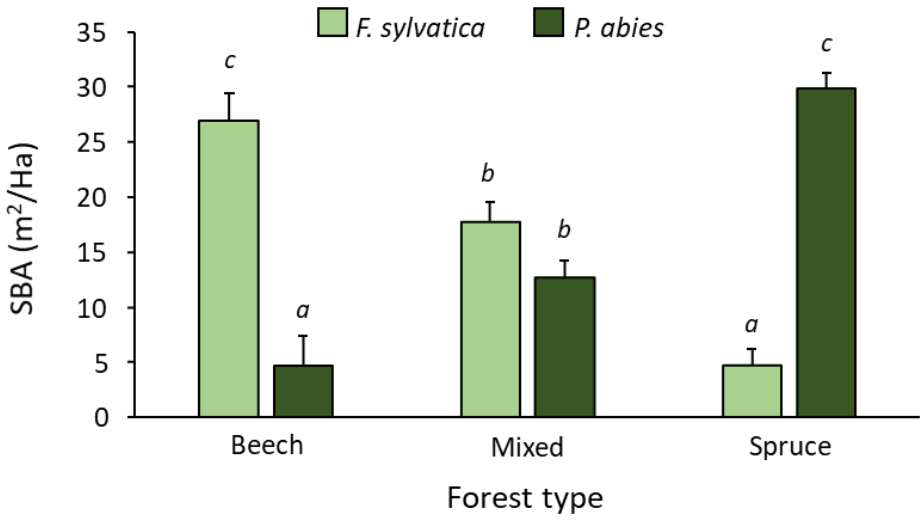


**Figure 2.1**. *Fagus sylvatica* and *Picea abies* Stand Basal Area. Data refer to mean and standard error of 4 replicated plots for each forest type. Different letters indicate significant pairwise differences of SBA between different combinations of forest type and tree species (*P < 0.05,* Duncan's post-hoc test after two-ways ANOVA, detailed statistics in Supplementary Tables 2.2. and 2.3.).

HI did not vary significantly across the explored vertical and horizontal gradients. Particle size (not performed in Oe and C horizons due to lack of fine fraction) showed increasing sand and decreasing clay and silt from A1 to A2 layers.

Considering data summed up for beech and spruce, stand basal area (SBA) did not substantially vary across the three forest types, ranging from 33.8 ± 5.6 $m^2$ $ha^{-1}$ in beech plots to 34.5 ± 1.7 $m^2$ $ha^{-1}$ in spruce ones with lowers value of 28.1 ± 3.5 $m^2$ $ha^{-1}$ in mixed (Fig. 2.1). In forest type dominated by either beech or spruce, the dominant tree species accounted for over 80 % of SBA, while in mixed forest species-specific SBA contributions were not significantly different (Fig. 2.1; Tables 2.2 and 2.3).

**Table 2.2**. Results of two-ways ANOVA testing for the effects of forest type (T: beech, mixed, spruce) and tree species (S: *Fagus sylvatica*, *Picea abies*) on specie-specific basal area ($m^2$/Ha) at the sampling plots. Significant P-values are marked in italic font.

| Effect | DoF | SS | MS | F | P |
|---|---|---|---|---|---|
| Forest Type (T) | 2 | 49.9 | 25.0 | 1.59 | 0.2319 |
| Species (S) | 1 | 3.2 | 3.2 | 0.21 | 0.6561 |
| T x S | 2 | 2390.2 | 1195.1 | 75.91 | *< 0.0001* |
| Error | 18 | 283.4 | 15.7 | | |

**Table 2.3**. Results of Duncan's post hoc test for the interactive effects of forest type and tree species on species-specific Stand Basal Area ($m^2$/ha) after two-ways ANOVA (Supplementary Table S1). P-values refer to pairwise comparisons between different forest types for each tree species (a) and between the two tree species within each forest type (b). Significant P-values are marked in italic font.

| | Stand Basal Area ($m^2$/ha) | | | Duncan's post-hoc test (*P*) | | |
|---|---|---|---|---|---|---|
| **a) Tree species** | **Beech (B)** | **Mixed (M)** | **Spruce (S)** | **B vs. M** | **B vs. S** | **M vs. S** |
| *F. sylvatica* | 26.87 ± 2.47 | 17.76 ± 1.75 | 4.70 ± 1.50 | *0.0004* | *< 0.0001* | *0.0014* |
| *P. abies* | 4.67 ± 2.82 | 12.66 ± 1.41 | 29.79 ± 1.41 | *0.0232* | *< 0.0001* | *< 0.0001* |

| **b) Forest type** | ***F. sylvatica* (F)** | ***P. abies* (Pa)** | **F vs. Pa** |
|---|---|---|---|
| Beech | 26.87 ± 2.47 | 4.67 ± 2.82 | *< 0.0001* |
| Mixed | 17.76 ± 1.75 | 12.66 ± 1.41 | 0.1946 |
| Spruce | 4.70 ± 1.50 | 29.79 ± 1.41 | *< 0.0001* |

With an insight into contributions to SBA values by different DBH classes, we found that the species-specific SBA pattern across the three forest types was substantially consistent also within each DBH class (Fig. 2.2). Moreover, largest contributions were ascribed to the lowest diametric class (DBH between 10 and 20 cm) in all forest types, with a consistent progressive decrease for larger diameter classes up to DBH > 40 cm, with the exception of a higher percent frequency of old trees (up to DBH = 54 cm) in spruce-dominated stands (Fig. 2.2).

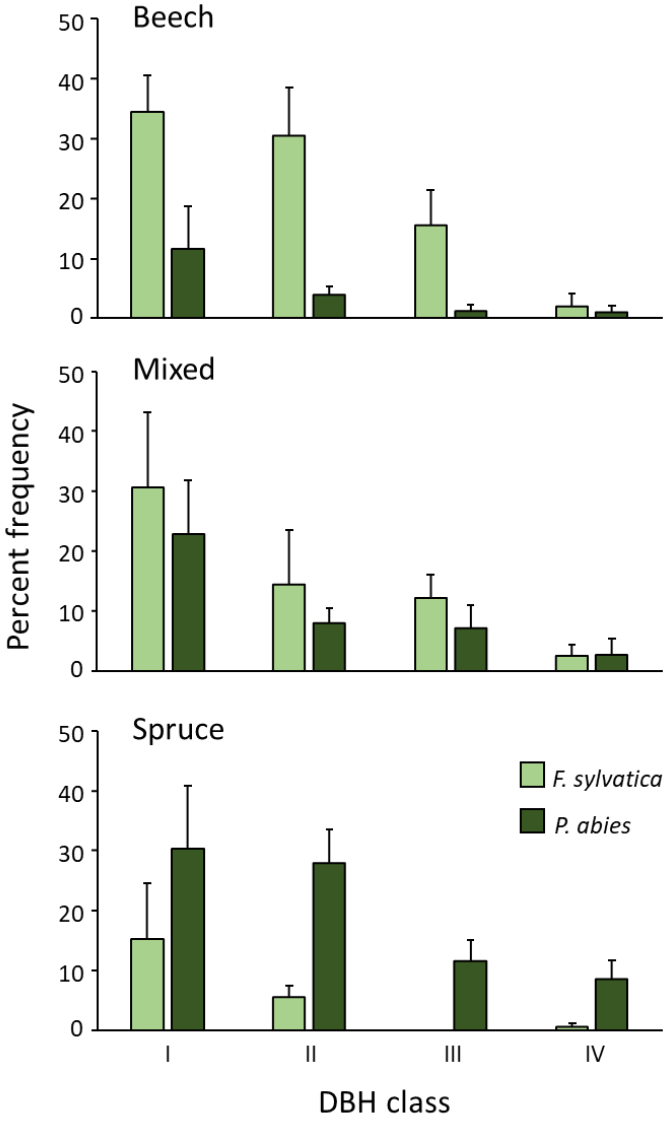


**Fig. 2.2** - Percent frequency of each diametric class of the two tree species (*F. sylvatica* and *P. abies*) in each forest type. DBH was aggregated in four classes as follows: (I) 10 - 19 cm; (II) 20 - 29 cm; (III) 30 - 39 cm; (IV) > 40 cm.

**Soil DNA distribution in relation to physical-chemical properties**

The two fractions of soil DNA were satisfactorily separated from the soil samples, although with different degree of intactness reflecting the degradation of exDNA, but not of iDNA (Fig. 2.3).

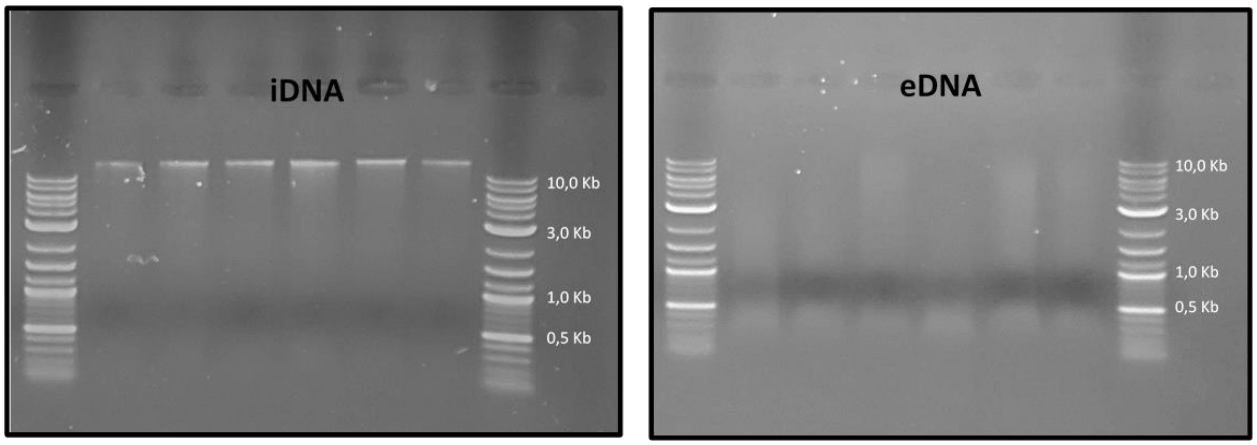


**Fig. 2.3.** The iDNA fraction, identified as the genetic material contained within a cell, displayed an abundant fraction of genomic material that is absent in exDNA, that appears fragmented at various length.

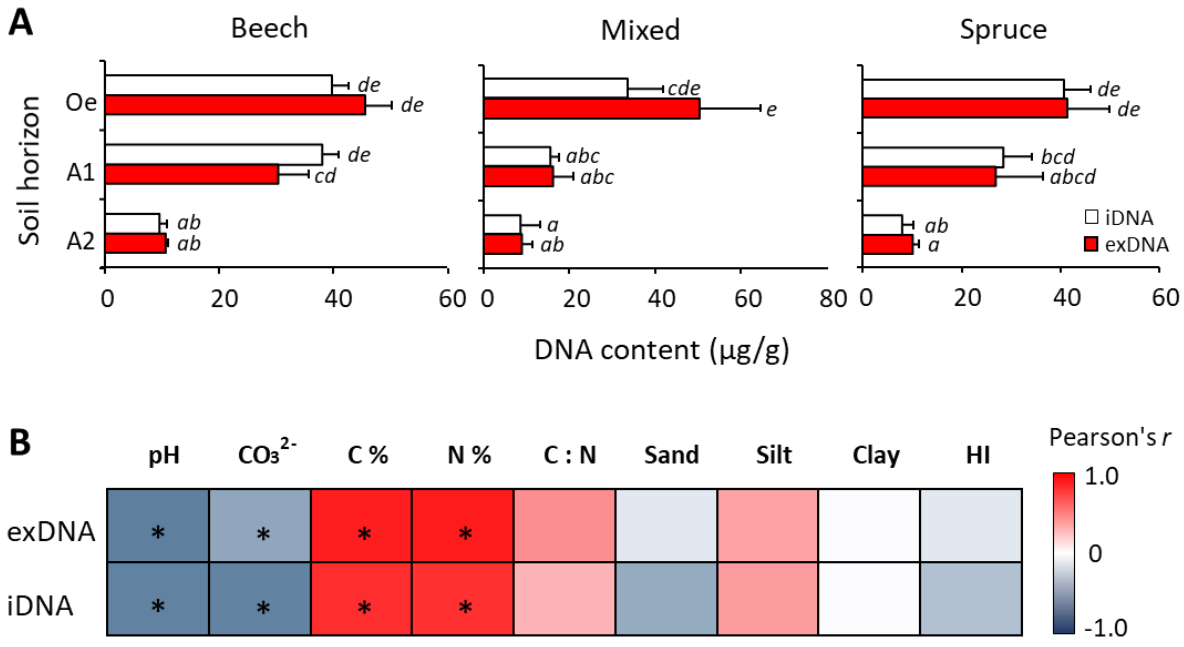


**Figure 2.4**. Vertical and horizontal distribution of soil intracellular (iDNA) and extracellular (exDNA) DNA fractions and relationships between soil DNA yield and physical-chemical properties at the study sites. **(A)** Data refer to mean ± standard error of DNA yield (µg/g) of 4 replicates for each forest type and pedologic horizon. Different letters within each panel indicate pairwise significant differences among combinations of DNA fraction and pedologic horizon (*P < 0.05,* Duncan's post-hoc test after three-ways ANOVA, detailed statistical results in Supplementary Tables S3 and S4). **(B)** Heatmap shows pairwise Pearson's correlation (r) between each physical-chemical variable and either intracellular (iDNA) or extracellular (exDNA) DNA yield (µg/g) measured in 12 soil samples (3 forest types x 4 replicates). Asterisks indicate significant p values (P < 0.05).

Both fractions persisted along the soil vertical profile, showing a progressive decrease with increasing depth (Fig. 2.4A). Interestingly, such pattern was consistent across the three forest types (Fig. 2.4A), as indicated by the non-significant terms T and T × H in the ANOVA model (Table 2.4). Differences of iDNA and exDNA abundance between Oe and A2 soil horizons were statistically significant, whereas A1 horizon showed intermediate values for both DNA fractions, in some cases not different from those of the underlying and/or overlying layers (Tables 2.4 and 2.5). Finally, within-layer differences between iDNA and exDNA abundances were not significant for all soil horizons and forest types. Interestingly, the largest difference between iDNA and exDNA abundances, though not statistically significant, was observed at the Oe horizon in mixed forests (33.6 ± 8.2 μg $g^{-1}$ vs. 50.4 ± 14.4 μg $g^{-1}$ for iDNA and exDNA, respectively).

The observed distribution of the two soil DNA fractions was associated to the soil chemical-physical properties. When such relationships were tested on all data pooled for the three forest types (Fig. 2.4B), we found a negative association of soil iDNA and exDNA abundance with pH and carbonate content, and a positive correlation with organic C and total N percent contents.

**Table 2.4**. Results of three ways ANOVA testing for main and interactive effects of Soil Horizon (H, three levels: Oe, A1, A2), Forest Type (T, three levels: Beech, Mixed, Spruce) and DNA source (D, two levels: exDNA, iDNA) on soil DNA content. Significant P-values are marked in italic font.

| Effect | DoF | SS | MS | F | P |
|---|---|---|---|---|---|
| Soil Horizon (H) | 2 | 12761.3 | 6380.6 | 45.26 | *< 0.0001* |
| Forest Type (T) | 2 | 541.1 | 270.5 | 1.91 | 0.1565 |
| DNA Source (D) | 1 | 72.3 | 72.3 | 0.51 | 0.4767 |
| H x T | 4 | 844.7 | 211.1 | 1.49 | 0.2156 |
| H x D | 2 | 348.7 | 174.3 | 1.23 | 0.2983 |
| T x D | 2 | 140.7 | 70.3 | 0.49 | 0.6097 |
| H x T x D | 4 | 209.8 | 52.4 | 0.37 | 0.8274 |
| Error | 54 | 7611.5 | 140.9 | | |

**Table 2.5**. Results of Duncan's post hoc testing for the 3rd order interactive effect of forest type, DNA pool and soil horizon on soil DNA content after three-ways ANOVA (Supplementary Table S3). P-values refer to pairwise comparisons between DNA content found in different soil horizons for each DNA pool within each forest type. Significant P-values are marked in italic font.

| Forest type and DNA source | DNA content (µg/g) | | | Duncan post-hoc test (P) | | |
|---|---|---|---|---|---|---|
| | Oe | A1 | A2 | Oe vs. A1 | A1 vs. A2 | Oe vs. A2 |
| *Beech* | | | | | | |
| iDNA | 39.7 ± 2.8 | 38.1 ± 2.7 | 9.4 ± 1.4 | 0.8457 | *0.0041* | *0.0025* |
| exDNA | 45.5 ± 4.8 | 30.2 ± 5.5 | 10.6 ± 0.4 | 0.1214 | *0.0434* | *0.0005* |
| *Mixed* | | | | | | |
| iDNA | 33.6 ± 8.2 | 15.6 ± 2.0 | 8.6 ± 4.7 | 0.0632 | 0.4731 | *0.0021* |
| exDNA | 50.4 ± 14.4 | 16.2 ± 4.8 | 9.0 ± 2.5 | *0.0006* | 0.4577 | *< 0.0001* |
| *Spruce* | | | | | | |
| iDNA | 40.6 ± 5.3 | 28.4 ± 5.9 | 8.0 ± 2.0 | 0.2112 | *0.0428* | *0.0014* |
| exDNA | 41.3 ± 8.6 | 26.8 ± 9.6 | 10.0 ± 1.2 | 0.1457 | 0.0789 | *0.0019* |

Considering data separately for each forest type (Fig. 2.5A) the general pattern of association between the abundance of DNA fractions and the soil chemical-physical properties still held for spruce forest soil, while the negative association with pH and carbonates did not emerge for beech and mixed forest soils, and the positive correlation of DNA with organic C and total N was not found for beech soil. On the other hand, when data were separately analysed for each soil horizon, the pattern of association between soil quality parameters and DNA fractions was mostly released (Fig. 2.5B). Finally, we found that the abundance of soil DNA was unrelated to C: N ratio, texture, particle size and humification index, both when the association was tested on all data pooled, and when the analysis was limited at single forest types or soil horizons (Figs. 2.4 and 2.5).

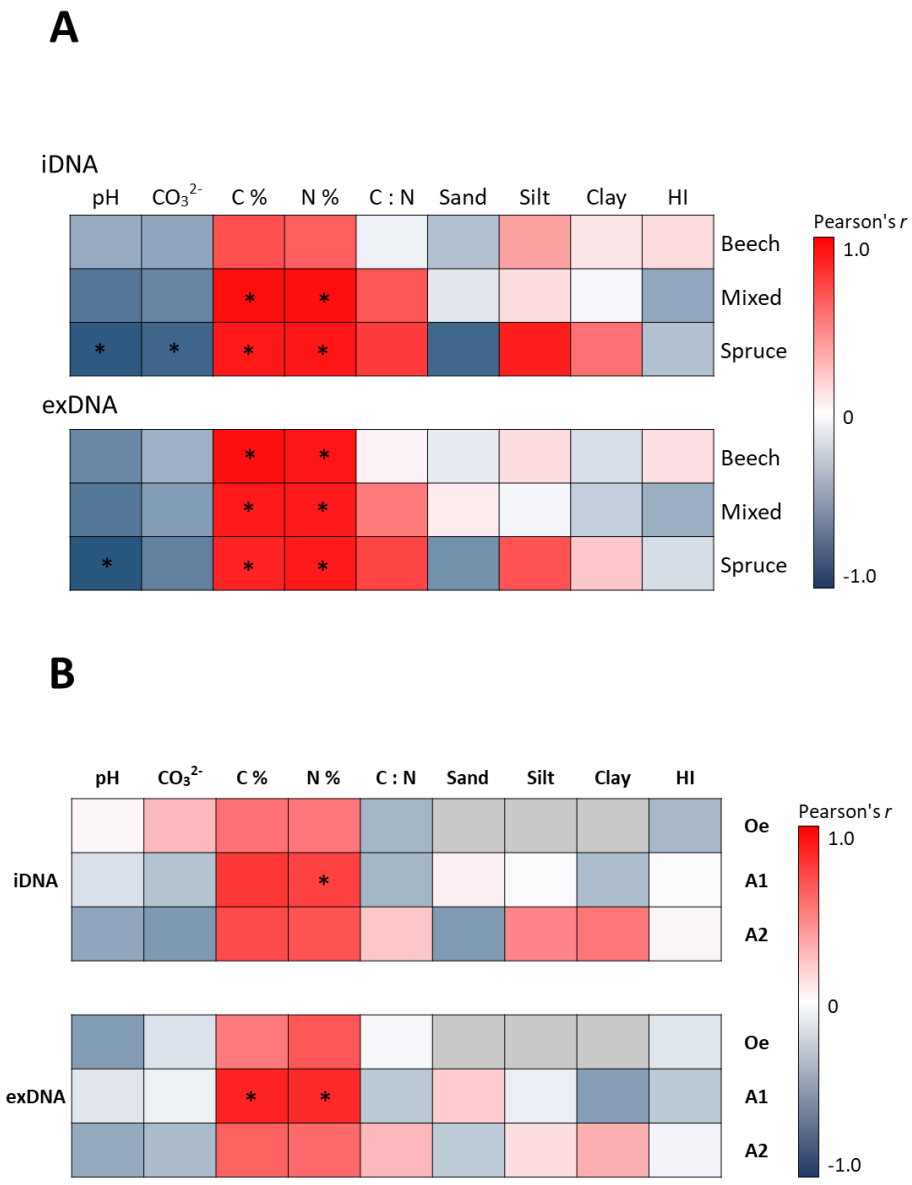


**Fig. 2.5.** - Relationships between soil DNA yield and physical-chemical properties at the study sites along the different forest type (**a**) and stratified for pedological horizon (**b**). Heatmap shows pairwise Pearson's correlation (r) between each physical-chemical variable and either intracellular (iDNA) or extracellular (exDNA) DNA yield (µg/g) measured in 12 soil samples (3 forest types x 4 replicates). Asterisks indicate significant p values ($P < 0.05$).

**Plant exDNA distribution and relationships with soil physical-chemical properties**

Out of a total of 4.6 million of unique sequenced *rbcLa* amplicons from exDNA, the taxonomic assignment produced a total of 386702 reads attributed at Streptophyta clade and 261082 reads assigned at plant order or lower rank taxa, out of which 223629 were taxonomically attributed at species level. Total reads numbers did not vary significantly neither among forest types ($F_{2, 27}$ = 0.343; P=0.713), nor among soil horizon ($F_{2, 27}$ = 0.008; P=0.991) or their interaction ($F_{4, 27}$ = 0.598; P=0.667), with high within group data variability (Fig 2.6).

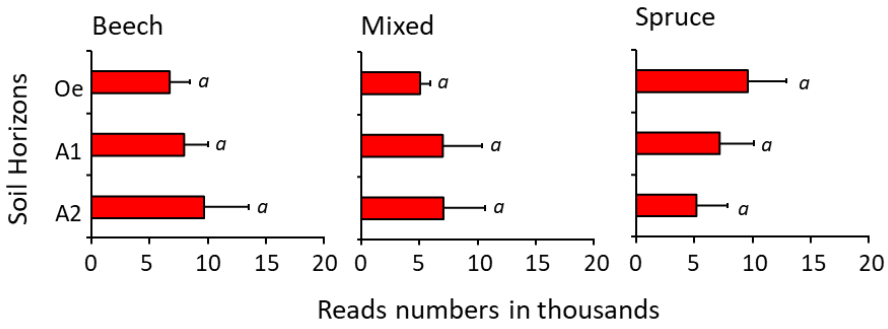


**Fig 2.6** - Vertical and horizontal distribution of exDNA reeds attributed to Streptophyta Clade in each forest type stratified for pedological horizon. Data refer to mean and standard error of 4 replicates. Lettering above bars indicate significant differences (P < 0.05, Duncan's post-hoc test).

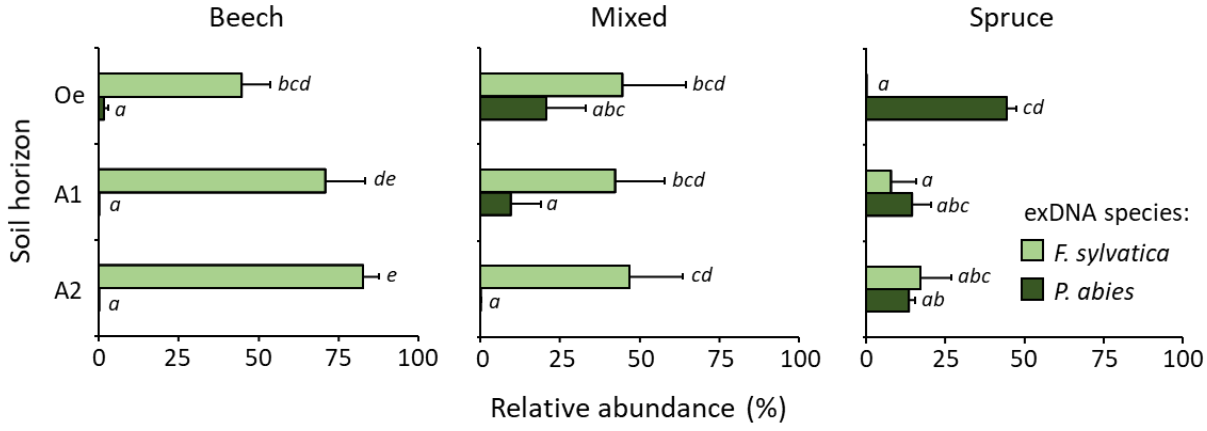


**Figure 2.7 -** Relative abundance of species-specific exDNA from *F. sylvatica* and *P. abies*, as assessed by metabarcoding in exDNA pool after amplification with Rbcl marker, across three different forest types (beech stands, spruce stands, or mixed forest) and along the vertical soil profile (Oe, A1 and A2 horizons). Data refer to mean and standard error of 4 replicates for each combination of dependent variables. Lettering above bars indicate significant differences between different forest types and tree species within each combination of soil horizon (P < 0.05, Duncan's post-hoc test after GLM, detailed statistical results in Tables 2.6 and 2.7)

**Table 2.6 -** Results of three-ways ANOVA testing for the effects of soil horizon (H, three levels: Oe, A1, A2), forest type (T, three levels: Beech, Mixed, Spruce) and tree species (S, two levels: *F. sylvatica*, *P. abies*) on the percent abundance of beech and spruce exDNA in soil at the sampling site.

| Effect | DoF | SS | MS | F | p |
|---|---|---|---|---|---|
| Soil horizon (H) | 2 | 78.8 | 39.4 | 0.1 | 0.8953 |
| Forest Type (F) | 2 | 3150.4 | 1575.2 | 4.4 | *0.0171* |
| Species (S) | 1 | 12733.8 | 12733.8 | 35.8 | *< 0.0001* |
| H × F | 4 | 1957.2 | 489.3 | 1.3 | 0.2560 |
| H × S | 2 | 3614.5 | 1807.2 | 5.0 | *0.0099* |
| F × S | 2 | 18049.0 | 9024.5 | 25.3 | *< 0.0001* |
| H × F × S | 4 | 427.2 | 106.8 | 0.3 | 0.8761 |
| Error | 48 | 17060.8 | 355.4 | | |

The distribution of *F. sylvatica* and *P. abies* exDNA show an interesting pattern (Fig. 2.7), significantly different among forest types and between the two species, also showing, for each of them, significant shifts with soil depth, as indicated by the significant terms forest type, species, forest x species, and species x horizon in the three-ways ANOVA model (Table 2.6). In detail, the contribution of the two tree species to the total DNA pool expectedly reflected the level of the tree species cover above ground (Fig. 2.7), with beech and spruce DNA fractions largely predominating in the soil DNA pool collected in beech and spruce forest stands, respectively. DNA samples from mixed forest showed higher content of beech DNA at all depths. Beech vs. spruce differences in exDNA relative abundance were significant in 6 out of 9 tested conditions, with beech DNA predominating at all depths in beech and mixed forest soils (with the exception of Oe in mixed forest), while spruce DNA was higher only at the superficial layer of spruce forest soil (Table 2.7). Interestingly, along the soil profile the persistence of exDNA from the two tree species followed different patterns: the abundance of *F. sylvatica* DNA showed a progressive increase along the vertical profile in beech forest (from 44.7 ± 8.8 % in Oe to 82.6 ± 5.2 % in A2) and non-significant variation in mixed and spruce forests, although in the latter case a large within-group variability could have masked a possible enrichment along the soil profile (Fig. 2.7, Table 2.7).

**Table 2.7 -** Results of Duncan's post hoc testing for the 3rd order interactive effect of forest type, tree species and soil horizon on the percent abundance of species-specific exDNA in soil after three ways ANOVA (Supplementary Table S5). P-values refer to pairwise comparisons between the percent abundance of *F. sylavatica* and *P. abies* exDNA within each combination of forest type and soil horizon (a), and between the percent abundance of exDNA of each tree species in the three soil horizons in each forest type (b). Data in (a) are means and standard errors of 4 replicates. Significant P-values are marked with asterisks (***: P < 0.001; **: P < 0.01; *: P < 0.05).

| | **Beech forest** | | | **Mixed forest** | | | **Spruce forest** | | |
|---|---|---|---|---|---|---|---|---|---|
| **a) Soil horizon** | ***F. sylvatica*** | ***P. abies*** | **P (Fs vs. Pa)** | ***F. sylvatica*** | ***P. abies*** | **P (Fs vs. Pa)** | ***F. sylvatica*** | ***P. abies*** | **P (Fs vs. Pa)** |
| Oe | 44.7 ± 8.8 | 1.5 ± 1.4 | 0.0111 * | 44.5 ± 19.9 | 20.6 ± 12.4 | 0.1151 | 0.1 ± 0.0 | 44.6 ± 3.1 | 0.0089 ** |
| A1 | 70.8 ± 12.7 | 0.1 ± 0.0 | < 0.0001 *** | 42.2 ± 15.4 | 9.5 ± 9.5 | 0.0441 * | 8.0 ± 7.9 | 14.6 ± 6.1 | 0.6759 |
| A2 | 82.6 ± 5.2 | 0.1 ± 0.0 | < 0.0001 *** | 46.7 ± 16.4 | 0.2 ± 0.2 | 0.0065 ** | 17.3 ± 9.7 | 13.5 ± 2.1 | 0.8010 |
| **b) Species** | **P (Oe vs. A1)** | **P (A1 vs. A2)** | **P (Oe vs. A2)** | **P (Oe vs. A1)** | **P (A1 vs. A2)** | **P (Oe vs. A2)** | **P (Oe vs. A1)** | **P (A1 vs. A2)** | **P (Oe vs. A2)** |
| *F. sylvatica* | 0.0843 | 0.4058 | 0.0155 * | 0.8732 | 0.7797 | 0.8869 | 0.6107 | 0.5622 | 0.3007 |
| *P. abies* | 0.9264 | 0.9998 | 0.9259 | 0.4871 | 0.5529 | 0.2206 | 0.0648 | 0.9407 | 0.0492 * |

Differently, spruce DNA abundance did not change significantly in beech and mixed forest soils, but showed a remarkable decrease along the soil profile in spruce forest (Fig. 2.7,Table 2.7), passing from 44.6 ± 3.1 % to 13.5 ± 2.0 % at the Oe and A2 horizons, respectively.

**Table 2.8 -** Correlation between the abundance of soil exDNA pools from different plant species (Fagus *sylvatica, Picea abies* and all plants) and soil physical-chemical properties at different forest types. For each exDNA pool and soil physical-chemical property, data refer to Pearson's r and associated p-value. Statistical significance was tested at α=0.0019 after application of the Bonferroni's correction for multiple comparison

| **Forest type** | **DNA pool** | **pH** | **$CO_3^{2-}$** | **N%** | **C%** | **C/N** | **Sand** | **Silt** | **Clay** | **HI** |
|---|---|---|---|---|---|---|---|---|---|---|
| Beech | All plants | 0.337 (0.284) | 0.642 (0.024) | -0.143 (0.658) | -0.141 (0.662) | -0.216 (0.500) | 0.761 (0.028) | -0.802 (0.017) | -0.619 (0.102) | -0.390 (0.210) |
| | *F. sylvatica* | 0.551 (0.079) | 0.571 (0.066) | -0.427 (0.191) | -0.413 (0.207) | -0.286 (0.393) | 0.733 (0.061) | -0.693 (0.084) | -0.769 (0.043) | -0.244 (0.470) |
| | *P. abies* | -0.516 (0.086) | -0.254 (0.425) | 0.578 (0.049) | 0.504 (0.095) | 0.066 (0.839) | 0.044 (0.918) | 0.053 (0.901) | -0.237 (0.572) | 0.303 (0.339) |
| Mixed | All plants | 0.422 (0.172) | 0.614 (0.034) | -0.111 (0.731) | -0.155 (0.631) | -0.445 (0.1479) | 0.733 (0.039) | -0.695 (0.056) | -0.764 (0.027) | 0.228 (0.476) |
| | *F. sylvatica* | 0.198 (0.560) | 0.273 (0.416) | -0.130 (0.702) | -0.136 (0.691) | -0.112 (0.742) | 0.535 (0.172) | -0.505 (0.201) | -0.560 (0.149) | 0.208 (0.540) |
| | *P. abies* | -0.388 (0.238) | -0.232 (0.493) | 0.566 (0.069) | 0.546 (0.0829 | 0.062 (0.856) | -0.088 (0.836) | 0.078 (0.854) | 0.099 (0.816) | -0.239 (0.479) |
| Spruce | All plants | -0.274 (0.389) | -0.332 (0.292) | 0.452 (0.140) | 0.398 (0.200) | 0.106 (0.743) | -0.204 (0.628) | 0.275 (0.510) | -0.056 (0.895) | -0.279 (0.380) |
| | *F. sylvatica* | 0.498 (0.119) | 0.367 (0.268) | -0.531 (0.093) | -0.525 (0.097) | -0.514 (0.106) | 0.236 (0.574) | -0.261 (0.533) | -0.116 (0.784) | 0.054 (0.874) |
| | *P. abies* | -0.781 (0.008) | -0.655 (0.040) | 0.821 (0.004) | 0.844 (0.002) | 0.612 (0.060) | 0.078 (0.868) | 0.067 (0.887) | -0.462 (0.296) | 0.078 (0.830) |

Considering the relationships between the abundance of species-specific exDNA and the physical-chemical properties of the forest soils samples, we did not found significant associations (Table 2.8) after controlling for multiple comparisons. However, the magnitude of some of the correlation scores and the associated p-values were very close to the corrected significance threshold, as in the cases of the abundance of spruce exDNA in spruce forest soils, where the abundance pattern was negatively related to soil pH (r = -0.781, P = 0.008) and positively to organic C (r = 0.821, P = 0.004) and total N (r = 0.844, P = 0.002) contents.

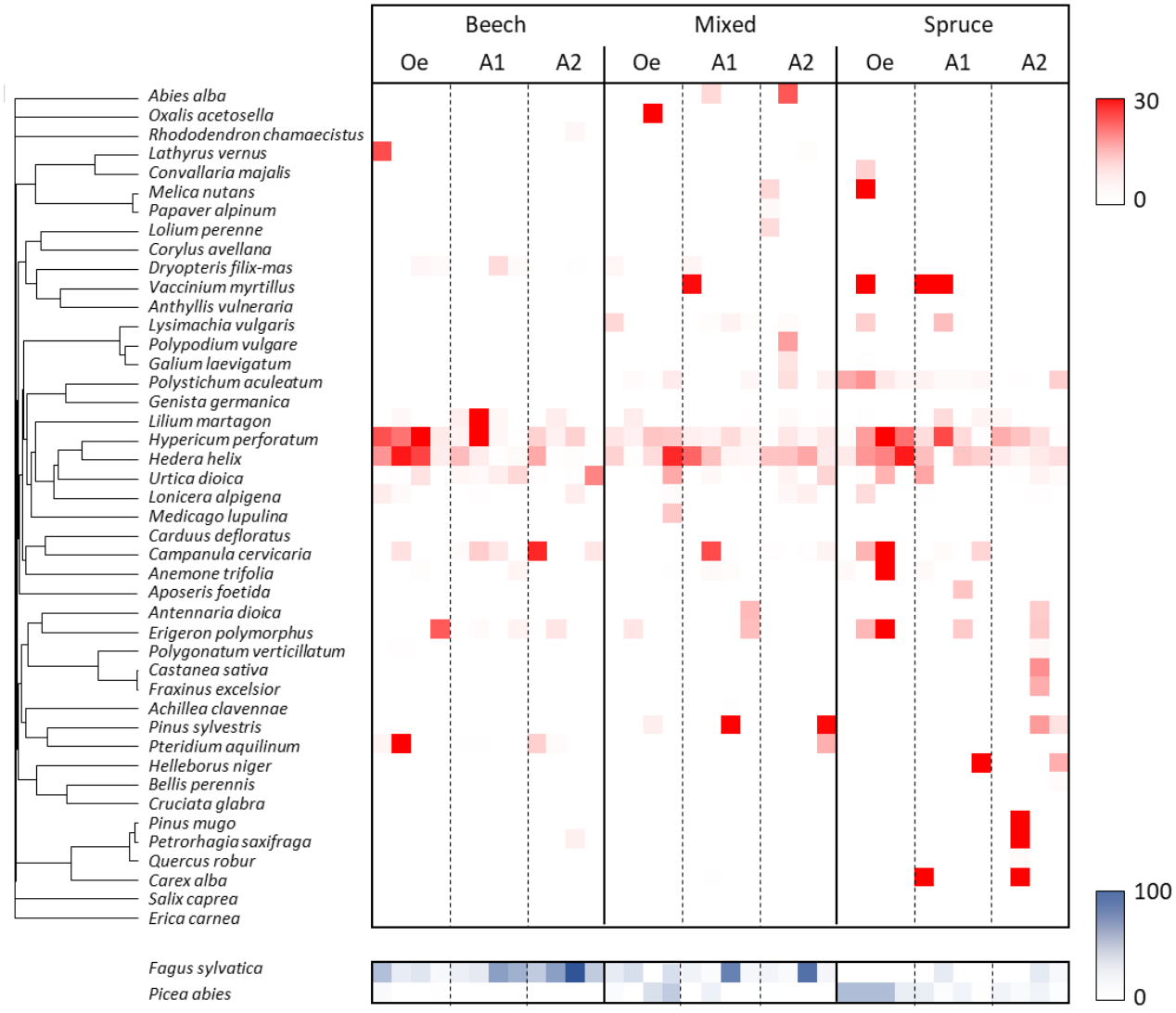


**Fig. 2.8 -** Heatmap showing the abundance of all the species identified in the plant community stratified for forest type (Beech, Mixed and Spruce) and pedological horizon (Oe, A1, A2).

**Plant community composition**

Metabarcoding analysis identified 47 species belonging to 30 families (Figure 2.8). Plant community composition, as assessed by exDNA metabarcoding, was significantly different among the three forest types, as indicated by the Permanova results (Table 2.9). Such compositional differences were well represented by the nMDS plot (Fig 2.9A), where the plant communities of beech and spruce forests were clearly separated. Indeed, beech vs. spruce forest compositional differences were statistically significant, both considering exDNA information separately for each soil horizon and all data pooled (Table 2.10). Mixed forest community expectedly showed intermediate composition, mostly closer to that of the beech forest (Fig 2.9A), although also showing significant compositional differences with respect to the spruce forest, when data pooled for the three soil horizons were considered (Table 2.10). Beech forest DNA corresponded to more conserved community as compared to the other two forest types, where the spreading along the two ordination axes indicated a much higher taxonomic variability (Fig 2.9A). Consistently, the Shannon's diversity index H' showed effects of forest type at the limit of statistical significance (Figure 2.9B), with significant difference between beech (H'= 1.20 ± 0.73) and spruce (H'= 1.95 ± 0.58) forest soils. Species richness as assessed by exDNA metabarcoding was not significantly affected by forest type and soil depth, as indicated by the Permanova results (Table 2.9) as well as by pairwise comparisons (Table 2.10). Finally, by considering the composition of plant communities at family (Fig. 2.10) and at species level (Fig. 2.8) level, as assessed by exDNA metabarcoding, a clear cut shift was observed passing from beech to spruce forest.

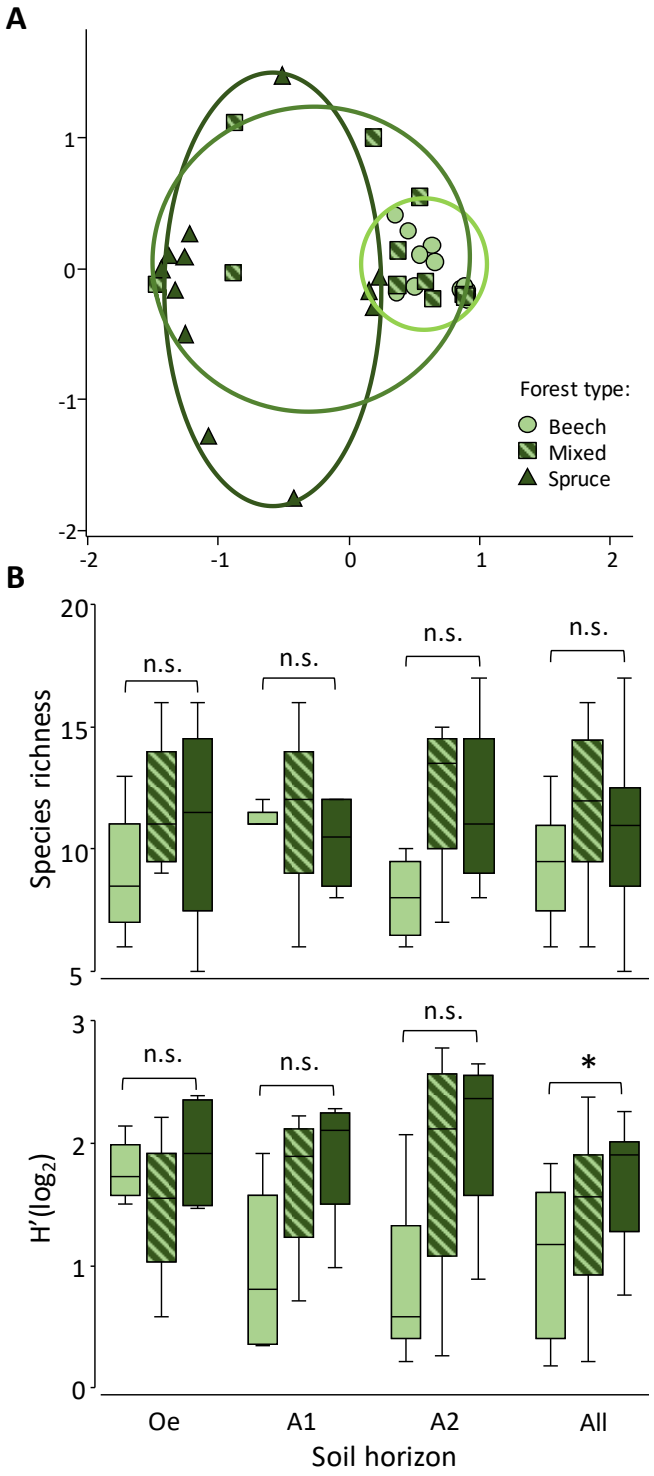


**Figure 2.9 -** Plant community metrics at the sampling sites as assessed by exDNA metabarcoding. (**A**): Non-metric Multidimensional Scaling (NMDS) ordination of exDNA samples based on Bray-Curtis similarity calculated on species abundance data, showing changes of plant community composition across the three forest types. 2D stress score for the ordination is 0.13. (**B**): Box and whisker plots of plant community metrics (species richness and Shannon's index H') across the forest types, separately calculated for each soil horizon and for all data pooled. Data refer to median, quartiles (boxes) and extremes (whiskers). Results of statistical testing of beech vs. spruce differences within each soil horizon are also reported (n.s.: not significant, *: $P < 0.05$, pairwise comparisons after Permanova analysis, detailed results in Table 2.10).

**Table 2.9 -** Results of Permanova testing for the effects of forest type (F), soil horizon (H) and their interaction on plant community composition, species richness and Shannon's index at the sampling sites. Community metrics were calculated based on the exDNA metabarcoding results, filtered considering only amplicons taxonomically attributed at species level (N = 223629).

| Effect | d.f. | SS | MS | Pseudo-F | P (perm) | Perms | P (MC) |
|---|---|---|---|---|---|---|---|
| *Community composition* | | | | | | | |
| Forest type (F) | 2 | 23870.0 | 11935.0 | 5.711 | ***0.001*** | 999 | ***0.001*** |
| Soil horizon (H) | 2 | 5574.1 | 2787.1 | 1.334 | 0.172 | 999 | 0.201 |
| F x H | 4 | 4806.6 | 1201.7 | 0.575 | 0.980 | 997 | 0.963 |
| Error | 27 | 56425.0 | 2089.8 | | | | |
| *Species richness* | | | | | | | |
| Forest type (F) | 2 | 36.167 | 18.083 | 1.752 | 0.195 | 999 | 0.184 |
| Soil horizon (H) | 2 | 1.167 | 0.583 | 0.056 | 0.949 | 999 | 0.936 |
| F x H | 4 | 26.667 | 6.667 | 0.646 | 0.610 | 997 | 0.610 |
| Error | 27 | 278.75 | 10.324 | | | | |
| *Shannon's index (H')* | | | | | | | |
| Forest type (F) | 2 | 3.407 | 1.703 | 3.280 | 0.052 | 999 | 0.050 |
| Soil horizon (H) | 2 | 0.293 | 0.146 | 0.282 | 0.761 | 999 | 0.764 |
| F x H | 4 | 2.025 | 0.506 | 0.975 | 0.427 | 997 | 0.438 |
| Error | 27 | 14.022 | 0.519 | | | | |

**Table 2.10 -** Result of Permanova pairwise significance test across forest types at the field sites, on plant plant community metrics based on exDNA diversity, calculated based on the exDNA metabarcoding results, filtered considering only amplicons taxonomically attributed at species level (N = 223629). In the permanova analysis, forest types and soil horizons were used as fixed factors, while replicated plots as random factor (N° of permutation 999). Significant values (P-value below 0.05) are marked in bold.

| Comparison | Composition | | Species richness | | Shannon's index (H') | |
|---|---|---|---|---|---|---|
| | t | P-value | t | P-value | t | P-value |
| *Soil horizon Oe* | | | | | | |
| Beech vs. Mixed | 1.020 | 0.389 | 1.288 | 0.254 | 0.836 | 0.423 |
| Beech vs. Spruce | 2.399 | ***0.010*** | 0.722 | 0.525 | 0.501 | 0.614 |
| Mixed vs. Spruce | 1.161 | 0.271 | 0.267 | 0.800 | 1.067 | 0.300 |
| *Soil horizon A1* | | | | | | |
| Beech vs. Mixed | 1.028 | 0.371 | 0.120 | 0.895 | 1.405 | 0.221 |
| Beech vs. Spruce | 2.109 | ***0.017*** | 0.943 | 0.365 | 1.870 | 0.119 |
| Mixed vs. Spruce | 1.205 | 0.243 | 0.542 | 0.612 | 0.425 | 0.669 |
| *Soil horizon A2* | | | | | | |
| Beech vs. Mixed | 0.933 | 0.469 | 2.109 | 0.076 | 1.391 | 0.225 |
| Beech vs. Spruce | 1.881 | ***0.041*** | 1.756 | 0.134 | 2.096 | 0.082 |
| Mixed vs. Spruce | 1.431 | 0.089 | 0.190 | 0.850 | 0.3627 | 0.730 |
| *All horizons* | | | | | | |
| Beech vs. Mixed | 1.331 | 0.122 | 2.016 | 0.060 | 1.469 | 0.159 |
| Beech vs. Spruce | 3.466 | ***0.001*** | 1.300 | 0.237 | **2.802** | **0.013** |
| Mixed vs. Spruce | 1.975 | ***0.009*** | 0.557 | 0.602 | 0.965 | 0.357 |

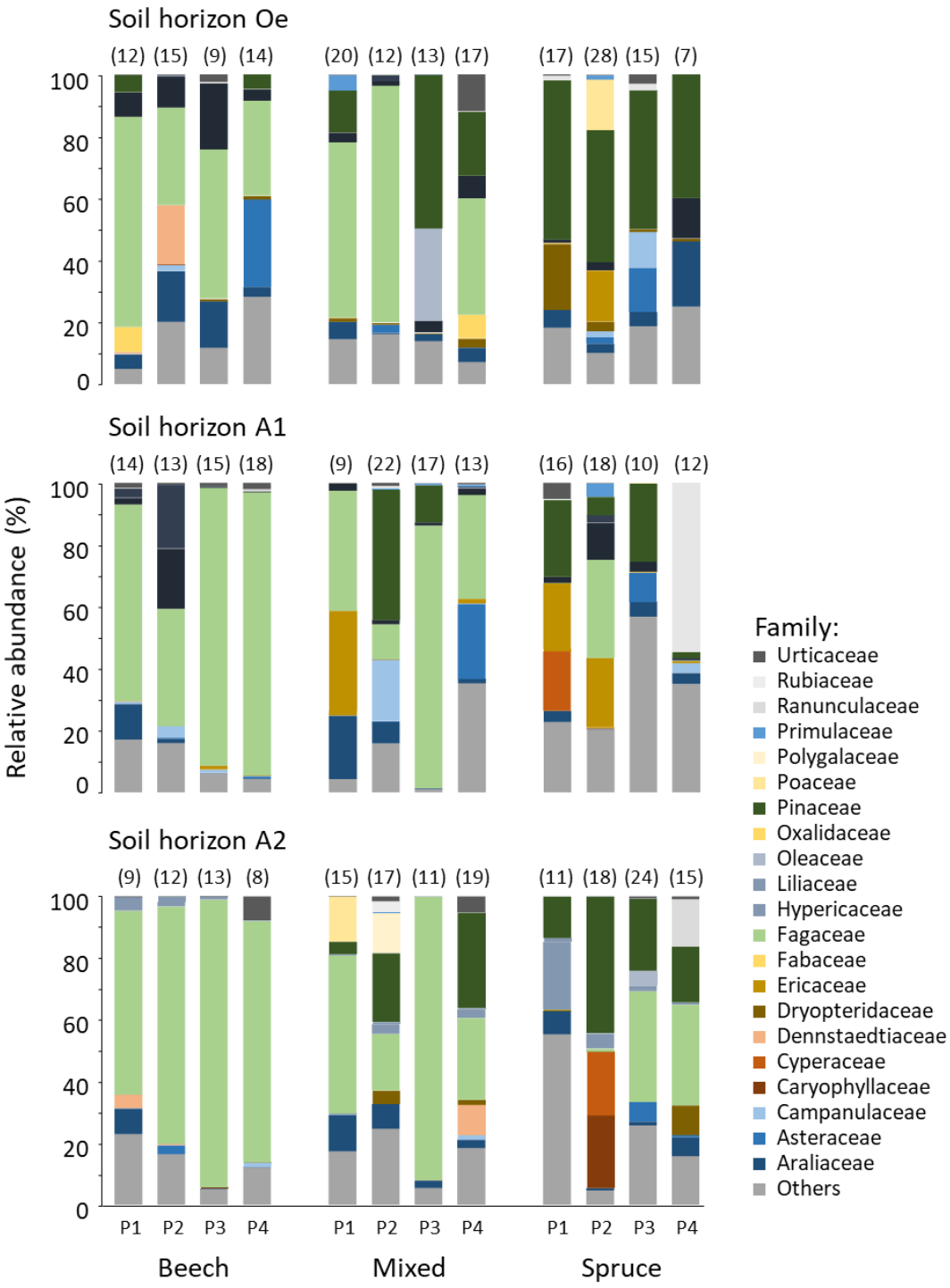


**Figure 2.10 -** Plant community diversity assessed by exDNA metabarcoding. Stacked bar chart of relative abundance of plant families in each sampling site stratified for forest type (beech, mixed and spruce) and pedological horizon (Oe, A1 and A2). Numbers in brackets refers to species identified inside each sampling site.

## Discussion

### Progress and problems on the use of the *rbcl* barcode

The use of metabarcoding is revolutionizing biodiversity studies, especially the detection of so-called hidden species (Taberlet et al., 2018). However, floristic studies based on the use of soil DNA metabarcoding are still rare (but see Yoccoz et al., 2012), mostly because of the difficulty of finding a unique, robust and effective barcode allowing to correctly detect and discriminate amplicons from DNA fragments of all plant species occurring in a given environmental sample (Hollingsworth, 2011; Hollingsworth et al., 2011). In 2009 the Consortium for the Barcode of Life (CBOL) Plant Working Group, proposed the use of two specific portions of two coding regions from the chloroplast genome - *rbcL* and *matK* - as a source for barcode for plants, that have been integrated with a great numbers of specific markers, belonging to ITS regions (Li D-Z et al., 2011; Cheng et al., 2016) or trnL P6 loop (Coissac et al. 2007; Yoccoz et al., 2012). Although the use of multiple markers can be recommendable (Fahner et al., 2016; Cheng et al., 2016), in our study, using only the *rbcla* marker, we obtained a satisfactory result. Indeed, we detected a total of 47 plant species, all known for the study area (Poldini, 1991; Nimis & Martellos, 2006). However, for a large fraction (32.5 %) of the 386702 unique amplicons attributed to *Streptophyta*, it was not possible to find a satisfactory taxonomic match, since the identification was limited to supra-generic ranks. Besides possibly related to intrinsic biases of the selected barcode, which has been previously criticized as showing good recovery and sequence quality but low species discrimination (Hollingsworth, 2011), our result possibly indicates that the reference database for the target barcode in plants is largely incomplete, at least for alpine and beech-spruce forest species. Therefore, further investigation and sequencing of rbcl barcode is needed, especially in alpine forest ecosystems, in order to increase database completeness and then improve our knowledge of the barcode performance.

Interestingly, 91.6 % of all the amplicons produced by *rbcla* in our samples (a total of 4.6 millions) and satisfactorily sequenced were even impossible to be taxonomically attribute within the plant kingdom. Such large predominance of unknown sequences resulting from the amplification of a barcode specific for plants, although unexpected, should not be surprising. Indeed, the length of *rbcla* sequence is negligible in comparison to the length and sequence diversity of all the possible exDNA fragments occurring in the soil environment. Therefore, it is reasonable that the *rbcla* primers, although specifically selected to amplify plant DNA and discriminate among different plant species, could also amplify by chance exDNA fragments of non-plant origin and hence not included in the reference database.

The possibility of using DNA metabarcoding to estimate species abundance is a subject of debate since the first studies based on this methodology (Matesanz et al., 2019). Indeed, translating the amplicons copy numbers to abundance is challenging, as the number of copies in the original sample can vary across cells, tissues, individual, species and probably time (Taberlet et al., 2018). Additionally, the efficiency of amplification for the same primers can change for different target sequences, as well as a function of the experimental conditions (Taberlet et al., 2018). However, such approach has been successfully adopted in previous studies showing that in appropriate conditions it is even possible to find a significant positive association between paired data of raw reads number and species-specific biomass (Yoccoz et al., 2012; Matesanz et al., 2019). In our study we cannot show the same level of correspondence between the cause (plant species biomass) and the effect (abundance of plant exDNA), although our result of reads numbers at the sampling plot for beech and spruce exDNA clearly reflect the expectations, considering that the highest abundance of *F. sylvatica* and *P. abies* exDNA was consistently found in beech and spruce forest soils, respectively, while in mixed forest soils we found intermediate abundance values of the exDNA of the two tree species.

**Soil DNA distribution along the vertical profile**

Our results showed that iDNA and exDNA contents in soil progressively decrease with depth. This is fully consistent with previous observations, based on the same approach to separate the two soil DNA fractions (Ascher et al., 2009) but carried out in different forest ecosystems, including silver fir pure forest (Agnelli et al., 2004), mixed black pine and silver fir forest (Ascher et al., 2009), downy oak and maritime pine mixed forest (Borgogni et al., 2019). We can explain such consistent observations across different ecosystems considering that the abundance of exDNA in soil dynamically changes as a function of inputs in the upper layer by litterfall and decay which feeds the soil microbiome turnover, and its persistence due to the interaction with the soil organic and mineral fractions (Levy-Booth et al., 2007). The pattern of decreasing abundance with depth corresponds to that of the soil organic matter, which in turns interplays with soil microbiome (Bonanomi et al., 2017). In other words, both fractions of soil DNA mostly corresponds to microbial DNA, which is most abundant in the upper layers where the amount of available organic compounds sustain the growth and development of microbial living cells (Agnelli et al., 2004), hence enhancing iDNA abundance, and the microbial turnover (Carini et al., 2016), which in turns increase exDNA input in the soil by cell death and lysis. As the process is recursive, larger microbial mass also make available larger amounts of organic compounds, with positive feedback effects limited by the overall soil nutrient resources. Accordingly, the of exDNA has been used as a proxy of microbial activity (Nagler et al., 2018), based on the evidence that when the microbial turnover is rapid exDNA is abundant in soil (Ascher et al., 2009), but when the soil is subjected to destructive events the microbial community is rapidly degraded and its activity is not detectable (Borgogni et al., 2019). Moreover, it is known that the soil microbiome undergoes a rapid turnover and variation with profundity (Sirois et al., 2019), mainly in association with the availability of organic carbon and nitrogen (Andersson et al., 2004; Šnajdr et al., 2008). Consistently, we found a significant positive

association between iDNA and exDNA abundance and soil acidity and carbonate content, which in forest soils frequently vary along the vertical profile (Abramo et al., 1998), as in the case of the survey area where a progressive increase of pH and carbonates with soil depth is related to the calcareous nature of the rock substrate. Finally, our results did not confirm previous indications that DNA persistence in the soil could be enhanced by its interaction with the mineral soil fraction, particularly with the clay minerals, and with the recalcitrant humic organic fraction (Ranjard & Richaume, 2001; Pietramellara et al., 2009). Indeed, the abundance of both iDNA and exDNA was unrelated to the clay content, as well as to the humification index in our samples. This could be explained by two possible non-mutually exclusive hypotheses, related to peculiar soil conditions at the sampling sites: i) a relative stability of these two soil chemical properties along the vertical profile; ii) the predominance of the soil biological processes over its chemical properties in controlling DNA dynamics and persistence, as previously shown under homogeneous chemical-physical conditions (Ranjard & Richaume, 2001).

**Plant exDNA horizontal and vertical distribution**

We found that the content of beech and spruce exDNA in the organic soil horizon reflected the species-specific tree density above ground. This result, which quantitatively depends on the species-specific DNA input by litter fall and decay, indirectly confirms the reliability of our methodological approach, as discussed in the previous section.

More interestingly, we found a progressive increase with depth of *F. sylvatica* exDNA under beech forest and, contrarily, a decrease with depth of *P. abies* exDNA abundance in spruce forest. Considering exDNA input by litter fall and decomposition, at the same site and basal area levels, beech and spruce can release yearly comparable amounts of leaf litter (Pedersen et al., 1999). Although in this study we did not directly measure the rate of species-specific litterfall, it is

reasonable to assume that, in the case of *P. abies*, litterfall is more evenly distributed throughout the year, while for *F. sylvatica* it is mostly limited to the fall period. However, it is clear that the two opposite species-specific patterns of vertical exDNA distribution cannot be uniquely related to exDNA input by litter fall, but litter decomposition dynamics must be also taken into account.

Litter decomposition represents the most important source of elements to the forest floor (Miller et al., 1979; Brinson et al., 1980). Once on the forest floor, litter is subjected to physical, chemical and biological degradation (Hättenschwiler et al., 2005), releasing organic compounds that, if hydrophilic, can diffuse both horizontally and vertically following water movements through leaching and percolation (Berg et al., 1981; Laskowski & Berg, 2006). The accumulation of soil organic matter depends on decomposition rates and progressive preservation of recalcitrant compounds, which include not only hydrophobic biomolecules (Attiwill & Adams, 1993; Piccolo, 2002), but also resistant hydrophilic molecules physically and chemically protected by the interaction with the soil mineral fraction, as well as hydrophobic domains (Incerti et al., 2017). Among these, exDNA under suitable environmental conditions can persist for a long time in the soil (Levy-Booth et al., 2007), ranging from weeks to even years (Carini et al., 2016). The major determinants of decomposition dynamics include climatic variables and plant litter molecular properties, which interact and selectively prevail at regional and local spatial scales, respectively (Liski et al., 2003; Bradford et al., 2016). This also applies in our study, where species-specific exDNA input in the upper soil layer is strictly controlled by spruce vs. beech litter decomposition rates, which in turns depend on species-specific litter quality. Although we didn't measure directly litter decay rates in this study, some previous studies reported that *P. abies* litter decompose at a fairly lower rate compared to *F. sylvatica* one (Bonanomi et al., 2013), as related to the spruce needles relatively high content of recalcitrant hydrophobic compounds such as resins, cutins and waxes (Incerti et al., 2011). Other, more recent studies, clearly showed that, more than an intrinsically

different decay rate linked to litter chemistry, spruce and beech litter decompose significantly more slowly in spruce stands, as compared to mixed and especially beech stands, associated to changes in soil acidity, soil structure and humus form (Albers et al., 2004; Berger & Berger, 2012; 2014). Independent on the local prevalence of the chemistry vs. micro-environment effects, our results are consistent with a higher total exDNA abundance in the upper soil layer in beech stands, lower in spruce stands, and intermediate in mixed forest. The mechanism of exDNA transport along the soil column is complex and not yet fully clarified (Ceccherini et al., 2007; Potè et al., 2007; Ascher et al., 2009; Potè et al., 2010). However, a more rapid decomposition in beech forest floor is likely to favour the plant exDNA vertical migration by percolation following water flow, while, under spruce stands, a slower litter decay may limit the release of exDNA and its vertical migration, resulting in the observed opposite vertical distribution pattern of species-specific exDNA abundance.

Finally, a further contribution to the observed pattern, and in particular to the higher beech exDNA at the highest sampled depth, could rely on the nucleic acids release by root exudates and turnover (Pietramellara et al., 2009). Although the role of such processes and their contribution to the nutrient cycle in forests has not yet been fully evaluated (Lukac, 2012), it is suggestively consistent with the different rooting depth ranges of beech vs. spruce, with the latter showing much lower exDNA abundance in the soil layers where its roots are less abundant, and vice-versa.

**Plant community composition and diversity by exDNA metabarcoding**

The plant community in the study area is ascribable to the association Anemono-Fagetum (Marincek et al., 1989), which includes all the Illyrian mixed forests dominated by *F. sylvatica* and *P. abies* on calcareous substrates in the mountain belt of the Querco-Fagetea class (Mucina, 1997). Our analysis by exDNA metabarcoding provided consistent information about the vegetation in the survey area as compared to the local phytosociology. Indeed, in addition to the two dominant tree species, we

found exDNA attributed to tree species occurring also at higher (*Pinus mugo*) or lower (*P. sylvestris, Abies alba*) altitude, scattered in some of our samples, mostly from mixed forest stands. Considering shrubs and herbaceous species, we found some characteristics entities of the association, such as *Vaccinium myrtillus*, *Carex alba, Anemone trifolia, Polystichum aculeatum* and *Erica carnea*, although unequally distributed among plots and sporadically in some cases. Other species such as *Hedera helix, Hypericum perforatum* and *Urtica dioica* were widespread in many of our samples, though being not characteristic of beech-spruce forests, as showing a cosmopolitan distribution. However, their presence is well-known locally (Poldini, 1991; Nimis & Martellos, 2006) and therefore exDNA transport by wind, water flow or animals (Yoccoz et al., 2012) from the surrounding areas cannot be excluded.

Considering compositional and diversity differences among the plant communities at the three forest types, we found that species richness was not significantly different among the forest types, as being highly variable among the plots of each forest type. This latter result could trivially reflect the variability of exDNA taxonomy at small spatial scale: for each plot in each forest type, at each soil horizon, we pooled 4 different small core subsamples systematically collected at fixed within-plot locations, which clearly contained the mixed exDNA pool resulting from the above-standing individual plants. Therefore, independent of the total list of species in each plot, considering the scattered within-plot distribution of most plant species (with the exception of the ubiquitous dominant tree species) we sampled only a small fraction of the total exDNA pool in each plot. This could also explain our apparently surprising finding of significantly higher compositional heterogeneity in spruce forests as compared to mixed and beech ones, as shown by both the plots spreading in the NMDS graph and the Shannon index H'. Indeed, a higher biodiversity would be expected from mixed forests, both in general (Felton et al., 2010; 2016) and in spruce-beech systems (Vacek et al., 2021). It is reasonable assuming that the small-scale variability intrinsic in our exDNA

assessment may have masked the general pattern, rather than considering our study plots a remarkable exception to a generalized phenomenon.

## Conclusions

In this study for the first time we provided a clear picture of total iDNA and exDNA, as well as species-specific plant exDNA abundance and distribution in spruce-beech mono-dominated and mixed forest soils. About total iDNA and exDNA, our study confirms previous observations from other different ecosystems, showing a progressive depletion with soil depth, independent of forest type, in relation to the interplay between soil chemical-physical features and microbial turnover.

In the case of our assessment on plant exDNA, our results clearly show the possibility to successfully use the metabarcoding approach to obtain a quantitative overview of species-specific exDNA abundance and distribution. While not producing direct mass balance evidence, our results demonstrate clear-cut different patterns of vertical distribution for beech exDNA vs. spruce exDNA, as likely related to species-specific differences of litter quality and micro-environmental conditions, both decisively affecting litter decomposition dynamics in the different forest types.

Finally, exDNA metabarcoding also provided a faithful, although incomplete, picture of the local plant diversity, indicating that such technique could positively integrate traditional biodiversity inventory studies base on expert field assessments.

# Chapter 3

# Production of $^{15}N$ labelled plant DNA

# 3 .1. N uptake, assimilation and isotopic fractioning control δ $^{15}$N dynamics in plant DNA: a heavy labelling experiment on *Brassica napus* L.

## 3.1.1. Introduction

In last decades a large body of evidence clarified the main sources of variation in nitrogen isotope composition (δ $^{15}$N) at intra-plant level (review in Evans et al., 2001), showing that isotopic fractionation occurs in relation to the chemical species, content and bioavailability of inorganic and organic N in the substrate, with known biochemical mechanisms (Yoneyama et al., 2019).

Plant isotope composition firstly relates to δ $^{15}$N and relative fractions in the substrate of different N sources, such as $NH_4^+$, $NO_3^-$, organic N, or $N_2$ in the case of species symbiotic with diazotrophic prokaryotes (Robinson et al., 1998). Plant δ $^{15}$N also varies compared to that of soil N due to different uptake mechanisms, assimilation pathways, and rates of N recycling, which can all discriminate against the heavy isotope (Evans et al., 2001). As an example, $NO_3^-$ uptake is mediated by either constitutive carrier system with high-substrate affinity or non-saturable transport mechanisms with low-substrate affinity, which act at low (0-500 μM) and high (> 500 μM) $NO_3^-$ concentrations in the substrate, respectively. Both transport systems produce isotopic fractionation (Yoneyama et al., 1991; Yoneyama et al., 2001; Kolb et al., 2003), although plant-to-soil δ $^{15}$N variation is larger when $NO_3^-$ is the primary N source, and smaller when $NH_4^+$ is used (Evans et al., 2001). On the other hand, species- and cultivar-specific effects can outcompete those of the N chemical species (Yoneyama et al., 2001; Craine et al., 2015), which led to measuring foliar δ $^{15}$N to understand the physiological mechanisms underlying N use differences among co-occurring species (Evans et al., 2001).

Considering discrimination against $^{15}$N during inorganic N assimilation (Robinson et al., 1998), several previous studies focused on the enzymatic fractionation by nitrate reductase and

glutamine synthetase (Haynes et al., 1986; Yoneyama et al., 1989; Werner et al., 2002; Tcherkez et al., 2006; Kalcsits et al., 2014) and its effects on intra-plant δ $^{15}N$ variation. Nitrate is assimilated both in roots and leaves, where the content of assimilation enzymes and the rate of assimilation can affect the resulting δ $^{15}N$ (Mariotti et al., 1982). However, the $NO_3^-$ available for assimilation in leaves is enriched relative to root $NO_3^-$ because it originates from a pool that has already been exposed to fractionation during root assimilation, leading to higher δ $^{15}N$ of leaves compared to roots (Evans et al., 2001). However, it has been recently reported that $NO_3^-$ can be enriched in $^{15}N$ in roots compared to leaves, due to nitrate circulation and compartmentalization, in particular by phloematic backflow from the leaves (Cui J et al., 2020) Differently, $NH_4^+$ is immediately assimilated in the root, therefore root vs. leaf δ $^{15}N$ differences are less affected by $NH_4^+$ assimilation, as organic nitrogen in shoots and roots is the product of a single assimilation event (Evans et al., 2001).

Further contributions to intra-plant δ $^{15}N$ variation rely on isotopic fractionation during xylematic (Yoneyama et al., 1998) and re-allocation (Yoneyama et al., 2003) flows, as well as N depletion by $NH_3$ and $NO_2$ volatilization, although the latter process, being limited to the leaf senescence stage, likely bears negligible effects (Kalcsits et al., 2014). Finally, the role of plant symbionts such as mycorrhizae and N-fixing rhizosphere bacteria were investigated in both field and controlled conditions (Hobbie et al., 2012), showing interesting dynamics (Makarov et al., 2019) but limited effects, in relation to their negligible mass compared to that of the plant (Högberg et al., 1999).

More recently, isotopic fractionation has been investigated along specific metabolic pathways by IRMS analysis after purification of different leaf metabolites, including amino acids, nucleic acids and chlorophylls (Gauthier et al., 2013) or by compound-specific stable isotope analysis (CSIA), where IRMS is coupled with GC-MS or LC-MS interface to separate different metabolites before isotopic analysis (Ostle et al., 1999; Zhang et al., 2016a; Tea et al., 2017). Differences of δ $^{15}N$

among different molecular N pools depend on isotopic discrimination by most enzymes of primary N metabolism (e.g. Glu synthase, transaminases, Asn synthetase, etc, Werner et al., 2002). Accordingly, Gauthier et al. observed by CSIA a different $\delta^{15}N$ in different N molecular pools in *Brassica napus* leaves, corresponding to a predominant effect of enzymatic discrimination in amino acid metabolic pathways, compared to that associated to the inorganic N source (Gauthier et al., 2013). Moreover, the N pool of leaf DNA, purified by standard methods, lyophilized and isotopically analyzed by EA-IRMS, was isotopically depleted compared to amino acids, consistent to discrimination associated with the synthesis of bases (Gauthier et al., 2013). Several enzymes involved in pyrimidine synthesis discriminate the heavy isotope, such as carbamoyl phosphate synthetase (Rishavy et al., 2000), aspartate carbamoyl transferase (Waldrop et al., 1992), dihydroorotase (Anderson et al., 2006), orotate phosphoribosyl transferase (Zhang et al., 2009). In the case of purine synthesis, amino acids such as Glu, Gln, Asp, and Gly are the N sources, but isotope effects along these metabolic ways are not yet fully clarified. Consequently, the $\delta^{15}N$ of leaf DNA is expected to be lower compared to other leaf N pools (Gauthier et al., 2013). Accordingly, lower $\delta^{15}N$ is expected in plant DNA compared to the source material from which it is purified, whose $\delta^{15}N$ value results from the average of different molecular pools, weighted by their relative mass fraction (Hayes et al., 2004). However, changes of $\delta^{15}N$ in plant materials during plant development could affect the expected pattern, as related to possible decoupling in time of DNA biosynthesis and inorganic N uptake and assimilation dynamics, and hence of their fractionation effects.

Therefore, in this study we set up a manipulative experiment in controlled conditions on *Brassica napus* var. *oleracea*, monitoring $\delta^{15}N$ of purified DNA and source leaf and root materials, over a 60-days growth period starting at d 60 after germination, in plants initially supplied with a heavy labelled ammonium nitrate solution and controlling for the labelled N species (either $NO_3^-$, $NH_4^+$ or both). We assumed that the magnitude of isotope effects is small enough that they generally

do not perturb plant growth dynamics when compared to the unlabelled control (Freund et al., 2017). Our specific hypotheses and expected outcomes were that: (1) leaf and root δ $^{15}N$ dynamics strictly depend on the labelled chemical species, as related to a limiting effect of $NH_4^+$ concentration on the uptake of $NO_3^-$ (Haynes et al., 1986). Accordingly, plants supplied with either labelled $NH_4^+$, labelled $NO_3^-$ or both labelled species (thereafter referred to as $\dot{N}H_4$, $\dot{N}O_3$ and $\dot{N}H_4\dot{N}O_3$, respectively) should initially show higher, lower and intermediate values, respectively. Then, the progressive $NH_4^+$ depletion from the nutrient solution should correspond to an increase of $NO_3^-$ uptake rate, with $\dot{N}H_4$ and $\dot{N}O_3$ plant materials showing progressively decreasing and increasing δ $^{15}N$, respectively. (2) In $\dot{N}H_4\dot{N}O_3$ plants, where δ $^{15}N$ is not affected by the labelled N chemical species, we tested the occurrence of isotopic fractionation associated to inorganic N uptake (Yoneyama et al., 1991; Evans et al., 1996; Kolb et al., 2003), expecting an increase of δ $^{15}N$ over time due to a progressive $^{15}N$ enrichment in the N pool residual in the pot solution. (3) Differences in assimilation rates in roots compared to leaves should produce, at a given observation stage, higher δ $^{15}N$ values in $\dot{N}H_4$ roots compared to $\dot{N}H_4$ leaves and in $\dot{N}O_3$ leaves compared to $\dot{N}O_3$ roots, with $\dot{N}H_4\dot{N}O_3$ materials showing intermediate values; (4) Consistent to expectations (Gauthier et al., 2013), DNA is depleted in $^{15}N$ compared to the other molecular N pools, and thus to the source plant material, due to enzymatic discrimination during purine biosynthesis.

## 3.1.2. Materials and methods

Our experimental design is summarized in Fig 3.1.1., including seed germination and potting, isotopic labelling, periodic destructive sampling, plant DNA extraction and purification and CHN-IRMS analysis.

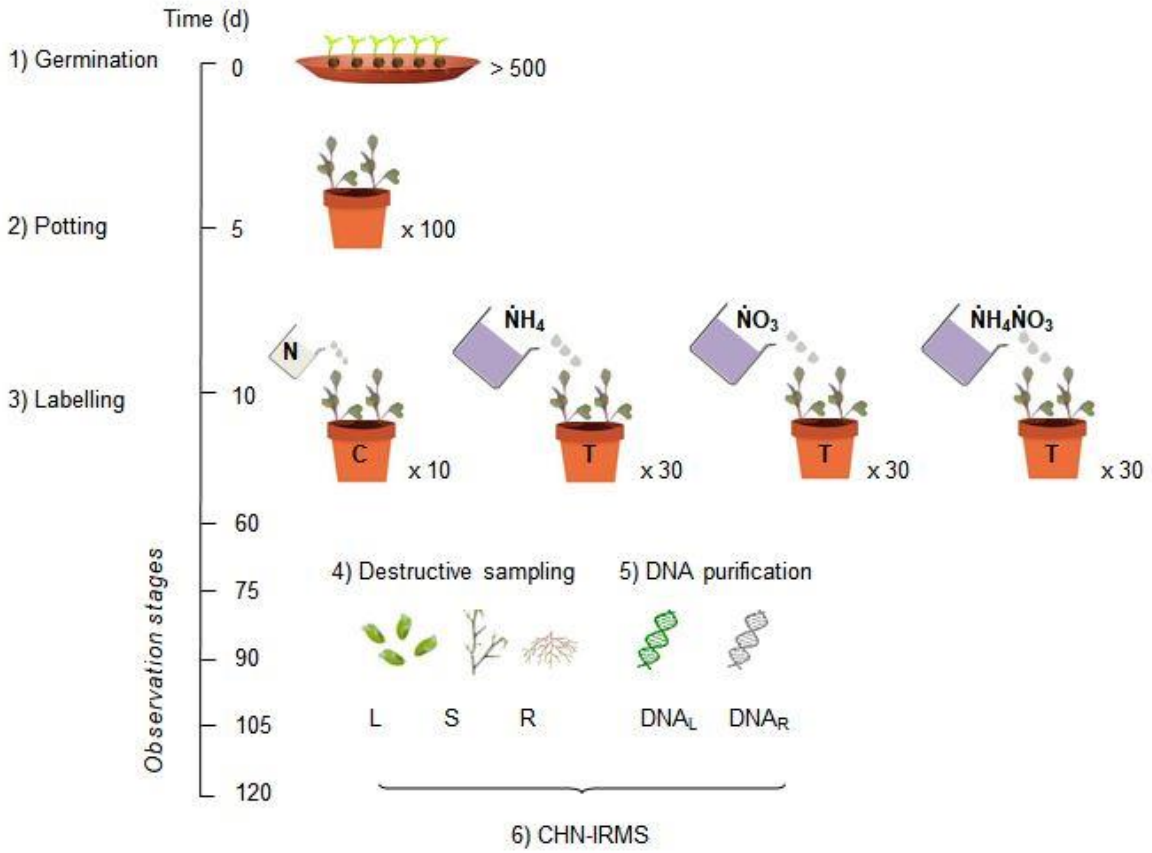


**Fig 3.1.1. -** Illustration of the experimental design. Six manipulative steps are shown along the experiment timeline: 1) seeds germination at d 0; 2) potting at d 5; 3) labelling with ammonium nitrate solution, including 3 treatments (T, each on 30 replicated pots) with the same isotopic ratio, i.e. $\delta^{15}N_{Air\text{-}N2}$ = 2100 mUr, but differing by the labelled chemical species ($\dot{N}H_4$, $\dot{N}O_3$ or $\dot{N}H_4\dot{N}O_3$), plus the untreated control (C, 10 replicates) administered with the same dose of unlabelled ammonium nitrate (N); 4) destructive sampling (6 replicates per treatment at each of 5 observation stages from d 60 to d 120) and separation of leaf, stem and root materials (L, S and R, respectively); 5) DNA extraction and purification from leaf ad root materials; 6) CHN-IRMS analysis of dry aliquots of plant materials and corresponding DNA samples. See methods for further details.

### Plant material, sowing and potting

Commercial seeds of *Brassica napus* var. *oleifera*, cultivar Gordon (KWS SAAT SE & Co. KGaA, Germany), were imbibed with Milli-RO water for 24 h into in 50 mL lab grade tubes, then transferred to plastic saucers filled with dried, quartz sand substrate (GESTECO Spa, Italy; physical-chemical

features Table 3.1.1) and kept in a growing room in controlled optimal conditions (T = 22/20 °C, photoperiod 12 h, RH = 50%, PAR 600 µmol photons $m^{-2}$ $s^{-1}$).

**Table 3.1.1.** Physical-chemical features of the quartz sand substrate used for potting.

| Characteristic | Value |
|---|---|
| Particle size | ≤ 1 mm |
| Density | 2.65 g/$cm^3$ |
| Cl content | < 0.01 % |
| Humus content | Absent |
| Total S content | < 1 % |
| $CO_3^=$ | 10.45 % |
| Total N | < 0.01 % |
| Light contaminants | Absent |

Five days after germination (i.e. d 5), seedlings were transplanted in pots (2 seedlings per pot) previously filled with 1.3 kg of substrate. Each pot had draining holes allowing drainage and preventing hypoxia, and was placed on a saucer to prevent nutrient loss. One-hundred pots were considered, corresponding to 30 replicates for each of 3 labelling treatments, plus 10 unlabelled controls.

**Labelling and nutrient solution**

Three different N labelling treatments with different labelled chemical species (either ammonium, nitrate or both) were considered, all with equal isotopic ratio (δ $^{15}N_{Air\text{-}N2}$ = 2100 mUr). Such extremely high value was used in order to ensure the detectability of $^{15}N$ depletion in leaf and root DNA along the observation period, in absence of previous quantitative evidence on the possible enzymatic discrimination coefficient (Gauthier et al., 2013). Ammonium nitrate solutions for labelling were prepared by mixing a water solution of commercial $NH_4NO_3$ (Sigma-Aldrich, USA, δ

$^{15}N_{Air\text{-}N2}$ = 0.7 mUr) with that of each labelled salt (Cambridge Isotope Labs, 98% of labelled atoms) in the opportune mixing ratio, following the equations reported in (Hayes et al., 2004).

At d 10, each of 30 potted seedlings for each labelling treatment was supplied with 50 mL of a 0.336 M ammonium nitrate solution (i.e. 470 mg of N per pot), corresponding to N requirements for a 180-days growing period, estimated according to previous reports on dynamics of leaf and root growth, N content and uptake efficiency (Diepenbrock et al., 2000; Rossato et al., 2001; Williams et al., 2013; Tribouillois et al., 2015). In this way, it was possible to assess the labelling dynamics of plant materials since an initial starting point of known δ $^{15}N$ of the nutrient solution.

Other macro- and micro-nutrient were also supplied at d 10, proportionally to N according to the following modified Hoagland solution (280 mL per pot): 10 mM $MgSO_4$, 1 mM Fe(Na)-EDTA, 20 µM KCl, 0,5 mM $H_3BO_3$, 40 µM $MnSO_4$, 40 µM $ZnSO_4$, 2 µM $CuSO_4$, 2 µM $(NH_4)_6Mo_7O_{24}$, 4 mM $CaSO_4$, 20 mM $K_2HPO_4$, 60 mM $K_2SO_4$. Before supply, the nutrient solution was buffered with MES (2-(N-morpholino)ethanesulfonic acid, 40 mM) and pH was corrected at 6.0±0.1 with HCl 4M. After d 10, no further nutrient was administered to the pots, with the exception of $CaSO_4$, since its low solubility made impossible to fulfill plant requirements with the initial dose at d 10. Therefore, $CaSO_4$ was supplied (at 4 mM per pot) over the growing period while watering (see next section), for a total of 1.524 g/plant.

It is worth noting that, all together, the ion strength of the nutrient solutions was extremely high (over 260 mM), especially immediately after the nutrient supply at d 10. While this posed issues related to osmotic stress, preliminary tests had showed that *B. napus* seedlings were capable to survive such stressing conditions. Therefore, although possibly misrepresenting physiological conditions during plant growth in nature/field, our approach, with most nutrient supply at the beginning of the growing period was the only choice allowing to monitor the labelling dynamics of plant tissues and DNA at medium term (120 d).

**Plant cultivation**

In the growing room, pots were randomly (i.e. independent of the labelling treatment) placed onto five trolleys. Trolley within the room and pots within each trolley were daily and weekly moved, respectively, to keep homogeneous exposure condition among replicates. Water loss by evapotranspiration was reintegrated by watering the pots every two days with milli-RO water. At d 15, pot thinning by uprooting the less developed seedling allowed to maintain a replicate for each treatment while avoiding possible confounding effects of within-pot intraspecific competition.

**Destructive sampling**

Starting at d 60 and every 15 days until d 120, 6 pots per treatment and 2 control pots were randomly selected and plants uprooted. Roots were gently washed with deionized water in order to remove sand particles and residues of nutrient solution. Afterwards, roots, stems (petioles) and leaf materials of each plant were separated.

Fresh plant materials (i.e. leaves, stems and roots) of each sampled pot were separately weighted. Afterwards, aliquots (i.e. 15 mm-diameter discs, 2 cm-long segments and a portion of the tip for each leaf blade, stem and root, respectively) were collected, fresh-weighted, dried in stove (24 hrs. at 60°C), dry-weighted, pulverized (TissueLyser II, Qiagen, Hilden, Germany) and kept in sterile plastic tubes for CHN-IRMS analyses. Residual, fresh plant materials (5 g each) were separately ground (Mill A11 basic, IKA, Saint Louis, Missouri, USA) in liquid nitrogen (T = -196 °C), placed in sterile 50 mL Falcon tubes and stored in freezer at -80 ° C for subsequent DNA extraction.

Since fresh plant materials are required for DNA extraction, the shoot: root ratio of each plant was determined as the ratio between the total dry leaf and stem biomass and the total dry root biomass of each sampled plant, with dry weights estimated based on the fresh weight: dry weight ratio of the aliquots.

**DNA extraction, purification and quantitation**

DNA extraction from fresh leaves and roots of each plant followed a modified version of the Doyle & Doyle protocol (Doyle & Doyle, 1987), as follows: a lysis buffer was prepared mixing 20 mL of CTAB (2.5 %), a spatula tip of PVP-40, 2 µl of Proteinase K (20 µg/µl) and 200 µl of β-mercaptoetanol (0,1%). The buffer solution was kept in agitation in a thermostatic bath at 65 °C (pbi, Braski, Bergamo, Italy), until PVP complete dissolution. For each source plant material, 20 mL of the buffer solution were added to the Falcon tube and the mixture was incubated at 65 °C for 30 min. and successively cooled in ice for 10 min. DNA purification was performed adding 20 mL of a mixture of chloroform : isoamyl alcohol (24:1) and gently shacking for 10 min. to homogenize. Falcon tubes containing the mixture were centrifuged at 6800 rpm at 4°C for 30 min, then gently pipetting out the aqueous supernatant fraction. Sodium acetate (1/10 starting volume, 3M), NaCl (3/10 starting volume, 4M) and pure Isopropyl alcohol (2/3 final volume) were added to the collected supernatant. The solution was incubated at -20°C for 30 min. and successively centrifuged as described above. As final step, after removing the supernatant and twice washing the pellet with 2 mL ethanol (80 %), the pellet was dried in a stove (10-15 min. at 37 °C) and re-suspended into an Eppendorf tube filled with 1.7 mL of sterile water for quantitation.

The concentration of extracted DNA in the resuspension medium was assessed by fluorimeter Qubit 3.0 (Life Technology, Carlsbad, California, USA). Finally, aliquots of 1.5 mL of each DNA sample were collected, frozen, lyophilized (55-4 Coolsafe, Scanvac, Allerød, Denmark) for 24 h (0.050 mbar, T = -57 °C) and kept in sterile plastic tubes at -20°C or subsequent CHN-IRMS analyses.

**CHN-IRMS analysis**

Dry, pulverized root and leaf samples as well as lyophilized DNA samples purified from the same materials were weighted at 2 ± 0.5 mg in cylindrical tin capsules (diameter 5 mm, height 9 mm)

(Säntis Analytical AG, Teufen, Switzerland) in duplicates. A total of 800 replicated samples (100 plants x 2 plant materials x 2 technical replicates x 2 N pools, i.e. total N and DNA) were processed by elemental analysis/isotope ratio mass spectrometry (EA/IRMS), using a Vario Micro Cube (Elementar GmbH, Langenselbold, Germany) elemental analyzer connected online in continuous flow mode to an IsoPrime 100 (Elementar UK Ltd, Cheadle Hulme, UK) isotope ratio mass spectrometer, using helium (He) as a carrier gas.

Flash combustion of all samples was conducted at 950 °C with a pulse of $O_2$ (30 ml/min for 70') into the He carrier gas in a quartz combustion column prepared following the manufacturer instructions. From bottom to top, the column was filled with: quartz wool (two layers, each of height 2.5 mm, separated by a 18 mm- thick layer of quartz chips), silver wool (25 mm), quartz wool (5 mm), CuO (65 mm), corundum balls (3 mm), an ash-finger tube with $Al_2O_3$ bottom, and a sheath tube. The combustion gas products ($CO_2$, $N_2$, $NO_x$ and $H_2O$) were passed through a reduction column at 500 °C to reduce the non-stoichiometric nitrous products to $N_2$ and to remove excess oxygen from the gas stream. The reduction column was prepared following the manufacturer instruction and filled with quartz wool at the bottom (5 mm height), elemental copper (295 mm), silver wool (20 mm). The plug of the reduction column was also filled with silver wool, to bind volatile halogen compounds contained in the combustion gas products. Reduced gases were then dried by passing through a 10 cm glass column filled with anhydrous SICAPENT® (Merk, Darmstadt, Germany), then passing into desorption columns to absorb the measurable components of the analysis gas mixture and then release each of them by controlling the desorption temperatures. Once released, the gases sequentially passed through a Thermal Conductivity Detector (TCD) and were vented out to the Isoprime diluter (Elementar UK Ltd, Cheadle Hulme, UK) for diluting $CO_2$ flow in the carrier helium flow at a rate of 100 ml/min before entering in the mass spectrometer. In parallel to this sample line, a second helium line is connected to the source of the mass spectrometer to carry the two

calibration gases ($CO_2$, $N_2$). Isotopic measurements and data processing were performed with the software IonVantage (Elementar UK Ltd, Cheadle Hulme, UK).

The nitrogen stable isotope composition in a given sample can be reported in the delta (δ) notation as variations of the molar ratio (R) of the heavy ($^{15}N$) to light isotope ($^{14}N$) in the sample relative to molecular nitrogen in air (Air-$N_2$) as international standard (Coplen et al., 2011). Accordingly, we used the following notation:

$$\delta^{15}N_{Air-N_2} = \frac{R(^{15}N/^{14}N)_{sample}}{R(^{15}N/^{14}N)_{Air-N_2}} - 1$$

The unit commonly used to express the delta value is permil (‰). However, the use of ‰ is debated as in conformity with the International System of Units (SI) and according to the guidelines and recommendations of the International Union of Pure and Applied Chemistry (IUPAC) - Commission on Isotopic Abundances and Atomic Weights (Brand et al., 2011; 2012), the unit of the delta values is the "urey" (symbol Ur). Therefore, we presented values of nitrogen isotopic composition with the unit notation mUr. However, as 1 mUr equals 1 ‰, for the sake of compliance with previous studies, we also presented isotopic composition values with the double unit notation of "mUr or ‰" limited to figures and tables, as often reported in the literature (e.g. Spangenberg et al., 2018; 2020).

Analytical results for nitrogen isotopic composition were calibrated using sulphanilamide (Elementar GmbH, Langenselbold, Germany, N = 16.26 %, C = 41.81%, 7 samples per batch) as a reference material. Analytical results for nitrogen isotopic composition were linearly corrected using the following international reference materials (International Atomic Energy Agency, Wien, Austria): IAEA-N1 (ammonium sulphate, $\delta^{15}N_{Air\text{-}N2}$ = 0.4 mUr, 4 samples per batch): IAEA-305A (ammonium sulphate, $\delta^{15}N_{Air\text{-}N2}$ = 39.8 mUr, 4 samples per batch), IAEA-310A (urea, $\delta^{15}N_{Air\text{-}N2}$ = 47.2 mUr, 2 samples per batch), USGS 26 (ammonium sulphate, $\delta^{15}N_{Air\text{-}N2}$ = 53.7 mUr, 2 samples per batch), IAEA-310B (urea, $\delta^{15}N_{Air\text{-}N2}$ = 244.6 mUr, 4 samples per batch), IAEA-305B (ammonium sulphate, $\delta^{15}N_{Air\text{-}N2}$ = 375.3 mUr, 4 samples per batch). Each analytic batch (120 positions) included 90 samples, 27

reference materials, 2 blanks consisting in empty tin capsules and 1 empty position. To avoid possible concerns of memory effects in the analytic results due to isotopically enriched samples, blanks were measured at the beginning of the batch and samples were sequentially placed in each batch according to the expected isotopic enrichment for different types of samples, thus minimizing the enrichment gaps between each sample type. Furthermore, duplicates of the same source sample were always placed in different batches to increase accuracy. The repeatability and intermediate precision of the EA/IRMS were determined by the standard deviation of separately replicated analyses and were better than 0.1 mUr.

**Data analysis**

All statistical analyses were performed using the software Statistica 7.0 (StatSoft inc, Tulsa, Oklahoma, USA). Generalized linear models (GLMs) were fitted for leaf, root and stem biomass and N content, considering main and interactive effects of the labelling treatment (three levels: $\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$, $\dot{N}O_3$), plant material (three levels: leaves, stems and roots) and plant age, the latter included in the model as a continuous covariate. For each plant material and age, significant differences between treatments and control plants were tested using Tuckey's HSD post-hoc test.

In order to compare $\delta\,^{15}N$ of purified DNA to that of the source plant materials, we calculated a Normalized Difference Index (NDI) for each replicate and treatment combination (i.e. labelling treatment, plant material and age), as follows:

$$NDI_{i,j,k,n} = \frac{\delta\,^{15}N(DNA)_{i,j,k,n} - \delta\,^{15}N(Source\ material)_{i,j,k,n}}{\delta\,^{15}N(DNA)_{i,j,k,n} + \delta\,^{15}N(Source\ material)_{i,j,k,n}}$$

where *i*, *j*, *k* and *n* indicates the labelling treatment, the type of source plant material (either leaf or root), plant age and the experimental replicate (individual plant), respectively. As such, NDI values range between -1 and 1, corresponding to unlabelled ($\delta\,^{15}N_{Air\text{-}N2}$ = 0 mUr) DNA and source plant

material, respectively, while NDI = 0 corresponds to equal δ $^{15}$N values of DNA and total N pool of the source plant material.

GLMs were fitted for δ $^{15}$N of DNA and source plant materials, and for NDI as well, considering main and interactive effects of the labelling treatment (three levels: $\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$, $\dot{N}O_3$), plant material (two levels: leaves, stems and roots) and plant age, the latter included in the model as a continuous covariate. For all GLMs, pair-wise significant differences between treatment combinations were evaluated by Tuckey's post-hoc HSD test at α = 0.05. Limited to NDI data, mean values of different experimental groups (i.e. unique combinations of plant material, age and labelling treatment) were also tested for significant difference from the reference zero value by one-sample *t* test at α = 0.01, thus reducing the conventional level of statistical significance of 0.05 in order to control for multiple comparisons. As such, significant negative and positive NDI mean values were interpreted as indicating $^{15}$N depletion and enrichment in DNA, respectively, compared to the total N pool of the source plant materials.

## 3.1.3. Results

### δ $^{15}$N dynamics in labelled plant leaves and roots

Labelling treatments did not affect plant growth (Fig 3.1.2, Table 3.1.2 and Table 3.1.4), percent N content (Fig 3.1.2, Table 3.1.4) and biomass allocation (Fig 3.1.3, Tables 3.1.5 and 3.1.6). Leaf, stem and root biomasses, as well as their percent N content, were not significantly different among labelling treatments and between them and the unlabeled control plants (Table 3.1.7), with the exception of leaf biomass at 120 d, which was significantly lower in plants of the three labelling treatments (Fig 3.1.2 and Table 3.1.7, Tuckey's HSD test: p-values < 0.0001 in all three pairwise treatment vs. control comparisons), possibly due to the interplay of individual variability and low

sample size of the control plants. Finally, labelling treatments did not affect shoot: root ratio (Fig 3.1.3., Tables 3.1.5 and 3.1.6).

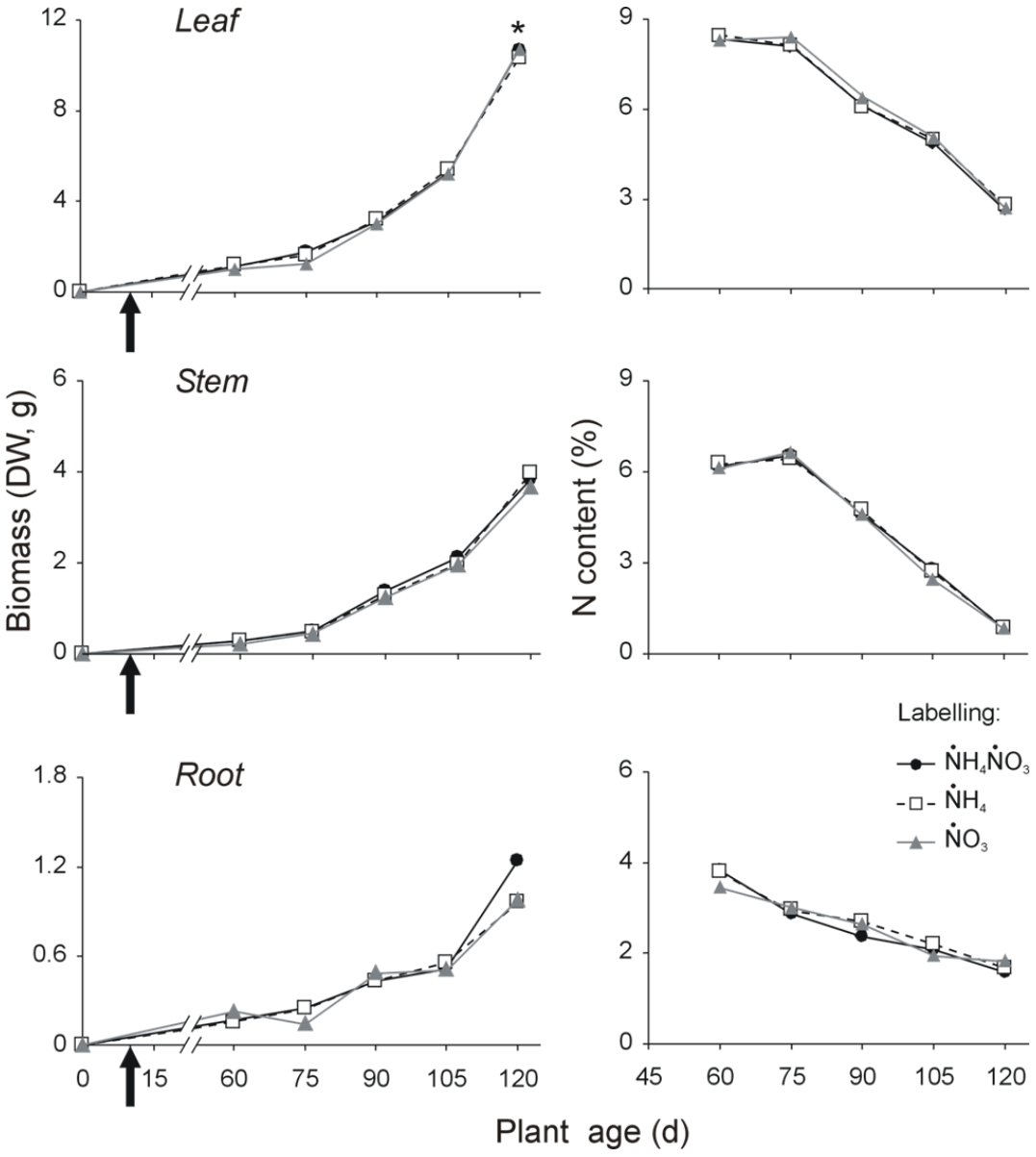


**Fig 3.1.2 -** Dynamics of dry biomass and percent N content in *B. napus* leaves, stems and roots of plants grown for 120 days in controlled conditions and fertilized with ammonium nitrate according to different N isotopic labelling treatments differing by the labelled chemical species ($\dot{N}H_4$, $\dot{N}O_3$, or both) but with the same isotopic ratio ($\delta^{15}N_{Air\text{-}N2}$ = 2100 mUr). Arrows on the left panels indicate the labelling administration date at plant age of 10 d. Data refer to mean of 6 plants for each treatment combination. Deviation bars are omitted to improve readability. Statistical support in 3.1.2, 3.1.3, 3.1.4.

**Table 3.1.2 - Results of GLM for *B. napus* biomass and N percent content.** GLMs include main and interactive effects of labelling treatment (L, three levels: $\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$, $\dot{N}O_3$), plant material (M, three levels: leaf, stem and root) and age (A, continuous covariate).

| Effect | DoF | SS | MS | F | *p* |
|---|---|---|---|---|---|
| ***Biomass*** | | | | | |
| Labelling treatment (L) | 2 | 0.462 | 0.231 | 0.244 | 0.7841 |
| Plant material (M) | 2 | 149.794 | 74.897 | 78.914 | < 0.0001 |
| Plant age (A) | 1 | 627.602 | 627.602 | 661.262 | < 0.0001 |
| L × M | 4 | 1.762 | 0.441 | 0.464 | 0.7620 |
| L × A | 2 | 0.254 | 0.127 | 0.134 | 0.8747 |
| M × A | 2 | 376.688 | 188.344 | 198.445 | < 0.0001 |
| L × M × A | 4 | 1.549 | 0.387 | 0.408 | 0.8027 |
| Error | 223 | 211.649 | 0.949 | | |
| ***N content (%)*** | | | | | |
| Labelling treatment (L) | 2 | 0.247 | 0.124 | 0.154 | 0.8572 |
| Plant material (M) | 2 | 239.095 | 119.547 | 148.999 | < 0.0001 |
| Plant age (A) | 1 | 645.382 | 645.382 | 804.378 | < 0.0001 |
| L × M | 4 | 0.174 | 0.044 | 0.054 | 0.9945 |
| L × A | 2 | 0.284 | 0.142 | 0.177 | 0.8378 |
| M × A | 2 | 110.420 | 55.210 | 68.812 | < 0.0001 |
| L × M × A | 4 | 0.187 | 0.047 | 0.058 | 0.9937 |
| Error | 234 | 187.747 | 0.802 | | |

**Table 3.1.3 - Result of Tuckey's post-hoc HSD testing for the interactive effect of plant age and labelling treatments ($\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$, $\dot{N}O_3$) on dry biomass of *B. napus* leaves, stems and roots.** Data refer to mean ± standard deviation of dry weight (g) of 6 plants for each treatment combination. Different letters indicate significantly different groups within each plant material ($P < 0.05$).

| Plant material | Plant age (days) | Dry biomass (g) | | |
|---|---|---|---|---|
| | | $\dot{N}H_4\dot{N}O_3$ | $\dot{N}H_4$ | $\dot{N}O_3$ |
| Leaf | 60 | 1.10 ± 0.20 *ab* | 1.20 ± 0.33 *abc* | 1.00 ± 0.05 *abc* |
| | 75 | 1.78 ± 0.32 *abc* | 1.61 ± 0.33 *abc* | 1.27 ± 0.09 *abc* |
| | 90 | 3.10 ± 1.34 *bcde* | 3.21 ± 1.54 *cdef* | 3.01 ± 1.16 *bcd* |
| | 105 | 5.27 ± 0.97 *f* | 5.41 ± 1.48 *ef* | 5.22 ± 1.62 *ef* |
| | 120 | 10.71 ± 3.68 *g* | 10.37 ± 2.88 *g* | 10.73 ± 0.85 *g* |
| Stem | 60 | 0.28 ± 0.05 *a* | 0.28 ± 0.07 *a* | 0.22 ± 0.02 *a* |
| | 75 | 0.49 ± 0.09 *a* | 0.48 ± 0.11 *a* | 0.44 ± 0.06 *a* |
| | 90 | 1.38 ± 0.59 *a* | 1.28 ± 0.78 *a* | 1.24 ± 0.53 *a* |
| | 105 | 2.11 ± 0.41 *ab* | 1.97 ± 0.49 *ab* | 1.94 ± 0.37 *ab* |
| | 120 | 3.84 ± 1.21 *bc* | 3.98 ± 1.48 *c* | 3.66 ± 0.22 *bc* |
| Root | 60 | 0.17 ± 0.07 *a* | 0.16 ± 0.05 *a* | 0.23 ± 0.12 *a* |
| | 75 | 0.25 ± 0.11 *a* | 0.25 ± 0.18 *a* | 0.15 ± 0.04 *a* |
| | 90 | 0.43 ± 0.21 *a* | 0.44 ± 0.27 *a* | 0.49 ± 0.22 *a* |
| | 105 | 0.51 ± 0.09 *a* | 0.56 ± 0.17 *a* | 0.51 ± 0.16 *a* |
| | 120 | 1.24 ± 0.52 *a* | 0.96 ± 0.29 *a* | 0.98 ± 0.36 *a* |

**Table 3.1.4 - Result of Tuckey's post-hoc HSD testing for the interactive effect of plant age and labelling treatments ($\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$, $\dot{N}O_3$) on percent N content in *B. napus* leaves, stems and roots.** Data refer to mean ± standard deviation of N content (%) of 6 plants for each treatment combination. Different letters indicate significantly different groups within each plant material ($P < 0.05$).

| Plant material | Plant age (days) | N content (%) | | |
|---|---|---|---|---|
| | | $\dot{N}H_4\dot{N}O_3$ | $\dot{N}H_4$ | $\dot{N}O_3$ |
| Leaf | 60 | 8.36 ± 0.44 *c* | 8.47 ± 0.4 *c* | 8.32 ± 0.53 *c* |
| | 75 | 8.09 ± 0.43 *c* | 8.15 ± 0.86 *c* | 8.43 ± 0.14 *c* |
| | 90 | 6.12 ± 1.51 *b* | 6.11 ± 1.32 | 6.41 ± 1.14 *bc* |
| | 105 | 4.85 ± 0.46 *b* | 4.98 ± 1.20 *b* | 5.06 ± 0.70 *b* |
| | 120 | 2.63 ± 1.11 *a* | 2.79 ± 0.90 *a* | 2.70 ± 0.26 *a* |
| Stem | 60 | 6.20 ± 1.06 *b* | 6.29 ± 0.76 *b* | 6.14 ± 0.68 *ab* |
| | 75 | 6.55 ± 0.24 *ab* | 6.44 ± 0.54 *ab* | 6.66 ± 0.62 *ab* |
| | 90 | 4.63 ± 1.57 *ab* | 4.75 ± 1.18 *ab* | 4.61 ± 1.07 *ab* |
| | 105 | 2.78 ± 0.59 *ab* | 2.71 ± 1.47 *ab* | 2.45 ± 0.13 *ab* |
| | 120 | 0.81 ± 0.46 *a* | 0.86 ± 0.59 *a* | 0.85 ± 0.14 *a* |
| Root | 60 | 3.81 ± 1.20 *cd* | 3.80 ± 0.71 *bcd* | 3.46 ± 1.69 *cd* |
| | 75 | 2.87 ± 0.60 *d* | 2.95 ± 0.79 *bcd* | 3.02 ± 0.63 *d* |
| | 90 | 2.37 ± 0.68 *bc* | 2.71 ± 0.83 *bcd* | 2.64 ± 0.53 *bc* |
| | 105 | 2.08 ± 0.22 *ab* | 2.20 ± 0.38 *a* | 1.96 ± 0.87 *ab* |
| | 120 | 1.57 ± 0.43 *a* | 1.68 ± 0.43 *a* | 1.84 ± 0.19 *a* |

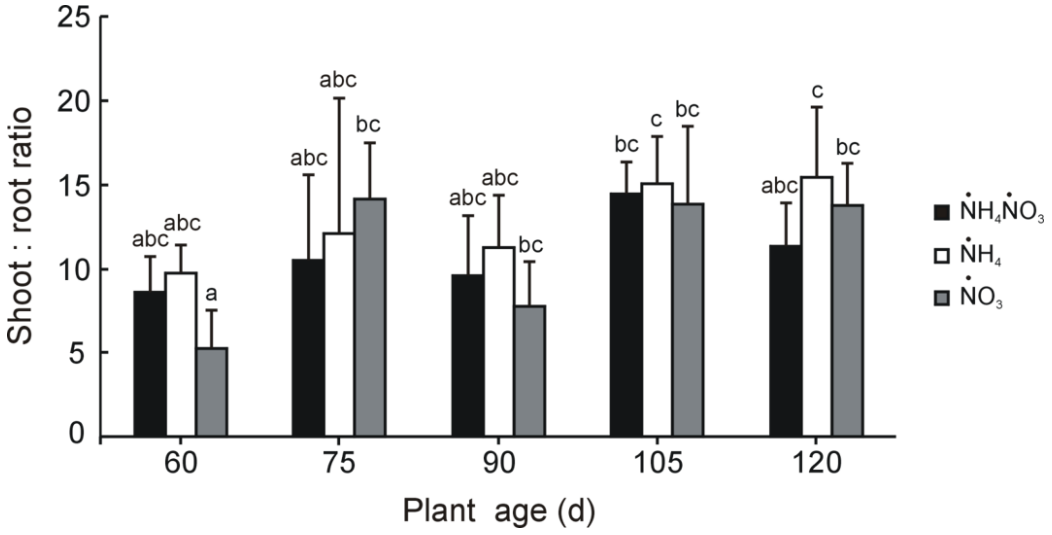


**Fig. 3.1.3 - Shoot : root ratio changes of in *B. napus* plants over the observation period, across the labelling treatments.** Different letters above bars indicate significant pair-wise labelling-dependent differences at equal plant age (Tuckey's post-hoc test after two-ways ANOVA, Tables 3.1.5 and 3.1.6).

**Table 3.1.5** Result of two-ways ANOVA testing for main and interactive effects of plant age and labelling treatment ($\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$, $\dot{N}O_3$) on the shoot : root ratio of *B. napus* plants.

| Effect | DoF | SS | MS | F | *p* |
|---|---|---|---|---|---|
| *Shoot/ root (S/R)* | | | | | |
| Plant age (A) | 4 | 547.94 | 136.99 | 10.9350 | < 0.0001 |
| Labelling treatment (L) | 2 | 63.61 | 31.80 | 2.5388 | 0.0857 |
| A x L | 8 | 132.66 | 16.58 | 1.3237 | 0.2450 |
| Error | 75 | 939.54 | 12.53 | | |

**Table 3.1.6 -** Result of Tuckey's post-hoc testing for the interactive effect of plant age and labelling treatment ($\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$, $\dot{N}O_3$) on the shoot : root ratio of *B. napus* plants.

| Plant age (days) | Labelling treatments | | |
|---|---|---|---|
| | **$\dot{N}H_4\dot{N}O_3$** | **$\dot{N}H_4$** | **$\dot{N}O_3$** |
| 60 | 8.6 ± 2.1 *abc* | 9.7 ± 1.6 *abc* | 5.3 ± 2.2 *a* |
| 75 | 10.5 ± 4.8 *abc* | 12.1 ± 7.6 *abc* | 14.2 ± 3.1 *bc* |
| 90 | 9.6 ± 3.4 *abc* | 11.3 ± 3 *abc* | 7.8 ± 2.5 *ab* |
| 105 | 14.5 ± 1.8 *bc* | 15 ± 2.7 *c* | 13.8 ± 4.4 *bc* |
| 120 | 11.3 ± 2.4 *abc* | 15.4 ± 4 *c* | 13.7 ± 2.4 *bc* |

**Table 3.1.7 -** Result of Tuckey's post-hoc testing for the interactive effect of plant age and labelling treatment (ṄH4ṄO3, ṄH4, ṄO3) on the shoot : root ratio of *B. napus* plants.

| Plant age (days) | Labelling treatments | | |
|---|---|---|---|
| | $\dot{N}H_4\dot{N}O_3$ | $\dot{N}H_4$ | $\dot{N}O_3$ |
| 60 | 8.6 ± 2.1 *abc* | 9.7 ± 1.6 *abc* | 5.3 ± 2.2 *a* |
| 75 | 10.5 ± 4.8 *abc* | 12.1 ± 7.6 *abc* | 14.2 ± 3.1 *bc* |
| 90 | 9.6 ± 3.4 *abc* | 11.3 ± 3 *abc* | 7.8 ± 2.5 *ab* |
| 105 | 14.5 ± 1.8 *bc* | 15 ± 2.7 *c* | 13.8 ± 4.4 *bc* |
| 120 | 11.3 ± 2.4 *abc* | 15.4 ± 4 *c* | 13.7 ± 2.4 *bc* |

δ $^{15}$N of leaves and roots largely varied with plant age and among labelling treatments (Fig 3.1.4), as indicated by the significant interaction term in the GLM model (Table 3.1.8). In leaves, initially (60 d) δ $^{15}$N did not show significantly different values among $\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$ and $\dot{N}O_3$ plants. At the second observation stage (75 d), a slight increase in $\dot{N}H_4$ and a decrease in $\dot{N}O_3$ plants were detected (Fig 3.1.4), but still not statistically significant compared to the previous stage in both treatments (Table 3.1.9). Since 75 d, δ $^{15}$N dynamics differed among the three treatments (Fig 3.1.4), with $\dot{N}H_4$ leaves showing a progressive decrease, down to -37.0% from 75 d to 120 d, while $\dot{N}O_3$ leaves showed the opposite pattern, increasing by 40.5% in the same time frame (Table 3.1.9). Differently, $\dot{N}H_4\dot{N}O_3$ leaves did not show significant δ $^{15}$N changes along the observation period (Table 3.1.9). Interestingly, after 120 d observation δ $^{15}$N in $\dot{N}O_3$ leaves significantly exceeded that of $\dot{N}H_4\dot{N}O_3$ ones by 31.1% and that of $\dot{N}H_4$ leaves by 61.5%, while the difference between the mean values of the two latter treatments was not statistically significant, with $\dot{N}H_4$ leaves showing high within-treatment variability (Table 3.1.9).

δ $^{15}$N dynamics in roots were qualitatively similar to those observed for leaves (Fig 3.1.4.). However, significant 2$^{nd}$ order interactive effects of the types of plant material and labelling treatment, and of the 3$^{rd}$ order interaction with plant age as well (Table 3.1.8), indicated that δ $^{15}$N values in plant roots, within each labelling treatment, followed quantitatively different dynamics

compared to leaves. In particular, significant differences among $\dot{N}H_4\dot{N}O_3$, $\dot{N}O_3$ and $\dot{N}H_4$ roots were observed since the beginning of the observation period (Table 3.1.9), with the latter treatment producing $\delta\ ^{15}N$ values exceeding those of $\dot{N}H_4\dot{N}O_3$ and $\dot{N}O_3$ roots by 49.0% and 88.8%, respectively. Such trend still held at 75 d, with corresponding percent differences of 51.1% and 94.0%, respectively.

**Table 3.1.8 - Results of GLM for δ $^{15}$N of plant materials and DNA extracted thereof.** GLMs include main and interactive effects of labelling treatment (L, three levels: $\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$ and $\dot{N}O_3$), plant material (M, two levels: leaf and root) and age (A, continuous covariate).

| Effect | DoF | SS | MS | F | *p* |
|---|---|---|---|---|---|
| ***δ $^{15}$N of plant materials*** | | | | | |
| Labelling treatment (L) | 2 | 17690704 | 8845352 | 124.538 | < 0.0001 |
| Plant material (M) | 1 | 132441 | 132441 | 1.865 | 0.1739 |
| Plant age (A) | 1 | 837738 | 837738 | 11.795 | 0.0007 |
| L × M | 2 | 1433748 | 716874 | 10.093 | < 0.0001 |
| L × A | 2 | 16619336 | 8309668 | 116.995 | < 0.0001 |
| M × A | 1 | 226831 | 226831 | 3.194 | 0.0758 |
| L × M × A | 2 | 649352 | 324676 | 4.571 | 0.0117 |
| Error | 168 | 11932294 | 71026 | | |
| ***δ $^{15}$N of DNA*** | | | | | |
| Labelling treatment (L) | 2 | 36223363 | 18111682 | 166.326 | < 0.0001 |
| Plant material (M) | 1 | 198035 | 198035 | 1.819 | 0.1793 |
| Plant age (A) | 1 | 4105552 | 4105552 | 37.703 | < 0.0001 |
| L × M | 2 | 3711058 | 1855529 | 17.040 | < 0.0001 |
| L × A | 2 | 27770355 | 13885178 | 127.513 | < 0.0001 |
| M × A | 1 | 382620 | 382620 | 3.514 | 0.0626 |
| L × M × A | 2 | 2607457 | 1303728 | 11.973 | < 0.0001 |
| Error | 168 | 18293926 | 108892 | | |

**Table 3.1.9 - Result of Tuckey's post-hoc HSD testing for the interactive effect of plant age and labelling treatments ($\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$, $\dot{N}O_3$) on N isotopic composition of *B. napus*.** Data refer to mean ± standard deviation of 6 plants for each treatment combination. Different letters indicate significantly different groups within each plant material (P < 0.05). Significantly different values between leaf and root within each combination of labelling treatment and plant age are indicated in bold.

| Plant material | Plant age (days) | N isotopic composition of plant materials (δ$^{15}$N/Air-$N_2$, mUr or ‰) | | |
|---|---|---|---|---|
| | | $\dot{N}H_4\dot{N}O_3$ | $\dot{N}H_4$ | $\dot{N}O_3$ |
| Leaf | 60 | 2213.4 ± 61.5 *abc* | **2587.6 ± 177.5 *cde*** | **2291.9 ± 262.9 *abcd*** |
| | 75 | 2186.8 ± 35.5 *abc* | 2801.8 ± 181.2 *de* | 2028.9 ± 169.0 *ab* |
| | 90 | 2233.0 ± 25.6 *abc* | 2236.4 ± 367.1 *abc* | 2380.5 ± 400.9 *bcde* |
| | 105 | 2242.3 ± 32.6 *abc* | 2002.7 ± 183.3 *ab* | 2681.1 ± 259.8 *cde* |
| | 120 | 2175.5 ± 41.2 *abc* | 1764.7 ± 360.3 *a* | 2851.3 ± 201.2 *e* |
| Root | 60 | 2193.3 ± 69.5 *bcde* | **3267.4 ± 139.8 *f*** | **1730.4 ± 255.9 *ab*** |
| | 75 | 2098.1 ± 54.0 *abcde* | 3170.8 ± 427.3 *f* | 1634.4 ± 161.1 *a* |
| | 90 | 2174.6 ± 24.1 *abcde* | 2549.4 ± 437.9 *e* | 2143.6 ± 455.0 *abcde* |
| | 105 | 2131.8 ± 36.7 *abcde* | 1957.5 ± 212.7 *abcd* | 2315.2 ± 384.4 *cde* |
| | 120 | 2054.7 ± 23.8 *abcde* | 1824.9 ± 501.4 *abc* | 2460.7 ± 212.8 *de* |

Thereafter, from 90 d to 120 d, root δ $^{15}$N dynamics were apparently similar to those observed in leaves (Fig 3.1.4), although $\dot{N}O_3$ roots did neither show statistically significant age-dependent variations, nor significant differences compared to the other treatments within each observation stage (Table 3.1.9). The only exception to such pattern was the significantly higher δ $^{15}$N values at 120 d in $\dot{N}O_3$ vs. $\dot{N}H_4$ roots (+34.8%), resulting from the decreasing age-dependent trend observed in this latter treatment (-42.4% from 75 d to 120 d). As observed in leaves, $\dot{N}H_4\dot{N}O_3$ roots did not show significant δ $^{15}$N changes along the observation period (Table 3.1.9). Finally, δ $^{15}$N differences between root and leaf materials within each labelling treatment at each observation stage were not statistically significant with the exception of $\dot{N}H_4$ and $\dot{N}O_3$ plants at 60 d, showing higher and lower values in roots, respectively (Table 3.1.9).

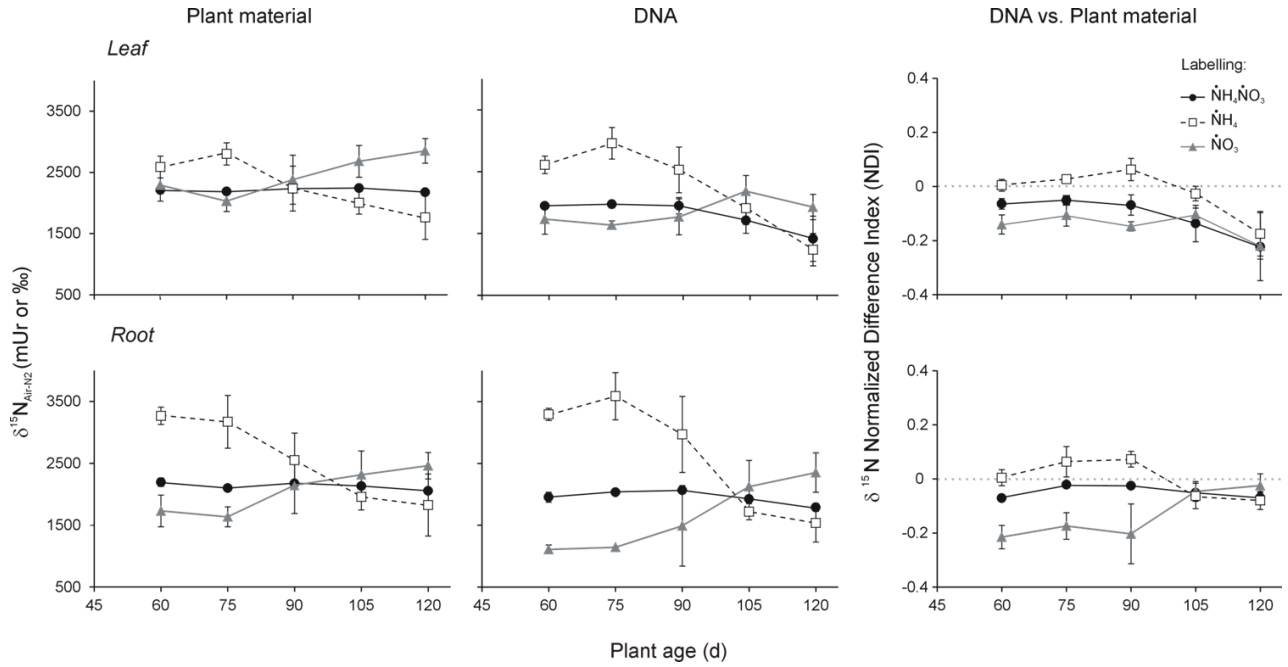


**Fig 3.1.4. Dynamics of N isotopic composition in *B. napus* leaves and roots (left) and DNA samples extracted therefrom (center) across the labelling treatments.** Right panels show the corresponding δ $^{15}$N Normalized Difference Index (NDI) dynamics. Data refer to mean of 6 plants for each treatment combination. Statistical support in Tables 3.1.8, 3.1.9, 3.1.10, 3.1.11 and 3.1.12

**δ $^{15}$N dynamics in plant DNA**

δ $^{15}$N of DNA purified from leaves and roots generally followed a similar pattern as compared to that observed in the source plant materials (Fig 3.1.4), resulting from the interaction of plant age and labelling treatment effects (Table 3.1.8), although with peculiar and interesting shifts along the observation period. In particular, in the case of leaf DNA, δ $^{15}$N of $\dot{N}H_4$ DNA was consistently higher compared to the other two treatments throughout the first 90 days of observation (Table 3.1.10), exceeding that of $\dot{N}H_4\dot{N}O_3$ DNA by 34%, 49.9%, and 29.8% at 60, 75 and 90 d, respectively, and that of $\dot{N}O_3$ DNA by 50.6%, 80.9% and 43%, respectively. Interestingly, such differences were released at 105 d, with δ $^{15}$N of DNA from all treatments showing converging dynamics up to that point, with a shift in time in comparison to what was observed for the δ $^{15}$N of the source plant materials (Fig 3.1.4). Thereafter, at 105 d and 120 d, δ $^{15}$N of DNA apparently decreased in all treatments, although such trend was statistically significant limited to $\dot{N}H_4$ plants (Table 3.1.10).

Different from leaf DNA, δ $^{15}$N dynamics in root DNA were much more similar to those observed for the source plant materials, with no significant within-treatment variation between 60 and 90 d, and $\dot{N}H_4$ DNA always showing higher levels compared to the other two treatments in the same time frame. In particular, δ $^{15}$N of $\dot{N}H_4$ DNA exceeded that of $\dot{N}H_4\dot{N}O_3$ DNA by 68.6%, 76.5%, and 43.7% at 60, 75 and 90 d, respectively, and that of $\dot{N}O_3$ DNA by 196.7%, 213.4% and 99.1%, respectively, corresponding to a larger magnitude of between-treatment variation compared to the source plant materials (Fig 3.1.4). From 90 d to 105 d, the δ $^{15}$N of $\dot{N}H_4$ DNA and $\dot{N}O_3$ DNA showed abrupt shifts corresponding to significant decrease and increase, respectively, while δ $^{15}$N did not significantly vary in $\dot{N}H_4\dot{N}O_3$ DNA (Fig 3.1.4, Table 3.1.10). Such trends were released at the final observation stage (120 d), as none of the three labelling treatments produced significant variation compared to the preceding stage (105 d) (Fig 3.1.4, Table 3.1.10).

**Table 3.1.10. Result of Tuckey's post-hoc HSD testing for the interactive effect of plant age and labelling treatments ($\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$, $\dot{N}O_3$) on δ $^{15}$N of *B. napus* leaf and root DNA.** Data refer to δ $^{15}$N mean ± standard deviation of 6 plants for each treatment combination. Different letters indicate significantly different groups within each plant material ($P < 0.05$). Significantly different values between leaf and root within each combination of labelling treatment and plant age are indicated in bold (*: DNA purified from root materials was pooled in order to provide the minimum sample amount for IRMS analysis).

| Plant material | Plant age (days) | N isotopic composition of DNA (δ$^{15}$N/Air-$N_2$, mUr or ‰) | | |
|---|---|---|---|---|
| | | $\dot{N}H_4\dot{N}O_3$ | $\dot{N}H_4$ | $\dot{N}O_3$ |
| Leaf | 60 | 1946.4 ± 45.1 *bc* | **2609.1 ± 140.9 *ef*** | **1732.8 ± 244.0 *abc*** |
| | 75 | 1974.7 ± 58.8 *bcd* | **2960.3 ± 255.3 *fg*** | 1636.3 ± 66.1 *abc* |
| | 90 | 1949.6 ± 132.0 *bc* | 2530.0 ± 369.6 *def* | 1768.9 ± 290.2 *abc* |
| | 105 | 1711.4 ± 208.5 *abc* | 1908.6 ± 264.1 *bc* | 2184.7 ± 253.6 *cde* |
| | 120 | 1415.3 ± 365.2 *ab* | 1238.7 ± 262.7 *a* | 1926.7 ± 204.7 *bc* |
| Root | 60 | 1954.6 ± 80.6 *bcde* | **3294.9 ± 98.8 *gh*** | **1110.5 ± 71.4 *a*** |
| | 75 | 2033.2 ± 46.0 *bcde* | **3587.9 ± 381.5 *h*** | 1144.7 ± 0.0*, *a* |
| | 90 | 2065.9 ± 47.4 *cde* | 2968.8 ± 615.3 *fg* | 1491.4 ± 650.7 *ab* |
| | 105 | 1924.8 ± 44.0 *bcde* | 1719.5 ± 131.9 *bcd* | 2125.7 ± 422.3 *de* |
| | 120 | 1789.2 ± 64.0 *bcde* | 1537.3 ± 307.9 *abc* | 2353.1 ± 317.1 *e* |

Similar to what observed for the total N pools of plant materials, δ $^{15}$N differences between root and leaf DNA within each labelling treatment at each observation stage were not statistically significant, with the exceptions of $\dot{N}O_3$ DNA at 60 d and $\dot{N}H_4$ DNA at 60 and 75 d, showing lower and higher values in roots, respectively (Table 3.1.10).

### δ $^{15}$N NDI dynamics: DNA vs total N pool of the source plant materials

A comparative analysis of δ $^{15}$N dynamics in DNA samples and in the corresponding source materials can be better clarified by observing δ $^{15}$N NDI patterns (Fig 3.1.4), which significantly changed in relation to the labelling treatment, plant material, age and their interactions (Table 3.1.11). In the case of leaves, δ $^{15}$N NDI values were consistently negative throughout the observation period for

$\dot{N}H_4\dot{N}O_3$ and $\dot{N}O_3$ plants, indicating that leaf DNA was always depleted in $^{15}N$ compared to the source plant material (Table 3.1.12). Such trend was found in $\dot{N}H_4$ leaves only at the final observation stage (120 d), while at the preceding stages δ $^{15}N$ NDI values did not significantly differ from the reference zero value, thus indicating that δ $^{15}N$ of $\dot{N}H_4$ DNA did not differ from that of the source plant material (Table 3.1.12). Correspondingly, δ $^{15}N$ NDI dynamics within each treatment did not show significant fluctuations up to the third or fourth observation stage (90 d or 105 d). Then, δ $^{15}N$ NDI significantly decreased, with negative values consistently observed at 120 d in all treatments, indicating that $^{15}N$ depletion in DNA at the final observation stage was independent of the labelling treatment

δ $^{15}N$ NDI dynamics in plant roots showed substantially the same pattern observed for leaves up to the first 90 d (Fig 3.1.4), with the only exception of $\dot{N}H_4$ roots showing a significant positive value at 90 d (Table 3.1.12). Differently, at the final observation stages (105 d and 120 d) δ $^{15}N$ NDI dynamics in plant roots showed a significantly different pattern compared to that observed for plant leaves, particularly in the cases of $\dot{N}H_4\dot{N}O_3$ and $\dot{N}O_3$ roots (Table 3.1.12). Indeed, $\dot{N}H_4\dot{N}O_3$ roots persistently showed negative δ $^{15}N$ NDI values, but without the decreasing trend observed in $\dot{N}H_4\dot{N}O_3$ leaves (Fig 3). $\dot{N}O_3$ roots showed an increasing trend (Fig 3.1.4), leading to δ $^{15}N$ NDI values non-significantly different form the reference zero value (Table 3.1.12). $\dot{N}H_4$ roots showed substantially the same pattern observed for $\dot{N}H_4$ leaves (Fig 3.1.4), with a significant decrease from 90 to 120 d corresponding to negative δ $^{15}N$ NDI value at the final stage (Table 3.1.12) indicating $^{15}N$ depletion in DNA.

Considering δ $^{15}N$ NDI values in roots and leaves within each treatment and observation stage, we found the only significant difference at the end of the observation period (120 d), with $\dot{N}H_4\dot{N}O_3$ and $\dot{N}O_3$ leaves showing lower values compared to the corresponding root materials (Table 3.1.12), thus indicating that $^{15}N$ depletion in DNA compared to the total N pool was larger in leaves than in roots.

**Table 3.1.11 - Results of GLM for δ $^{15}$N NDI.** GLM includes main and interactive effects of labelling treatment (L, three levels: $\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$ and $\dot{N}O_3$), plant material (M, two levels: leaf and root) and age (A, continuous covariate).

| Effect | DoF | SS | MS | F | *P* |
|---|---|---|---|---|---|
| Labelling treatment (L) | 2 | 0.322 | 0.161 | 43.443 | < 0.0001 |
| Plant material (M) | 1 | 0.091 | 0.091 | 24.483 | < 0.0001 |
| Plant age (A) | 1 | 0.057 | 0.057 | 15.469 | 0.0001 |
| L × M | 2 | 0.044 | 0.022 | 5.922 | 0.0033 |
| L × A | 2 | 0.182 | 0.091 | 24.531 | < 0.0001 |
| M × A | 1 | 0.127 | 0.127 | 34.362 | < 0.0001 |
| L × M × A | 2 | 0.042 | 0.021 | 5.614 | 0.0044 |
| Error | 166 | 0.615 | 0.004 | | |

**Table 3.1.12 - Result of Tuckey's post-hoc HSD testing for the interactive effect of plant age and labelling treatments ($\dot{N}H_4\dot{N}O_3$, $\dot{N}H_4$, $\dot{N}O_3$) on δ $^{15}$N NDI.** δ $^{15}$N NDI indicates differences of isotopic composition between leaf or root DNA and the total N pool of the source plant material. Data refer to δ $^{15}$N NDI mean ± standard deviation of 6 plants for each treatment combination. Different letters indicate significantly different groups within each plant material (P < 0.05). Mean values significantly different from zero, as assessed by one sample t-tests at P < 0.01, are marked with an asterisk (*). Significantly different values between leaf and root within each combination of labelling treatment and plant age are indicated in bold.

| Plant material | Plant age (days) | δ $^{15}$N Normalized Difference Index (NDI) | | |
|---|---|---|---|---|
| | | $\dot{N}H_4\dot{N}O_3$ | $\dot{N}H_4$ | $\dot{N}O_3$ |
| Leaf | 60 | -0.0641 ± 0.020*, *cde* | 0.0045 ± 0.022 *ef* | -0.1404 ± 0.035*, *abc* |
| | 75 | -0.0511 ± 0.016*, *cde* | 0.0253 ± 0.016 *ef* | -0.1059 ± 0.041*, *bcd* |
| | 90 | -0.0686 ± 0.038*, *cde* | 0.0627 ± 0.041 *f* | -0.1471 ± 0.017*, *abc* |
| | 105 | -0.1368 ± 0.067*, *abc* | -0.0264 ± 0.027 *def* | -0.1030 ± 0.023*, *bcd* |
| | 120 | **-0.2226 ± 0.126***, *a* | -0.1747 ± 0.083*, *ab* | **-0.2195 ± 0.046***, *a* |
| Root | 60 | -0.0711 ± 0.013*, *c* | 0.0044 ± 0.030 *cd* | -0.2151 ± 0.044*, *a* |
| | 75 | -0.0216 ± 0.012*, *cd* | 0.0635 ± 0.056 *d* | -0.1739 ± 0.049*, *ab* |
| | 90 | -0.0257 ± 0.010*, *cd* | 0.0729 ± 0.029*, *d* | -0.2033 ± 0.111*, *a* |
| | 105 | -0.0511 ± 0.012*, *c* | -0.0632 ± 0.047 *c* | -0.0462 ± 0.036 *c* |
| | 120 | **-0.0693 ± 0.018***, *c* | -0.0798 ± 0.033*, *bc* | **-0.0248 ± 0.044** *cd* |

## 3.1.4. Discussion

**Effects of N uptake on δ $^{15}$N dynamics in leaf and root**

We found that the isotopic composition of plant roots and leaves largely varied along the vegetative growth period, with early-to-medium dynamics corresponding to $^{15}$N enrichment and depletion in $\dot{N}H_4$ and $\dot{N}O_3$ plants, respectively, and with an opposite pattern at later stages. Such trend, more evident in roots compared to leaves, was independent of labelling treatments effects on plant growth and N content and biomass allocation dynamics. The substantially specular dynamics of δ $^{15}$N in $\dot{N}H_4$ vs. $\dot{N}O_3$ plants, clearly indicated that uptake fluxes of the two N chemical species were decoupled over time, with plants mostly using $NH_4^+$ up to an age of 90 days and $NO_3^-$ afterwards. This is consistent with the expected outcomes, since the relative abundance and the isotopic composition of different chemical species in the substrate are the most controlling factors of plant δ $^{15}$N dynamics (Evans et al., 1996). In this respect, it is worth noting that our experimental design is novel as compared to previous studies, where a single labelled N species and single harvesting shortly after the labelling treatment were used (Bergersen et al., 1988; Yoneyama et al., 1991; Evans et al., 1996). Differently, by adopting a factorial combination of different labelled N sources and harvesting plants over a prolonged observation period, we assessed the relative importance of uptake fluxes by comparing enrichment dynamics between $\dot{N}H_4$ and $\dot{N}O_3$ plants. Additionally, we could evaluate the associated isotopic fractioning by monitoring labelling dynamics in $\dot{N}H_4\dot{N}O_3$. As such, our results clearly confirm that the effects of the N source largely overcome that of isotopic discrimination during N uptake in controlling plant δ $^{15}$N dynamics.

Consistently, δ $^{15}$N did not significantly change over time in $\dot{N}H_4\dot{N}O_3$ roots and leaves, as not affected by the uptake dynamics of the two labelled N chemical species. Such finding may be surprising, as at least a slight increase over time in δ $^{15}$N of $\dot{N}H_4\dot{N}O_3$ plants, and particularly of their roots, would be expected as a result of isotopic discrimination associated to $NH_4^+$ and/or $NO_3^-$

uptake (Evans et al., 2001), due to a progressive enrichment in $^{15}N$ in the pot solution and hence in plant roots. The magnitude of isotopic discrimination (ε) associated to N uptake was previously estimated both for $NO_3^-$ (Kohl et al., 1980; Mariotti et al., 1982; Bergersen et al., 1988) and $NH_4^+$ (Evans et al., 1996; Yoneyama et al., 2001) Reported values ranges between 0 (i.e. absence of discrimination) and -12,6 mUr (Yoneyama et al., 1991; Evans et al., 1996) in the case of $NH_4^+$, and between 0 and -9,6 mUr (Kohl et al., 1980; Bergersen et al., 1988; Evans et al., 1996) for the uptake of $NO_3^-$. Such variability could be likely related to different experimental conditions considered in those previous studies. Among these, the target species and/or cultivar (Craine et al., 1986) and plant age (Evans et al., 2001) may play a major role, as well as N concentration in the substrate, which at low values triggers active uptake transporters (Haynes et al., 1986; Yoneyama et al., 1991) and positively affects the magnitude of isotopic discrimination. Therefore, it is not surprising that we could not detect the occurrence of isotopic fractioning associated to N uptake, considering that we started to monitor δ $^{15}N$ values in N-rich plants of 60 d.

**Effects of N assimilation on δ $^{15}N$ dynamics in leaf and root**

We expected isotopic enrichment in $\dot{N}H_4$ roots compared to leaves and in $\dot{N}O_3$ leaves compared to roots at each observation stage, and intermediate values in $\dot{N}H_4\dot{N}O_3$ materials, due to differences in assimilation rates of the two inorganic N chemical species in the two plant organs.

$NO_3^-$ is readily assimilated in the roots after uptake, being firstly reduced to $NO_2^-$ by the Nitrate Reductase enzymatic complex and then to $NH_4^+$ by the Ferrodoxin-Nitrite reductase (Haynes et al., 1986). The first enzyme discriminates the heavy isotopic form (Yoneyama et al., 1989; Evans et al., 2001), with a generally accepted fractionation value of –16 mUr (Tcherkez et al., 2006; Cui et al., 2020). Then, the $NO_3^-$ available for assimilation in leaves, after xylematic transport, is enriched in $^{15}N$ compared to that assimilated at root level (Evans et al., 2001). Our results confirmed the

expected pattern only for the first observation stage (60 d). This apparently contrasts with the enhanced content of assimilation enzymes at root level (Zanin et al., 2015), which is expected to result in progressive enrichment of unassimilated $NO_3^-$ that is then transported to the leaves. However, our results can be easily explained considering the remarkable N availability in the pot solution and the consequent low $NO_3^-$ assimilation flux in the roots. In such conditions, biomass and N allocation were extremely unbalanced between roots and leaves. For instance, at a plant age of 75 d, mean N content values in $\dot{N}O_3$ leaves and roots were 111.8 mg and 3.9 mg, respectively, while the same values at 90 d were 177.4 mg and 12.3 mg. This corresponded to 65.6 mg N allocated to the leaves, while the net N allocation to the roots in the same time frame was one order of magnitude lower, equal to only 8.4 mg. Therefore, $^{15}N$ enrichment in the leaves due to fractioning effects of $NO_3^-$ assimilation in the roots (Evans et al., 2001) was likely lower than expected, as related to the low $NO_3^-$ assimilation flux in the roots. In addition, a $^{15}N$ enrichment in roots due to the backflow of nitrate to roots via the phloem, as recently suggested by a modelling work by (Cui et al., 2020), cannot be excluded.

In the case of $NH_4^+$ assimilation, N is firstly incorporated into organic molecules such as Glutamine and Glutamate in the roots (Haynes, et al., 1986; Yoneyama et al., 2016) through a three-stages assimilation process mediated by the GS-GOGAT enzymatic complex. Since the first stage of such process, mediated by the Glutamine Synthetase enzyme, discriminates the heavy N isotopic form [e.g. Yoneyama et al., 1993; Kalcsits et al., 2014), the organic products of the $NH_4^+$ assimilation transported to the leaves are depleted in $^{15}N$. Therefore, $\delta\ ^{15}N$ in $\dot{N}H_4$ roots is expected to be higher than in $\dot{N}H_4$ leaves. Our results for $\dot{N}H_4$ plants were consistent with such expectation only at the first observation stage (60 d). Interestingly, at later stages, we found large within-group variability, particularly at 90 d. At this stage, $\delta\ ^{15}N$ also did not differ among $\dot{N}H_4$ and $\dot{N}O_3$ materials, indicating that plant materials had acquired the same amount of $NH_4^+$ and $NO_3^-$, irrespective of the labelling

treatment. Since at this stage slight variations in the uptake rates of either N source produces large δ $^{15}$N variations, the high within-group variability of δ $^{15}$N values, which prevented from detecting significant root vs. leaf differences, could be ascribed to a certain asynchrony in labelling dynamics among replicates within each treatment. Finally, a possible role of ammonia volatilization, at least for some replicates, and its effect on δ $^{15}$N dynamics cannot be excluded (Kalcsits et al., 2014). It is well known that ammonia volatilization rates increase with temperature and with leaf N content, particularly during senescence (Schjoerring et al., 2001; Johnson et al., 2013). In our experiment, mild temperatures and absence of senescence processes likely contributed to limit ammonia volatilization. On the other hand, the remarkable percent N content in plant leaves up to the end of the observation period, might have enhanced N loss by volatilization.

**$^{15}$N depletion dynamics in plant DNA**

Following Gauthier et al. (2013), we hypothesized that isotopic fractioning along the purine and/or pyrimidine biosynthesis pathways leads to a depletion of $^{15}$N in plant DNA, hence expecting lower δ $^{15}$N values in leaf and root DNA samples compared to those of the source plant materials.

Dynamics of δ $^{15}$N values in DNA samples, decreasing and increasing in $\dot{N}O_3$ and $\dot{N}H_4$ treatments, respectively, were substantially consistent to those of the source materials up to 105 d. Then, at the final observation period, δ $^{15}$N dynamics in all DNA samples were completely different from that of the source materials, with a consistent decrease in most cases. Dynamics of δ $^{15}$N NDI provided a clue to explain such pattern, consistently showing negative values for $\dot{N}H_4\dot{N}O_3$ and $\dot{N}O_3$ (i.e. $^{15}$N depletion in DNA samples compared to the total N pool of the source plant materials) for both leaves and roots. For such treatments, our findings fully support the occurrence of isotopic fractioning along the purine and/or pyrimidine biosynthesis pathways (Gauthier et al., 2013). On the other hand, δ $^{15}$N NDI dynamics observed in the case of $\dot{N}H_4$ leaves and roots, showed values not

significantly different from zero (i.e. equal δ $^{15}$N in DNA samples and the source plant materials up to 105 d), and even a positive value for roots at 90d. Such result may be explained considering the interplay of isotopic fractioning during DNA biosynthesis, N uptake and assimilation in the three labelling treatments. Up to 90 d, the negative δ $^{15}$N NDI values of $\dot{N}H_4\dot{N}O_3$ plants, as not affected by the labelled N species, relied only on $^{15}$N depletion in DNA. Differently, δ $^{15}$N NDI values were significantly different between $\dot{N}O_3$ and $\dot{N}H_4$ plants, for both leaves and roots. This indicates that the progressive isotopic enrichment and depletion in $\dot{N}O_3$ and $\dot{N}H_4$ leaves and roots, respectively, were exacerbated in DNA samples compared to the source materials. Moreover, the increase and decrease of δ $^{15}$N values in $\dot{N}O_3$ and $\dot{N}H_4$ DNA samples, respectively, were delayed compared to the corresponding plant materials. This may be attributed to a temporal decoupling of N incorporation in purine and pyrimidine precursors with respect to nucleotide assemblage into DNA molecules, with $^{15}$N signature of DNA samples at a given stage reflecting that of the source material at previous stages. Accordingly, in the case of $\dot{N}H_4$ DNA samples, the effect of isotopic fractioning in purine and pyrimidine biosynthesis (Gauthier et al., 2013) might have been masked by labelling dynamics of the source materials.

After 90 d, δ $^{15}$N NDI values were consistently negative, and mostly decreasing, for leaves of all treatments, confirming the occurrence of $^{15}$N fractioning during leaf DNA biosynthesis (Gauthier et al., 2013). The results for root materials was less straightforward, for different possible reasons. First, root growth rate between 105 and 120 d was one order of magnitude smaller compared to leaves (mean and standard deviation of all treatments was $k_B$ = 0.036 ± 0.011 $g_{DW}$ $d^{-1}$ and $k_B$ = 0.353 ± 0.020 $g_{DW}$ $d^{-1}$ for roots and leaves, respectively). In these conditions, DNA biosynthesis rate was higher in leaves, compared to roots and to the preceding stages, hence magnifying the effect of isotopic fractioning associated to purine and pyrimidine biosynthesis. Second, in the same observation period (between 105 and 120 d) mean daily increases of N mass in leaves ($k_N$ = 0.58 ±

0.1 $mg_N$ $d^{-1}$) and roots ($k_N$ = 0.18 ± 0.03 $mg_N$ $d^{-1}$) showed more similar magnitude as compared to the corresponding $k_B$ values. Hence, at this stage, the utilization efficiency (Moll et al., 1982) of newly up taken N (i.e. $k_B$ / $k_N$) in the leaves (i.e. 0.222 $g_{DW}$ $mg_N^{-1}$) was larger compared to the roots (0.077 $g_{DW}$ $mg_N^{-1}$) and to the preceding stages (i.e. 0.012, 0.013, 0.024 and 0.031 $g_{DW}$ $mg_N^{-1}$ at 60, 75, 90 and 105 d, respectively), with DNA bases biosynthesis likely relying more on this N pool rather than that previously taken up but not used and stored in reserve pools such as vegetative proteins (Rossato et al., 2001). Finally, we cannot exclude a possible and decisive role of N translocation from leaves to roots (Yoneyama et al., 2016), which however should have played the major role at the initial stages of observation, when percent N content in leaves was far higher than physiological values commonly reported (e.g. Rossato et al., 2001).

### 3.1.5. Conclusions

In this study we confirmed previous evidence on the effect of the labelled chemical species on leaf and root δ $^{15}N$ dynamics. Under the tested conditions, higher uptake rate of $NH_4^+$ and its limiting effect on the uptake of $NO_3^-$ were the main causal factors of the observed outcomes, with $\dot{N}H_4$, $\dot{N}O_3$ and $\dot{N}H_4\dot{N}O_3$ plants initially showing higher, lower and intermediate δ $^{15}N$ values, respectively, then progressing towards the opposite trend when $NH_4^+$ depletion from the nutrient solution corresponded to increasing $NO_3^-$ uptake rate.

Although it is well known that isotopic fractionation during inorganic N uptake, associated to $^{15}N$ enrichment of the N pool residual in the substrate solution, results in progressive isotopic enrichment of plant tissues, our study did not provide conclusive results, even in the case of $\dot{N}H_4\dot{N}O_3$ plants, unaffected by uptake rates of the two chemical species. However, possibly unsuitable

experimental conditions, in terms of excessive N availability, might have hampered active inorganic N uptake mechanisms, decisively affecting our observations.

Evidence from previous studies on leaf vs. root isotopic enrichment due to enzymatic fractionation during inorganic N assimilation were only partially confirmed, limited to $\dot{N}O_3$ plants at the early observation stages. At later stages and for $\dot{N}H_4^+$ plants, the predominant effects of $NH_4^+$ and $NO_3^-$ uptake rates in the tested conditions, as well as the reduced root development and the extremely high leaf N content, with the associated possible confounding effects of nitrate phloematic backflow and ammonia volatilization, likely masked the expected outcome.

Considering the hypothesis of $^{15}N$ depletion in DNA compared to the source plant materials, possibly due to enzymatic discrimination during purine biosynthesis, our findings provide confirmatory evidence. However, we did not provide a direct evidence of $\delta\ ^{15}N$ variation between molecular products such as nuclei acids and their precursors according to known biochemical pathways. Indeed, addressing such issue would require more detailed characterization of the involved N molecular pools and additional experiments to accurately estimate the fractionation coefficient of each enzymatic step during DNA biosynthesis. However, as an added value of our original experimental design, we were able for the first time to specifically report about the dynamics of specific plant molecular pools, such as leaf and root DNA, over a long observation period.

## 3.2. Semiautomatic fertirrigation system for $^{15}N$ labelling beech DNA

### 3.2.1 Introduction

Plant residue is the main source of organic matter for microorganisms in forest ecosystems. During decomposition a wide range of chemical compounds is released from plant litter, like nutrients which can be beneficial (Staaf, 1980) or detrimental to subsequent generations of plants (Facelli & Pickett, 1991) or secondary metabolites or DNA from decomposing that can cause litter-mediated negative plant-soil feedback (Mazzoleni et al., 2015; Veen et al., 2019a). The DNA released from litter decomposition (review in Levy-Booth et al., 2007; Pietramellara et al., 2009) can persist by binding to soil minerals and humic substances (Crecchio & Stotzky, 1998), be degraded by microbial DNases and used as a nutrient for plant and microbial growth (Paget et al., 1992; Macfadyen et al., 2001; Ceccherini et al., 2003), and/or be incorporated into a bacterial genome as a possible source of genetic information (de Vries et al., 2001; de Vries & Wackernagel, 2004).

The study of the soil exDNA has been generally directed towards microbial DNA dynamics, both because it is the most abundant exDNA fraction in natural and agro-ecosystem soils and it is subjected to interesting processes of wide application interests and public concern. Among these, the phenomenon known as Horizontal Gene Transfer (HGT, Syvanen & Kado, 2001) poses serious concern in public health and agriculture due to the onset of resistant microbial pathogens which acquire resistance to antibiotics and pesticides by HGT (e.g. Poté et al., 2003). Similarly, microbial exDNA by HGT can spread transgenic genomes of possible environmental concern, as in the case of modified Genetically Modified Organisms (GMO, Keese, 2008).

All these studies on microbial exDNA methodologically rely on molecular biology techniques, often aimed at identifying specific genomes, transgenic sequences or target genes or transcripts in environmental samples (Taberlet et al., 2018). Differently, a different approach has been previously used to follow the fate and functions of microbial exDNA in the soil or in mesocosms, using DNA

labelled with stable isotopes by $^{13}C$-or $^{15}N$-enriched culture medium, which is now a common tool for microbial ecology research (Radajewski et al., 2000; Cadish et al., 2005; Morris et al., 2015). The possibility of labelling the DNA with stable isotopes has opened the possibility to clarify different processes of the DNA biogeochemical cycle, such as the reuse of the nitrogen-rich constitutive elements of DNA (Ingraham et al., 1983; Katahira & Ashihara, 2002).

In several applications of molecular life sciences, material sciences, forensics, medical diagnostics and therapeutics, $^{15}N$ labelled dsDNA is directly produced in vitro by random amplification of target templates by PCR techniques, using commercially available $^{15}N$-labelled nucleotides as building blocks, which in turn are produced by extraction from labelled microbial cells followed by purification by high-tech chromatography such as HPLC (review in Nelissen et al., 2016).

However, the possibility to produce only small DNA fragments (100-200 bp) makes this production approach suboptimal when the purpose is to produce whole labelled genomes, especially when the target organism is less simple than a bacterial cell, as in the case of higher plants. In this case, the production of labelled DNA in vivo, by growing the plant in presence of a isotopically enriched fertilizer (for $^{15}N$) or a isotopically enriched atmosphere (for $^{13}C$), may be a more sustainable and reliable approach. Both these techniques have been successfully employed in previous studies aimed at labelling plant tissues in the past decades (e.g. Zeller et al., 1998; 2000; Bromand et al., 2001) and more recently specific methodological protocols have been shared among researchers to produce uniformly labelled plant materials (Ćeranić et al., 2020). However, much less is known, under a quantitative perspective, about the molecular processes controlling the level and dynamics of plant DNA labelling, when the plant is continuously exposed to stable isotopes but see (Gauthier et al., 2013). Then, in a very recent paper (see section 3.1) Foscari et al. (2021) explored the dynamics of $^{15}N$ incorporation into plant root and leaf DNA as compared to the source plant materials, in individuals of *Brassica napus* L. fertilized with $^{15}N$-enriched ammonium nitrate in

different conditions, highlighting the importance of the type of fertilizer, the labelled chemical species, the harvest timing and enzymatic isotopic fractioning o the resulting level of $^{15}N$ enrichment in plant DNA. Besides an intrinsic interest of pure knowledge, such previous study provided fundamental indications about the possibility to upscale the process of production of $^{15}N$ labelled plant DNA, from pot-cultivated herbaceous species to tree species in controlled field.

Then, in this follow-up application, applied the outcomes by Foscari et al. (2021) by fertilizing 56 four-years old beech juveniles with heavily enriched $\dot{N}H_4\dot{N}O_3$ in a semi-automatic system, for two consecutive growing seasons. Specific aims were to: (i) produce of a large amount of heavily labelled leaf materials to be used in follow-up studies to track N transfer pathway in natural soil-plant-atmosphere systems. As such, a very heavy labelling level was planned, taking into account dilution effects in the future applications in the field; (ii) verify the labelling signature of the plant material and, especially, of the DNA extracted.

## 3.2.2. Materials and methods

**Plant material and potting**

A controlled field application was carried out at Azienda Agraria "A. Servadei" (Udine) in an automated fertirrigation implant. The target species was *F. sylvatica*, with 56 four-years-old plants (diameter 1.85 ± 0.34 cm, height 151 ± 30 cm), provided by the Regional Forest Nursery of the Friuli Venezia Giulia Region (Vivaio Pascolon, Maniago, Italy). Each plant was individually transplanted in December 2017 in a 90 L pots containing a substrate composed by quartz sand (GESTECO Spa, Povoletto (UD), Italy) mixed at 50% v/v of expanded perlite (Perlite Italiana Srl, Corsico (MI), Italy). These potting substrate was chosen due to a negligible, if any, nitrogen content, a high stability over time, a neutral pH and absence of parasitic microorganisms. Before potting, the pots were buried in field along two lines (28 pots each), under which a drainage system run to avoid water stagnation

and to collect the percolation solution containing the fertilizer. The drainage collector was weekly inspected and drainage solution re-collected and re-used to watering (see next section). The system was left free to receive rainwater.

**Labelling and fertirrigation treatment**

Ammonium nitrate was utilized for $^{15}N$ labelling and fertilization treatment. The labelled fertilizer was prepared by mixing a water solution of commercial $NH_4NO_3$ (Sigma-Aldrich, USA, δ 15NAir-N2 = 0.7 mUr) with $\dot{N}H_4\dot{N}O_3$ (Cambridge Isotope Labs, 5% of labelled atoms) in the opportune mixing ratio, following the equations reported in (Hayes et al., 2004), in order to reach $\delta\ ^{15}N_{Air\text{-}N2}$ = 1500 mUr. In 1 April 2018, before leaves sprouting, each plant was manually supplied with 15 g of the labelled fertilizer (i.e. 5.4 g of N per plant) opportunely diluted in 500 ml of milli-Q water. Each pot was covered with a plastic sheet after $^{15}N$ administration and for the following 36 h to avoid excessive ammonia volatilization.

Fertirrigation was provided with an automated system (Fig. 3.2.1), composed by a tank (2500 L) connected to drip irrigation system that provides at each plant the amount of nutrient solution. At each tank re-filling cycle (see next section), it was added with 5 L of a modified Hoagland solution without nitrogen, composed by the following macro- and micro-nutrient: 10 mM MgSO4, 1mM Fe(Na)-EDTA, 20 µMKCl, 0.5 mM H3BO3, 40 µMMnSO4, 40 µMZnSO4, 2 µMCuSO4, 2 µM (NH4)6Mo7O24, 4 mM CaSO4, 20 mM K2HPO4, 60 mM K2SO4. The nutrient solution was buffered with MES (2-(N-morpholino)ethanesulfonic acid, 40 mM) and pH was corrected at 6.0±0.1 with HCl 4M.

The drip irrigation system was controlled by a CR1000X data logger (Campbell Scientific Inc, Logan, UTAH, USA) connecting a weather station assembled on site composed by the following

sensors (all from Campbell Scientific Inc, Logan, UTAH, USA): temperature and relative humidity probe Vaisala HMP45C; wind speed & direction Windsonic-Lc, Global radiation sensor SMP10.

Irrigation was based on previous day evapotranspiration estimated utilizing the Penman-Monteith equation (McNaughton et al., 1984) and an original software for monitoring and control of irrigation valve and pump was coded to provide an optimal quantitative of nutrient solution and avoiding excessive irrigation and consequently loss of nutrient solution. Generally, there were 3-6 irrigation x 1-10 min daily with a flow rate 4 L/h. In addition, a water content sensor (CS616, Campbell Scientific Inc, Logan, UTAH, USA) and a soil temperature probe (CS109, Campbell Scientific Inc, Logan, UTAH, USA) were plugged into each pot to stop the irrigation system whenever one of the pots reached the water field capacity value (i.e. 22%, separately assessed for the used substrate at the lab by gravimetric method), thus avoiding possible excessive watering and consequent nutrient an $^{15}N$ loss by percolation due to unequal actual evapo-transpiration at each pot, as the plant size were relatively heterogeneous. A further control to stop irrigation and limit possible $^{15}N$ loss by percolation due to rainfall was implemented by placing a wet-leaf sensor (Rain-detector, Kemo Gmbh, Germany) at the end of each collector line, automatically switching irrigation pump to off when wet. Finally, irrigation was also stopped when the tank sent the “empty” signal. The tank was weekly checked, to ensure that re-filling was not needed, and re-filled when needed.

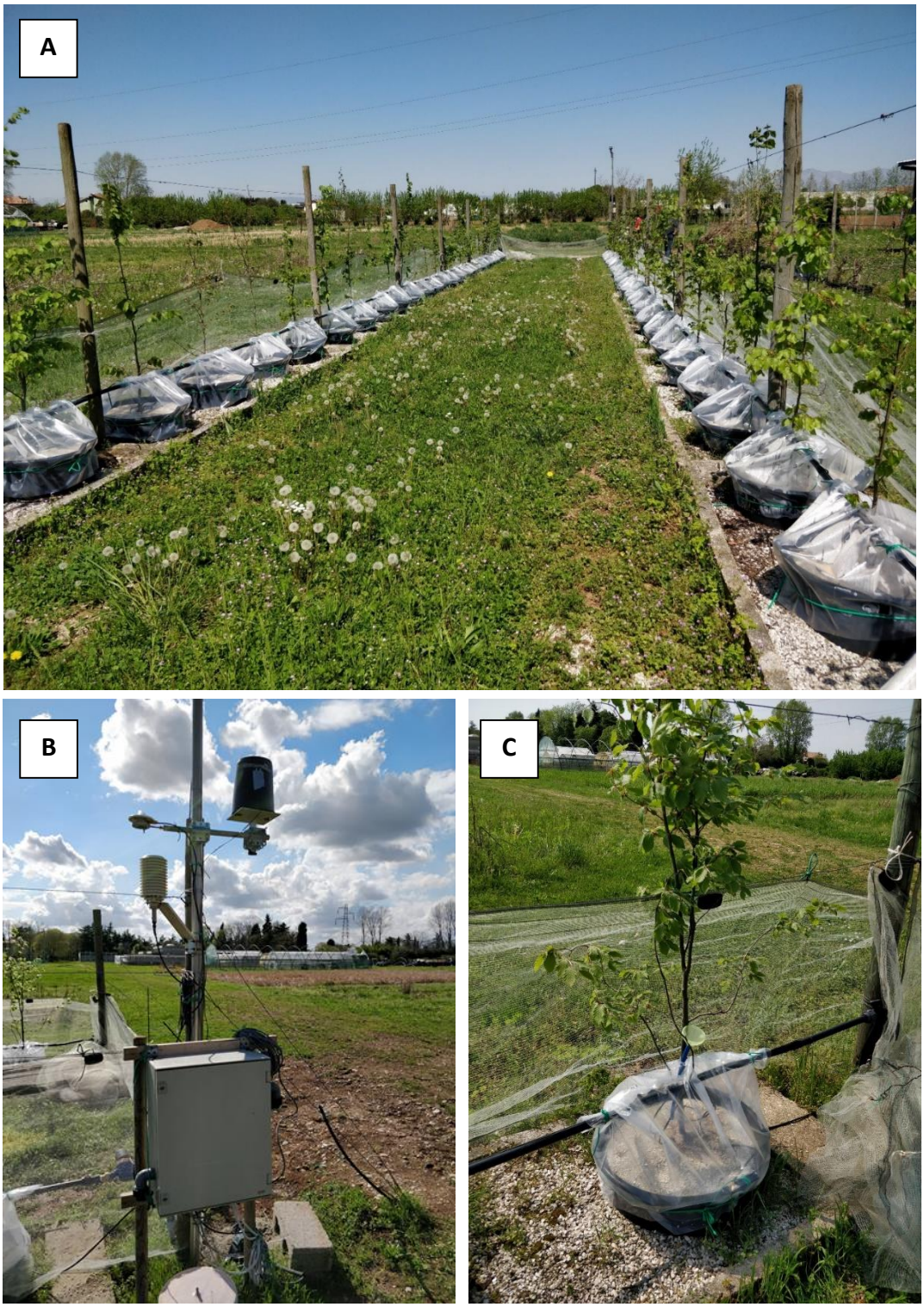


**Fig 3.2.1.** Pictures of the semi-automatic fertirrigation systems. (**A**) Photo of the controlled field with the two rows of beech juveniles; (**B**) Particular of the fertirrigation controlling system. CR1000X data logger is contained in the grey box and connected at different environmental sensors; (**C**) Particular of a pot covered with plastic sheet to avoid ammonia volatilization after the labelling treatment, which contained also the dripping line connected to the fertirrigation system. The visible funnel was used to administrate the $^{15}N$ fertilizer.

**Plant cultivation and harvesting**

The labelling fertilizer was administered to the beech juveniles only at the beginning of the growing season of the year 2018, while the nutrient solution was also administered during the full growing season of the year 2019. At the end of both seasons. During the growing seasons, the controlled field was surrounded by a plastic net fence, to prevent possible physical damage and/or intrusion by rodents and hares, relatively frequent in the area. Weeds were manually removed from each pot every 15 days. During summer, in order to limit the evaporation and the rising temperatures, pots were covered with a shade cloth. Each year, starting week 1 of September, fresh leaves were separately collected for each beech plant before the natural senescence, in order to avoid re-translocation of nitrogen and macromolecules (Staaf et al., 1986; Zeller et al., 1998). Considerable effort was maintained throughout sampling to ensure clean, uncontaminated samples, including use of gloves during sample collection and decontamination of equipment prior to and during sampling.

Leaves were stored in plastics bags at -20° for subsequent DNA extraction and CHN-IRMS analysis. The fertirrigation system was kept active each year from spring to the end of the harvesting, while during autumn and winter the limited plant water requirements of were supplied by natural precipitations.

**Destructive sampling**

At the lab, fresh leaves from each sampled plant were weighted. Afterwards, aliquots (1 g) were collected, fresh-weighted, dried in stove (24 hrs. at 60˚C), dry-weighted, pulverized (TissueLyser II, Qiagen, Hilden, Germany) and kept in sterile plastic tubes for CHN-IRMS analyses. The remaining fresh material was subdivided into aliquots of 5 g each, which were were separately grounded (Mill

A11 basic, IKA, Saint Louis, Missouri, USA) in liquid nitrogen (T = -196 ˚C), placed in sterile 50 mL Falcon tubes and stored in freezer at -80 ˚C for subsequent DNA extraction.

**DNA extraction**

DNA extraction from fresh leaves followed a modified version of the protocol by Doyle & Doyle (1987), as follows: a lysis buffer was prepared mixing 20 mL of CTAB (2.5 %), a spatula tip of PVP-40, 2 µl of Proteinase K (20 µg/µl) and 200 µl of β-mercaptoetanol (0,1%). The buffer solution was kept in agitation in a thermostatic bath at 65 °C (pbi, Braski, Bergamo, Italy), until PVP complete dissolution. For each source plant material, 20 mL of the buffer solution were added to the Falcon tube and the mixture was incubated at 65 °C for 30 min. and successively cooled in ice for 10 min. DNA purification was performed adding 20 mL of a mixture of chloroform: isoamyl alcohol (24:1) and gently shacking for 10 min. to homogenize. Falcon tubes containing the mixture were centrifuged at 6800 rpm at 4°C for 30 min, then gently pipetting out the aqueous supernatant fraction. Sodium acetate (1/10 starting volume, 3M), NaCl (3/10 starting volume, 4M) and pure Isopropyl alcohol (2/3 final volume) were added to the collected supernatant. The solution was incubated at -20°C for 30 min. and successively centrifuged as described above. As final step, after removing the supernatant and twice washing the pellet with 2 mL ethanol (80 %), the pellet was dried in a stove (10-15 min. at 37 °C) and re-suspended into an Eppendorf tube filled with 2 mL of sterile water for quantitation.

DNA samples were quantified by fluorimeter Qubit 3.0 (Life Technology, Carlsbad, California, USA) and the quality was assessed by NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The fragment length distribution was assessed by 0.8% agarose gel electrophoresis. Finally, pooled DNA samples for each beech plant were collected, frozen,

lyophilized (55-4 Coolsafe, Scanvac, Allerød, Denmark) for 24 h (0.050 mbar, T = -57 °C) and kept in sterile plastic tubes at -20°C or subsequent CHN-IRMS analyses.

**CHN-IRMS analysis**

Dry, pulverized leaf samples as well as lyophilized DNA samples were weighted at 2 ± 0.5 mg in cylindrical tin capsules (diameter 5 mm, height 9 mm) (Säntis Analytical AG, Teufen, Switzerland) in duplicates. A total of over 1000 replicated DNA samples (56 plants x 2 years x 3 to 20 replicates ) were processed by elemental analysis/isotope ratio mass spectrometry (EA/IRMS), using a Vario Micro Cube (Elementar GmbH, Langenselbold, Germany) elemental analyzer connected online in continuous flow mode to an IsoPrime 100 (Elementar UK Ltd, Cheadle Hulme, UK) isotope ratio mass spectrometer, using helium (He) as a carrier gas. For a detailed explanation of the procedure see Foscari et al. (2021) and the previous section 3.1.

## 3.2.2. Results

**Monitoring and control system**

The semi-automatic fertirrigation system allowed to optimize the plant growth and labelling dynamics, satisfactorily recording the environmental conditions at the controlled field, thus correctly activating and deactivating the irrigation valve and pump thus limiting water consumption and avoiding the loss of the heavily labelled fertilizer. However, some unexpected incidents affected the completeness of the data recordings, as in the case of blackouts by meteorological summer storms (Fig. 3.2.2).

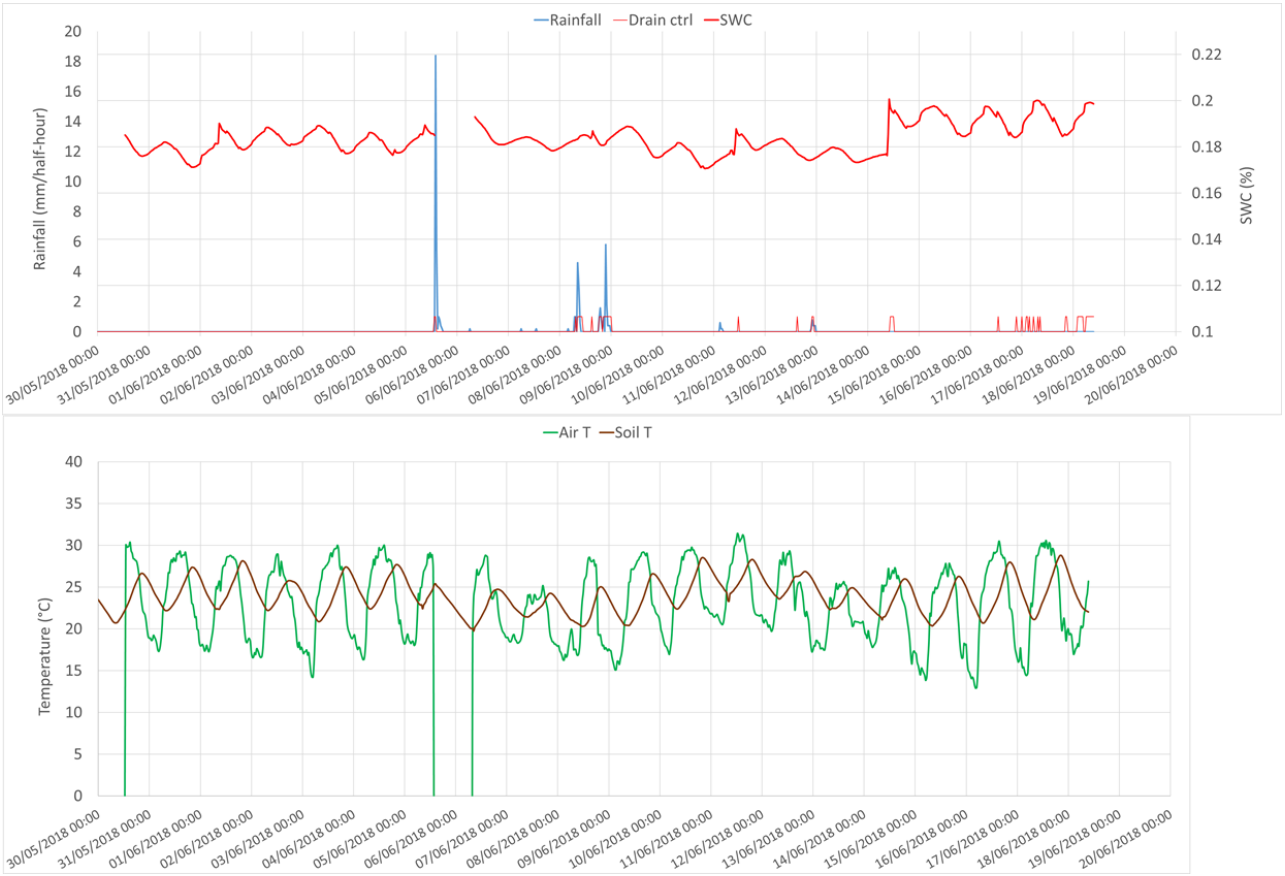


**Fig 3.2.2.** Example of data extrapolated from the environmental sensors placed in the cultivation site. In the 20 days of observation are visible different rain events and an unexpected blackout.

### Litter production and labelling

In the two cultivation years we were able to collect over 4 kg per year of fresh leaf litter from the 56 beech plants involved in the experiment (Table 3.2.1A), with a slightly lower production in 2018 (4113.1 g) as compared to 2019 (4415.0 g). CHN-IRMS analyses carried out on leaf materials showed values of carbon and nitrogen percent content consistent among plants (Table 3.2.01), with %C content ranging between 41.1 and 50.6% at the end of the growing season 2018 and between 36.3 and 44.9% the following year, while the corresponding ranges for %N were 1.1 ÷ 4.6%, and 1.1 ÷ 5.3%. Differently, $\delta^{15}N_{AIR\text{-}N2}$ ‰ was significantly different between the two growing seasons (Fig. 3.2.3), with a higher mean $^{15}N$ labelling at the end of the 2018 season (846.6 ± 606.9‰, with a

maximum recorde value of 2258.7‰), and a lower mean value at the end of the 2019 season (262.7 ± 181.2‰), when the maximum observed $\delta^{15}N_{AIR\text{-}N2}$ ‰ values reached was 800.5‰. (Table 3.2.1A).

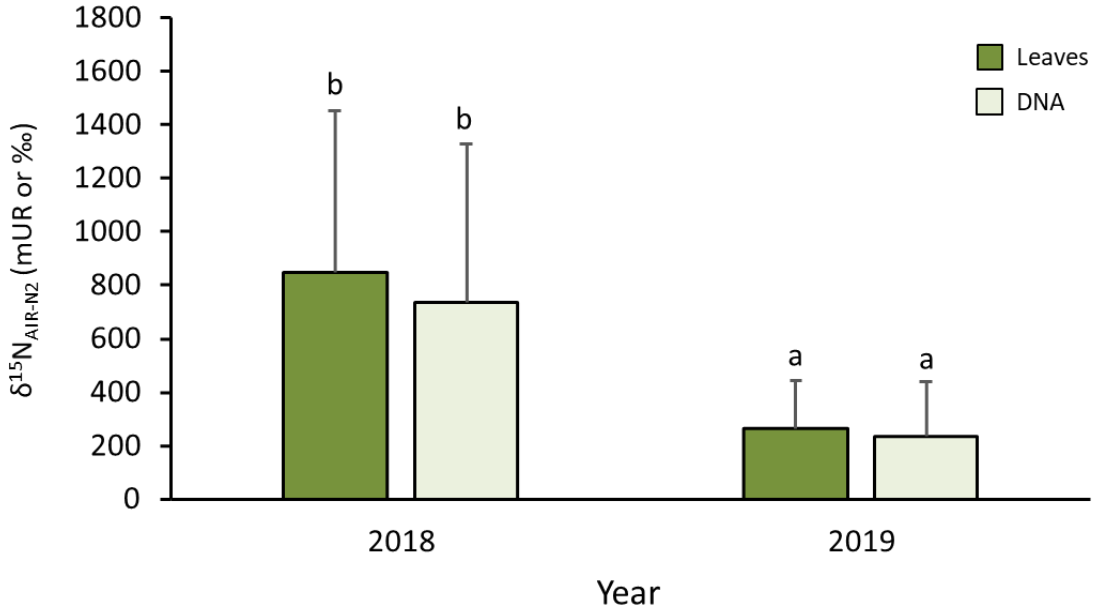


**Fig. 3.2.3 -** Value of δ15NAIR-N2 (mUR or ‰) in both leaves material and DNA extracted in the two years of cultivation. Letters refers to significant differences between the materials based on post hoc Duncan tests from a one way Anova.

**Table 3.2.1:** Summary of the different parameters measured for leaves material (**A**) and DNA (**B**) in the two different cultivation years.

| a) Leaves | Total Weight (FW g) | C % | N % | $\delta^{15}N_{AIR\text{-}N2}$ ‰ - Min | $\delta^{15}N_{AIR\text{-}N2}$ ‰ - Avr | $\delta^{15}N_{AIR\text{-}N2}$ ‰ - Max |
|---|---|---|---|---|---|---|
| 2018 | 4013.1 | 44.8 ± 2.0 | 2.0 ± 0.0 | 80.5 | 846.6 | 2258.7 |
| 2019 | 4415.0 | 46.5 ± 1.8 | 1.9 ± 0.1 | 17.7 | 262.7 | 800.5 |

| b) DNA | Yield $_{Nanodrop}$ (µg/g FW) | Yield $_{Qubit}$ (µg/g FW) | $\delta^{15}N_{AIR\text{-}N2}$ ‰ - Min | $\delta^{15}N_{AIR\text{-}N2}$ ‰ - Avr | $\delta^{15}N_{AIR\text{-}N2}$ ‰ - Max |
|---|---|---|---|---|---|
| 2018 | 181.3 ± 5.3 | 22.4 ± 4.7 | 49.0 | 736.8 | 2023.0 |
| 2019 | 179.0 ± 1.5 | 22.9 ± 6.7 | 1.0 | 234.3 | 890.1 |

**DNA quantitative and labelling overview**

The quantitative analysis on the DNA extracted from leaf fresh materials showed similar extraction yield (µg/g FW) between the two cultivation years (Table 3.2.1B), both considering the estimates based on the spectrophotometric method (Nanodrop) and those produced by the fluorimetric method (Qubit).

As in the case of the leaf materials, DNA samples showed $\delta^{15}N_{AIR\text{-}N2}$ ‰ values significant different between the two cultivation years (Fig. 3.2.3), with highest $^{15}N$ labelling at the end of the 2018 growing season (736.8 ± 693.1‰) compared to the following one (234.3 ± 202.1‰) (Table 3.2.1B).

## 3.2.4. Discussion

The total leaf production in the two cultivation years was ~ 8.5 Kg of fresh material for 56 plants, with a starting age of 4 years. Leaf production in young beeches is proportionally lower compared to adult individuals (Pajtík et al., 2011), highly variable depending on the environmental conditions (Schall et al., 2012) and directly correlated to plant basal area and age (Lebret et al., 2001). It is clear that, considering our aim to test a massive production of isotopically labelled litter and DNA, individual trees of higher age and larger size could have been selected (see Zeller et al., 1998 for comparison). However, transplanting adult trees would have implied a certainly very painstaking work, not mentioning the intrinsically higher cost in terms of labelled fertilizer requirements to sustain the yearly growth of Adult trees. Moreover, the adaptation of beech adult plants out of the species normal ecological range would have been more critical for adult plants as compared to juveniles seeded and developed in conditions similar to those of our controlled filed, in the Regional Forest Nursery.

Interestingly, during both growing seasons the levels of $\delta^{15}N_{AIR\text{-}N2}$ ‰ in the leaf materials were remarkably variable among plants. These is consistent previous observations (Zeller et al., 1998), although the latter were carried out in different conditions and with a different labelling method (i.e. leaf spraying of labelled urea). All in all, the results of this and previous studies indicate that leaf labelling is a complex phenomenon directly controlled by N mobilization within (Staaf & Stjernquist, 1986; Glavac & Jochheim, 1993) and between plants (Staaf & Stjernquist, 1986). Additionally, the roles of possible leaching (Meisinger & Delgado, 2002), ammonia volatilization (Jones et al., 2013) and sequestration of nitrogen by weeds (Yang et al., 2007) cannot be completely excluded and, even if we purposely adopted methodological strategies to control for these confounding effects, could have amplified between-pots differences.

Interestingly, in some pots, leaf materials as well as leaf DNA reached levels of $\delta^{15}N_{AIR\text{-}N2}$ ‰ remarkably higher that that of the treatment solution (i.e. $\delta^{15}N_{AIR\text{-}N2}$ ‰ = 1500). Is it clear that foliaging during the growing season proceeds continuously and, due to isotopic discrimination during root N uptake and assimilation (e.g. Mariotti et al., 1982; Yoneyama, 1991), late leaves receive inorganic N residual in the pot solution more heavily labelled compared to that uptaken and assimilated in the early leaves. Therefore, asynchrony in the sprouting among beech juveniles could have exacerbated differences among plants, even producing apparently anomalously high $\delta^{15}N_{AIR\text{-}N2}$ ‰ values.

Finally, $\delta^{15}N_{AIR\text{-}N2}$ ‰ levels in leaf DNA from the two cultivation years were highly variable among plants, but not dissimilar from the source leaf materials, clearly suggesting a negligible, if any, effect of isotopic fractionation phenomena (Foscari et al., 2021).

### 3.2.5. Conclusions

The results of this application test clearly demonstrated that it is possible to produce a remarkable amount of heavily labelled leaf material, which constitute an important resource for the research group where I worked during my PhD studentship, for a possible use in follow-up studies to track N transfer pathway in natural soil-plant-atmosphere systems.

Consistent with previous observation, the labelling signature of the plant material can highly vary as a function of several physiological and environmental processes. The same holds true for DNA labelling levels, which however strictly reflect those of the source leaf materials.

While the knowledge and the simplicity of the reference system are much lower compared to microbial systems, the use of stable isotopes to label plant tissues and nucleic acids appears as convenient, low-cost technique, which produces easy-to-detect signal, for tracing the distribution, transformations and effects of nutrients, organic compounds and metabolites in the environment, which, in the case of exDNA, are still far from being clarified.

# Chapter 4

# Negative feedback by self-DNA inhibition on beech seedlings

## 4.1. Introduction

The ability of a plant to modify or adapt to his growing substrate can be determinant for its fate. Indeed, the growth of a plant, its reproductive success and in general its capability to coexist with other plants are influenced by the nature of the soil where the plant expresses his fitness (Van der Putten et al., 2013). Moreover, the magnitude of modification made by a plant during its growth to its biotic and abiotic soil environment can lead to a feedback loops of different directions, in terms of either beneficial or inhibitory effect on the plant itself or to its progeny, which however generally tend to be negative, thus maintaining stability conditions (Bever, 2003; review in Kulmatiski et al., 2008). In particular, negative intraspecific plant–soil feedback (NF) may result in the degeneration of the plant itself (Van der Putten et al., 1988), or it may cause high seedling mortality near the mother plant (Packer & Clay 2000; Mangan et al. 2010), thus contributing to stabilize a diversified plant community (Van der Putten et al., 2013).

It has been long recognized that NF can be mediated via direct interactions between plants and rhizosphere communities, as well as indirect interactions between plants and decomposer communities driven by litter inputs (Wardle et al., 2004; Ehrenfeld et al., 2005; Kardol et al., 2015). Plant-soil feedback effects mediated via the biotic community in the rhizosphere are known to be driven by species-specific relationships between plant species and organisms inhabiting the rhizosphere (Veen et al., 2019a). The precarious balance between mutualists and pathogens often can be shifted towards negative effects by not opportune cultivation methods, like the repeatedly growing the same crop in a field that can lead to the build-up of plant species-specific soil pathogens and root herbivores (McDonald et al., 2016). This phenomenon, also known as ‘soil sickness’ or ‘soil fatigue’ (Huang et al., 2013), has led to the practice of crop rotation (Dias et al., 2015). While in cultivated system the effects of litter mediated plant soil feedback are limited due the removal of

crop from the field, in natural systems, litters may leave different negative legacies in the soil that have a strong impact on plant growth (Ehrenfeld et al., 2005; Elgersma et al., 2012).

The physical effects of plant litter on the soil environment have strong potential to feed back to plant performance through effects on seed germination, seedling establishment, and initial plant growth (Olson & Wallander, 2002), in fact the litter layer can have negative impacts on seed germination because it can reduce the amount of light reaching the soil surface (Asplund et al., 2018).

When litter fall to the ground, an entire community composed by litter-fragmenting or detritivores transforms a large part of plant litter increasing the surface area for microbial decomposition, which often results in accelerated litter breakdown (Hattenschwiler & Gasser, 2005; Joly et al., 2018). Different mineralization rates depend of different feeding preferences of saprotrophic fungi and bacteria, and the variation of the soil community in time contribute to litter-mediated feedbacks by altering soil nutrient availability (Joly et al., 2018) and liberating secondary metabolites from plant litter (Facelli et al., 1991), which in turn affect the plant performance.

During decomposition a wide range of chemical compounds is released from plant litter. On one hand, nutrients released from rapidly decomposing litter can increase the soil nutrient stock and become available for the subsequent plant generation, thus producing positive feedback effects. On the other hand, litter materials with a high content of structural carbohydrates, lignin and other recalcitrant compounds such as resins, cutins, and waxes, decompose at lower rates, with less evident beneficial effects or even NFs (Hodge et al., 2000; Vahdat et al., 2011). In addition, litter-mediated feedback via chemical compounds may strongly differ between above- and belowground plant organs, especially considering the different decomposition rates of shoots, stems and roots (Hobbie et al., 2010). Compared to current evidence on the biological effects of leaf litter, little is known on the impacts of root litter (but see Zhang et al., 2016b), and nothing is known about

possible differential impacts and contributions to NF by root and leaf litter, both considering the overall litter production at community scale, and at species-specific level. Plant litter also contains a range of secondary metabolites, including phenolic compounds (Li et al., 2010), tannins (Kraus et al., 2003) and terpenes (Chomel et al., 2016). Most of these short-chain organic molecules can have ephemeral life and disappear during the early decomposition stages (Siegrist et al., 2010), while others can persist in the soil and inhibit the growth or germination of co-ocurring and next-generation plants (Bonanomi et al., 2011b), producing a series of inhibitory effect commonly known in agroecosystems as allelopathy (Muller, 1966; Rice, 1984).

While allelopathy effects are not bound to species-specific litter and show a clear time dependent pattern with inhibition generally removed after the early decomposition stages, in the case of conspecific litter species-specific autotoxic and long-lasting inhibition effects have been reported and reviewed (Inderjit, 1996; Singh et al., 1999; Cesarano et al., 2017). Autotoxicity has been also associated to the accumulation and persistence of conspecific extracellular DNA (self-DNA) in the litter layer and underlying soil during litter decomposition (Mazzoleni et al., 2015a). The effects of fragmented self-DNA were demonstrated *in vitro* (Mazzoleni et al., 2015a; Duran-Flores & Heil, 2018) and greenhouse experiment (Mazzoleni et al., 2015a), and self-DNA was found occurring in soil DNA consistently with a spatial pattern of inhibition of conspecific seed germination (Mazzoleni et al., 2015a) but the accurate quantification of selfDNA in natural soil conditions, as well as the reliability of its functional NF effect in filed conditions is still much less known (but see Chapter 2 of this PhD thesis). The accumulation of self-DNA in soil as a causal agent of NF could have many implications at ecosystem scale (Cartenì et al., 2016) including the maintenance of high tree diversity in tropical forest (Givnish et al., 1999), where self-inhibition clearly could leave free room for heterospecifics. Oppositely, monospecificity or monodominance in plant community characterized by permanent or transient water submersion, such as kelp forest, marine phanerogams prairies, or

mangrove forest, could be explained by the displacement of phytotoxic and autotoxic agents from the proximity of plants by water flow, as well as by a dilution to ineffective concentrations (Cartenì et al., 2016). Differently, the existence of monodominated communities in boreal and in mountain temperate forests (Hart et al., 1989) may not be affected by self-DNA accumulation in the upper soil layer, due to the cold-dependent low litter decomposition rate, paired to seasonal washing by soil thawing, followed by water runoff and infiltration to the deep layers. Moreover, adult individuals of the dominant tree species in these forest communities can indirectly positively affect conspecific recruitment and seedling survival by the so-called *home-field advantage* (HFA, Veen et al., 2018; 2019b). Indeed, according to HFA, plant litter decomposes at highest rate in the *home* soil, where the plant producing the litter is rooted, due to the highest fitness of the decomposer community and microbiota to the local litter quality and environmental conditions. Due to higher decay rates, a correspondingly high nutrient release rate consistently maintains high nutrient availability, which translates into higher seedling survival probability, although with different magnitude depending on the species involved (Wurst et al., 2015). In the frame of the balance between positive vs. negative litter-dependent effects, as possibly produced by self-DNA and HFA effects, the net combined outcome of these processes is not yet fully clarified, especially in beech-spruce forests, where an interesting species substitution dynamics occur (Fox, 1977; Oliver & Larson, 1990) with site-specific turnation period (Del Favero, 1998; 2004). Such phenomenon has been originally related to not-well specified shifts in soil chemical-physical properties (Susmel et al., 1951), but then left unclear. Moreover, a recent study by Foscari & Incerti (see Chapter 2 of this PhD thesis) has demonstrated that in beech-spruce forest soil, in NE Italian Alps, beech and spruce extracellular DNA differently accumulate along the soil profile, consistently to species-specific litter dynamics, with higher content of *F. sylvatica* DNA in the upper layer, where seedlings are rooted, but the net effects on seedlings has not yet been assessed.

This study aims to test the effects of rhizosphere- and litter-mediated feedback on seedling growth, as well as their interplay with possible self-DNA inhibition. To this aim, the effects of decomposed conspecific litter (separately from leaves and roots) is used as a growth substrate in comparison to natural field soil from beech forest in bioassays on beech seedlings under controlled conditions. In this way, the effect of root- and leaf-litter specific microbiomes and molecular composition can be compared to that of the natural home soil. In order to disentangle the microbial effect to that of possible phytotoxic chemicals contained in the litters, substrate treatments to separately remove each causal factor, such as sterilization and addition of activated carbon are factorially applied. Then, each factorial combination (substrate type and treatment) is separately tested without and with the addition of beech self-DNA *sensu* Mazzoleni et al. (2015a), in order to assess possible non-additive effects. The specific underlying hypotheses are: (i) the home-field advantage (HFA) holds for beech seedlings by comparing their performance in home soil vs. litter-conditioned substrate; (ii) litter chemistry, as well as litter-specific microbiomes, have a negative effect on seedling performance; (iii) self exDNA has a negative effect *per se* also exacerbating that of litter chemistry and microbiome.

## 4.2. Material and methods

### Collection of soil and plant material

Forest soil used for the experiment was collected at Fusine lakes (46°30'15" N, 13°38'26" E), in NE Alps near the Italian border with Slovenia and Austria, within the forest compartments belonging to the regional government, in May 2019. Soil was collected in the organic pedological horizon (Oe) in forest stands under adult *Fagus sylvatica* individuals, homogenized manually in the field, and sieved (< 2 mm) at field moisture. Fine soil fraction was placed in plastic bags and stored at 4°C. During sieving, beech roots (< 5 mm diameter) were collected and other coarse materials including leaves and bark fragments were discarded. At the same sites nets were placed under randomly selected beech plants (n > 20) to collects seeds and freshly abscised leaves. Plant litter types (leaves and roots) were separately placed in plastic bags, dried at room temperature in a ventilated chamber until a constant weight was reached, and then stored at room temperature.

Beech seeds belong to intermediate/deeply dormant species and must be pre-treated to ensure successful germination (Packham et al., 2012). Seed pre-treatment included surface sterilization (1 min in 70% alcohol, 3 min in 50% bleach, 1 min in 70% alcohol, 1 min in distilled water) followed by mixing with moist peat and sand in 1:1 v/v, then storage in a loosely-tied polythene bag to allow gas exchange, placed in the main compartment of a refrigerator at 4°C for 4 months. Seeds were weekly controlled until emersion of the first rootlets.

### Litter decomposition

Plant leaf and root litter materials were separately fragmented and placed inside plastic trays (size 30 x 50 x 50 cm). A microbial field inoculum was prepared by mixing 10 g of soil taken from the field from which litter was collected (top 10 cm) and 90 g of water and nebulized on litter in order to

improve the start-up of the decomposition process. The material was kept at a temperature of 24 ± 2°C in a controlled chamber for 120 d, mixed weekly and kept moist with distilled water to holding capacity, in order to avoid anoxic decomposition.

**Substrate chemical-physical analysis**

Soil pH was measured potentiometrically with a sureflow combine glass-calomel electrode in $H_2O$ solution 1:5 solid: liquid ratio (McLean, 1982). Organic carbon (C) and total nitrogen (N) content and corresponding C to N ratio were measured using a using a Vario Micro Cube (Elementar GmbH, Langenselbold, Germany) elemental analyzer in triplicated aliquots of 10 ± 0.5 mg of each sample weighed in a silver capsule and treated with HCl to eliminate carbonates (Nieuwenhuize et al., 1994).

**Self-DNA solution preparation**

DNA was extracted from 800 g of fresh, young leaves of *F. sylvatica* collected at the filed site in May 2019. Extraction followed a modified version of the Doyle & Doyle (1987) protocol as described in Chapter 3.1. Additionally, at the end of the extraction, in order to remove undesired RNA residues in the samples, DNA was subjected to an additional purification. DNA solution was incubated with RNase A (25 µL, 1 ng/µL) and kept in agitation at 37 °C (1 h, 300 rpm), then adding to the solution 3M sodium acetate (10 % v/v), 4M NaCl (30% v/v), and pure Isopropyl alcohol (66% v/v) prior to overnight incubation at -20°C and centrifugation (4°C, 30 min, 7500 g). After removing the supernatant, the residual pellet was twice washed with 2 mL ethanol (80 %). Finally, DNA pellets were dried in stove (15 min. at 37 °C) and re-suspended in 2 mL sterile water.

The inhibitory effect of self-DNA is limited to < 1000-bp-long fragments, corresponding the molecular size observed in natural condition and produced by chemical-physical degradation after plant debris decomposition, while genomic DNA has no effect (Mazzoleni et al., 2015a). Then, in

order to replicate that condition, DNA was sonicated using a Bioruptor Plus® (Diagenode SA, Seraing, Belgium), with 0.5 ml (12 samples) tube holder & temperature controlled system Bioruptor® Water Cooler (Diagenode SA, Seraing, Belgium). Each tube vas filled with 100 µl of purified DNA solution, vortexed (10-15 sec) and centrifuged (10 sec) before shearing. Cycle condition was 60/30 (On/Off times in sec.) at high power and 4°C, all repeated 8 cycles (12 min total) in order to obtain a fragmentation size between 500 and 100 Bp. A total of 180 mL of self-DNA solution at a concentration of 100 ng $\mu L^{-1}$ was produced.

DNA samples were quantified by fluorimeter Qubit 3.0 (Life Technology, Carlsbad, California, USA) and the quality was assessed by NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The fragment length distribution was assessed by 0.8% agarose gel electrophoresis.

**Substrate preparation**

Substrates for the bioassays were prepared by mixing either the home forest soil, or leaf or root decomposed litter with a sterilized commercial potting mix COMPO SANA® (Compo GmbH, Münster, Germany), in 1:1 v/v in order to ensure sufficient nutritional supply to beech seedlings. According to the producer, the potting mix has a pH value of 5.0 - 6.5, salt content (g $dm^{-3}$) of 1.0 -1.5, nutrient content: 50 - 250 N, 80 - 300 $P_2O_5$, 100 - 350 $K_2O$.

Each substrate type was either kept untreated as control, or subjected to one of the following treatments: (i) AC+, mixing with 1:10 v/v of activated carbon (Sigma-Aldrich Co, St. Louis, USA); (ii) ST+, sterilization by autoclaving at 121 °C for 20 min three times with 24 h interval; (iii) both AC+ and ST+; (iv) AC. For each of the 12 resulting substrate types, mesocosms consisting of 50 mL falcon tubes were filled with 20 g of substrate. One pre-germinated beech seed was placed into each mesocosm, on the substrate surface, with the rootlet placed into the substrate. After seeding,

and a 2-days of taking root period, half of the replicated mesocosms for each substrate type were additioned with 20 ml of self-DNA solution diluted at a concentration of 10 µg/ml (treatment DNA+), while the remaining mesocosms were additioned with 20 mL distilled water (DNA-). Overall, 144 mesocosms were set up, corresponding to 6 replicates for each of the 24 factorial combinations of substrate type (3 levels: home field soil, leaf itter, root litter), treatment (4 levels: AC-ST-, AC+ST-, AC-ST+, AC+ST+) and DNA addition (either DNA+ or DNA-).

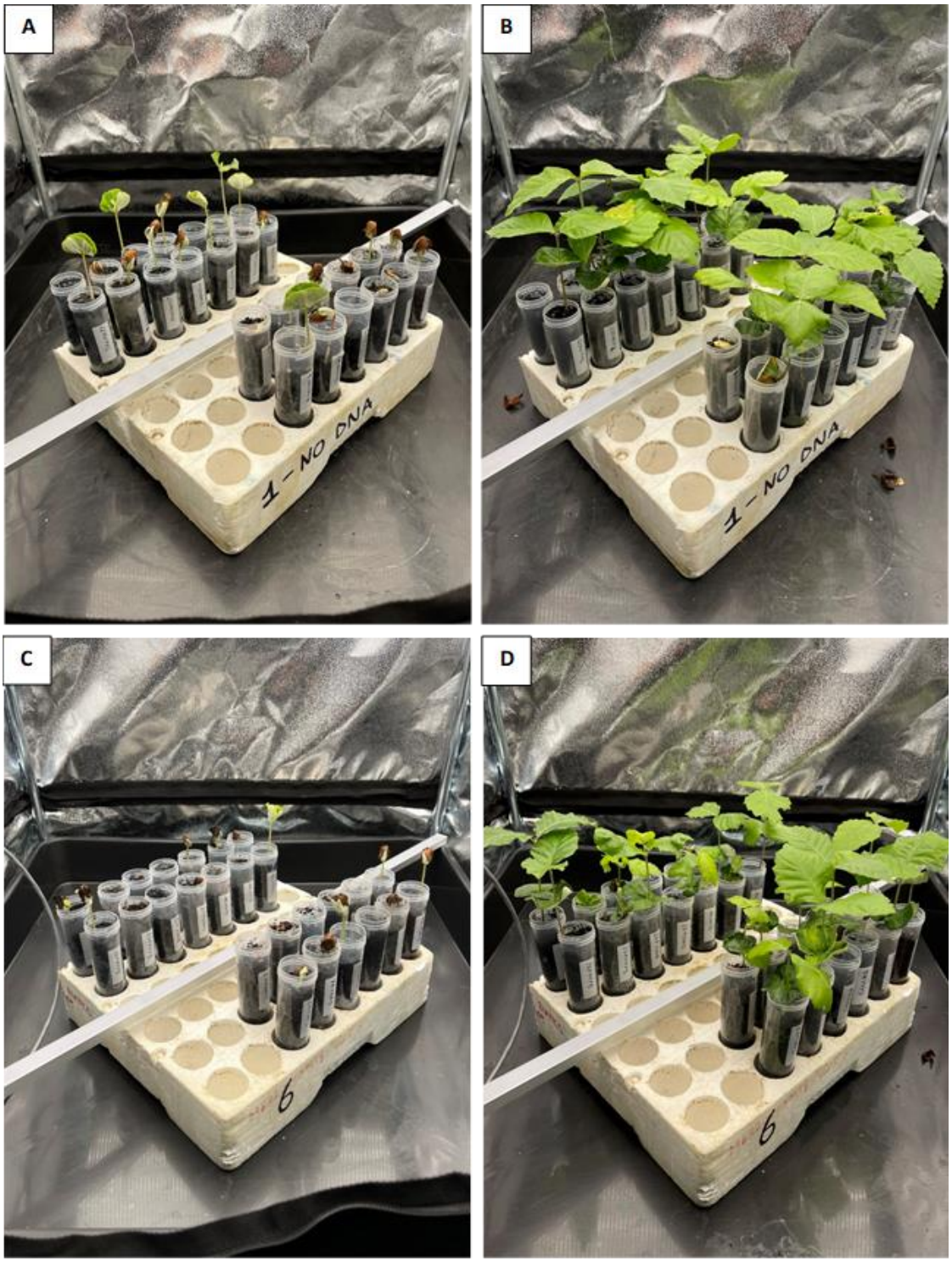


**Fig 4.1.** Experimental sub unit of plants placed in the growing chamber. Each polystyrene seed tray contains falcon tubes with different treatments. (**A - B**) refers to treatment without self exDNA addition respectively ad T0 and T30; (**C - D**) refers to treatment with self exDNA addition respectively ad T0 and T30.

**Plant growth**

All mesocosms were placed in a growing chamber in controlled conditions (T = 25/20 ˚C, photoperiod 12 hr: 12 hr day: night, RH = 50%, PAR 600 µmol photons $m^{-2}$ $s^{-1}$) and weekly rotated to maintain homogeneity of exposure condition among replicates (Fig 4.1). Water loss by evapotranspiration was reintegrated by watering the falcon tubes every two days with milli-RO water at holding capacity. Beech seedlings were grown in the bioassay for 30 days.

**Destructive sampling**

At the end of the growing period, each seedling was gently extracted from substrate and separated in roots, leaves, steam and cotyledons (Fig 4.2). Roots were gently washed with deionized water in order to remove substrate particles and dried on absorbing filter paper sheet to eliminate excess of water, dried in stove (24 hrs. at 60˚C) and dry-weighted. Substrate was collected and homogenised individually for each treatment group and stored at -20°C for further analysis.

**Statistical analysis**

In order to assess HFA effects, main effects of substrate type (three levels: Field soil, leaf litter, root litter) on root mass and total plant mass (DW g) was tested by one-way ANOVA models for each dependent variable, limited to AC-, ST- and DNA- treatment combinations. To evaluate litter chemical effects, main effects of substrate type on root mass and total plant mass were tested by one-way ANOVA, limited to AC+, ST- and DNA- treatment combinations. To evaluate litter microbiome effects, main effects of substrate type on root mass and total plant mass were tested by one-way ANOVA, limited to AC-, ST+ and DNA- treatment combinations. At last, to evaluate self-DNA addiction effects, main effects of substrate type on root mass and total plant mass were tested by one-way ANOVA, limited to AC-, ST- and DNA+ treatment combinations. For all ANOVA models,

pairwise comparisons between combinations of independent factors were tested by Duncan's post hoc test at $\alpha$=0.05, in all tested comparisons.

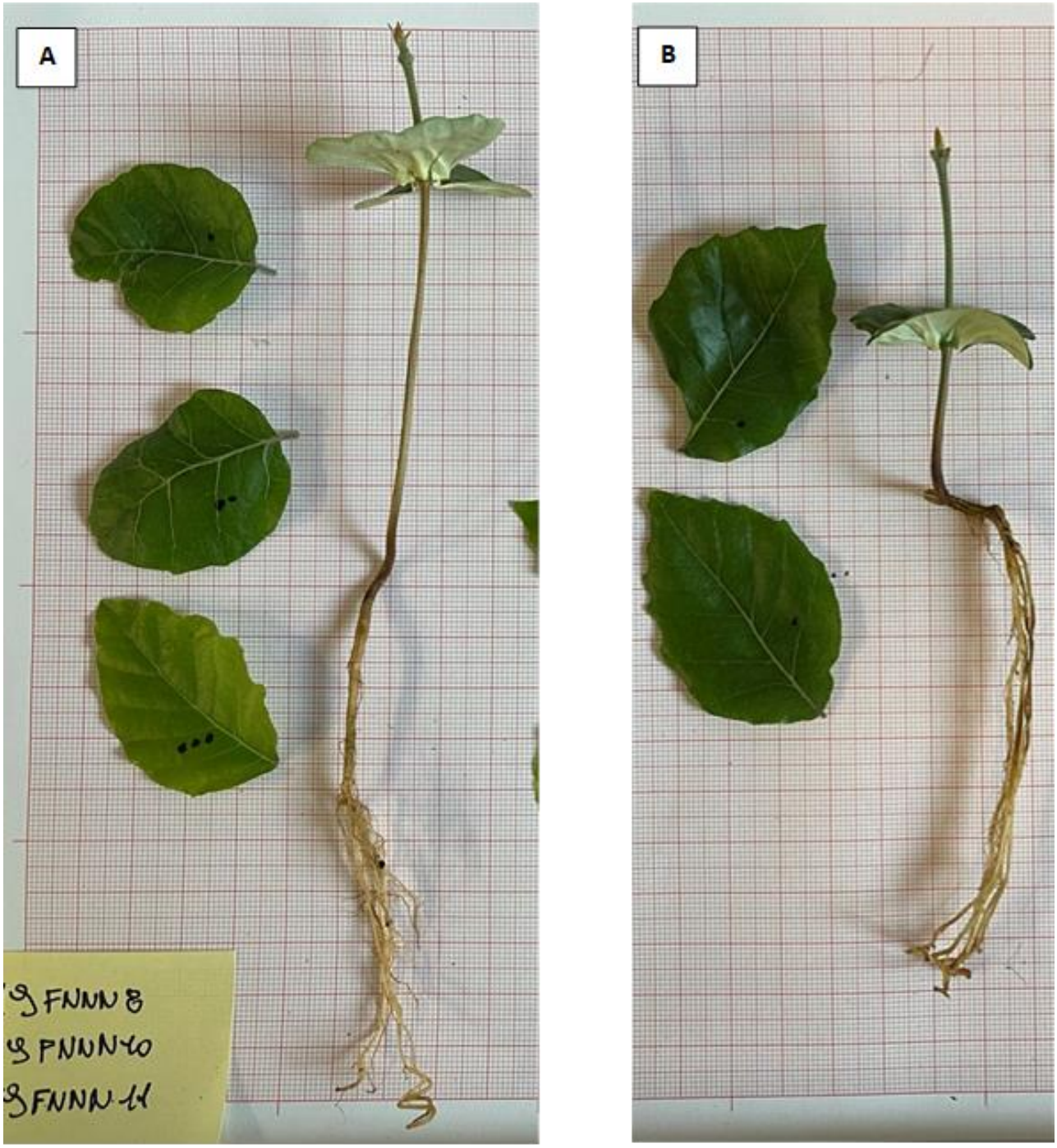


**Fig 4.2.** Seedlings of *F. sylvatica* after 30 days of growth in different substrate and treatments. (**A**) plant growth in field soil; (**B**) plant growth in field soil with addition of self exDNA.

## 4.3. Results

### Substrate basic chemistry

The main chemical characterization of the substrate used for conditioning are summarized in Table 4.1. The organic carbon content (C%) was equal or higher than 30% in all soil conditioners, showing higher mean value for root litter and similar values in leaf litter and field soil. Total nitrogen (N%) ranged between 1.1. and 2.5 %, showing maximum in leaf litter, minimum in root litter, and intermediate values in field soil. The corresponding values of the C to N ratio were much higher in root litter compared to the other two conditioners, which instead showed similar values. Such differences were greatly reduces considering the three substrates obtained by mixing the conditioners with the commercial potting mix.

**Table 4.1.** list of different parameters measured for the and substrate and conditioners used in the experiment.

| Material | | C % | N % | C : N | pH |
|---|---|---|---|---|---|
| Substrate | Potting mix | 25.1 ± 1.8 | 1.5 ± 0.3 | 16.7 ± 0.5 | 6.5 ± 0.5 |
| Conditioner | Field Soil | 29.6 ± 1.8 | 1.7 ± 0.1 | 17.5 ± 0.7 | 5.3 ± 0.3 |
| | Leaves Litter | 32.1 ± 0.2 | 2.5 ± 0.1 | 12.8 ± 0.5 | - |
| | Root Litter | 40.4 ± 0.1 | 1.1 ± 0.2 | 36.3 ± 0.2 | - |

### Litter inhibitory effects

We observed significantly different effects by different substrate types, consistent for root and total plant biomass (Table 4.2). In particular, for both dependent variables seedlings grown on plant litters showed consistently lower biomass compared to those grown on the home filed soil, with root litter substrate producing the most evident inhibitory effect (Fig. 4.3). In detail, the root mass of seedlings grown in litters was 32.2% and 47.2% lower for leaf and root materials, respectively, compared to that observed over home field soil. The corresponding values for the total plant mass, were 39.2% and 32.8%, respectively.

**Table 4.2**. Results of one-way ANOVA testing for the effects of substrate type (Field Soil, Leaves Litter, Root Litter) on root and total plant mass. Significant P-values are marked in italic font.

| Effect | DoF | SS | MS | F | P |
|---|---|---|---|---|---|
| *Root* | | | | | |
| Substrate type | 2 | 0,057 | 0,002 | 19,354 | *0.0008* |
| Error | 8 | 0,004 | 0,000 | | |
| | | | | | |
| *Total plant* | | | | | |
| Substrate type | 2 | 0,047 | 0,023 | 53,678 | *< 0.0001* |
| Error | 10 | 0,004 | 0,000 | | |

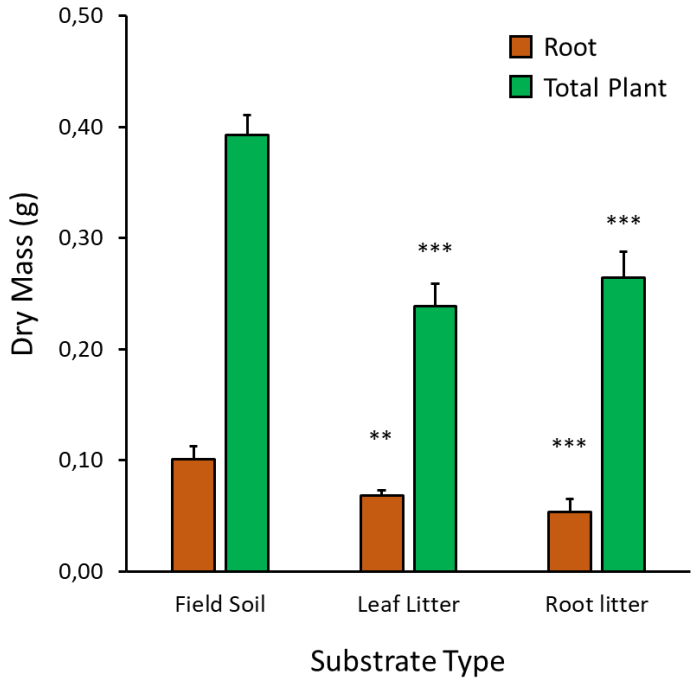


**Fig. 4.3:** Effects of three different substrate type (Field soil, Leaf Litter and Root Litter) on root and whole plant dry mass of beech seedling (*F. sylvatica*) after 30 days of growth. Separately for root and whole plant data are marked with asterisks to indicating significant differences compared with each field soil data (***, P < 0.001; **, P < 0.01; *, P < 0.05; post hoc Duncan tests from a one way Anova in Table 4.2).

**Substrate chemicals vs. microbiome effects**

After removing the effect of litter chemicals by adsorption on active carbon, seedling inhibition by the litter materials mostly released, as showed by the significant first-order and/or interactive AC terms in the ANOVA models for root and total plant mass (Table 4.3). In particular, compared to the AC- treatments, AC+ seedlings consistently showed higher root and total biomass in both root and leaf litter substrates, which was statistically significant in all cases, with the only exception of total biomass over leaf litter (Fig. 4.4). Interestingly, AC+ seedlings also showed biomass values non-significantly different from those of AC- seedlings grown on home field soil. Finally, in the home field soil the treatment with AC did not affect seedling performance.

**Table 4.3**. Results of two-ways ANOVA testing for the effects of substrate type and AC treatment on root and total plant mas. Significant P-values are marked in italic font.

| Effect | DoF | SS | MS | F | P |
|---|---|---|---|---|---|
| *Root* | | | | | |
| Substrate type (T) | 2 | 0,000 | 0,000 | 1,031 | 0,3790 |
| AC (A) | 1 | 0,004 | 0,004 | 10,480 | *0,0051* |
| T x A | 2 | 0,006 | 0,003 | 6,992 | *0,0065* |
| Error | 16 | 0,007 | 0,000 | | |
| | | | | | |
| *Total plant* | | | | | |
| Substrate type (T) | 2 | 0,048 | 0,024 | 7,438 | *0,0036* |
| AC (A) | 1 | 0,002 | 0,002 | 0,849 | 0,3672 |
| T x A | 2 | 0,029 | 0,014 | 4,468 | *0,0241* |
| Error | 21 | 0,068 | 0,003 | | |

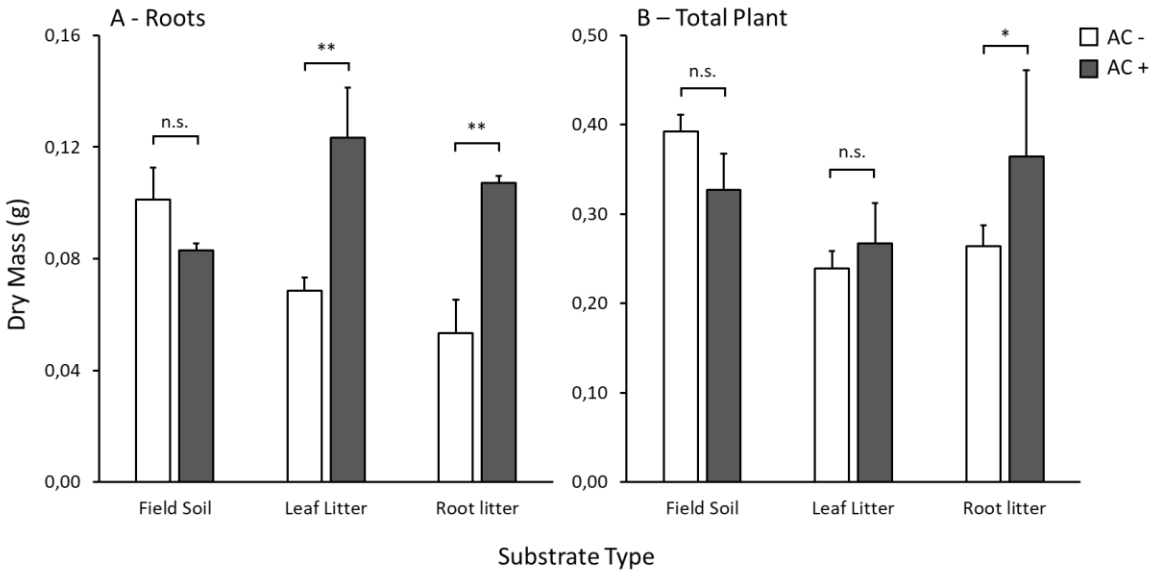


**Fig. 4.4:** Interactive effects of Activated carbon (AC) with three different substrate types on (**A**) root and (**B**) total plant dry mass of beech seedlings after 30 days of growth. Data are marked with asterisks within each substrate type to indicating significant differences (***, P < 0.001; **, P < 0.01; *, P < 0.05; n.s. > 0.05; post hoc Duncan tests from a one way Anova in Table 4.3).

The substrate sterilization treatment produced significant effects only in interaction with the substrate type (Table 4.5). In particular, ST+ seedlings grown over litter substrates did not show significantly different performance as compared to ST- seedlings, with the exception of total plant mass over leaf litter, which was slightly but significantly higher in ST+ seedlings (Fig. 4.6). More interestingly, home filed soil effect was completely different between ST- and ST+ treatments, with the latter showing a remarkable negative effect due to the removal of the home filed microbial community.

**Table 4.5**. Results of two-ways ANOVA testing for the effects of Conditioning and Sterilization on Root and on total plant. Significant P-values are marked in italic font.

| Effect | DoF | SS | MS | F | P |
|---|---|---|---|---|---|
| *Root* | | | | | |
| Substrate type (T) | 2 | 0,000 | 0,000 | 2,579 | 0,1089 |
| Sterilization (S) | 1 | 0,000 | 0,000 | 1,109 | 0,3088 |
| T x S | 2 | 0,004 | 0,002 | 11,442 | *0,0009* |
| Error | 15 | 0,002 | 0,000 | | |
| | | | | | |
| *Total plant* | | | | | |
| Substrate type (T) | 2 | 0,006 | 0,003 | 2,955 | 0,0808 |
| Sterilization (S) | 1 | 0,001 | 0,001 | 1,527 | 0,2343 |
| T x S | 2 | 0,045 | 0,022 | 19,597 | *< 0,0001* |
| Error | 16 | 0,018 | 0,001 | | |

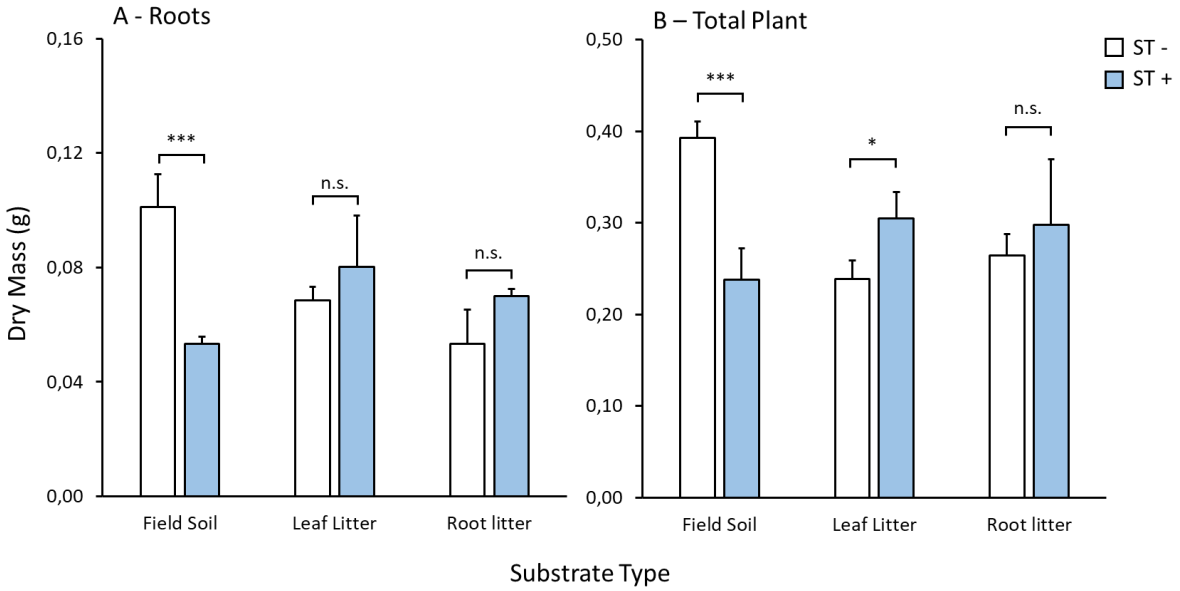


**Fig. 4.6:** Interactive effects of Sterilization (ST) with three different substrate types on (**A**) root and (**B**) total plant dry mass of beech seedlings after 30 days of growth. Data are marked with asterisks within each substrate type to indicating significant differences (***, P < 0.001; **, P < 0.01; *, P < 0.05; n.s. > 0.05; post hoc Duncan tests from a one way Anova in Table 4.5).

**Self-DNA effects**

Addition of DNA to the mesocosms produced apparently contrasting results, showing a significant first order effect on root mass, but not on total plant biomass, while the opposite patter held for the interaction DNA × substrate type (Table 4.4). In particular, for both root and total plant mass, DNA+ seedlings showed a significantly worst performance compared to DNA- ones only on the home filed soil (Fig. 4.5), while in the case of the litter substrates the self-DNA effects were not statistically significant with the possible exception of the root biomass in DNA+ seedlings grown over root litter, which showed lower mean values compared to the corresponding DNA- ones, but the pairwise Duncan's test comparing the two means produced a borderline p-value (P=0.06).

**Table 4.4**. Results of two-ways ANOVA testing for the effects of substrate type and DNA on root and total plant mass. Significant P-values are marked in italic font.

| Effect | DoF | SS | MS | F | P |
|---|---|---|---|---|---|
| *Root* | | | | | |
| Substrate type (T) | 2 | 0,005 | 0,002 | 21,667 | *< 0.0001* |
| DNA (D) | 1 | 0,001 | 0,001 | 15,208 | *0,0014* |
| T x D | 2 | 0,000 | 0,000 | 1,61 | 0,2322 |
| Error | 15 | 0,001 | 0,000 | | |
| | | | | | |
| *Total plant* | | | | | |
| Substrate type (T) | 2 | 0,036 | 0,018 | 7,962 | *0.0033* |
| DNA (D) | 1 | 0,001 | 0,001 | 0,678 | 0,4209 |
| T x D | 2 | 0,027 | 0,013 | 6,041 | *0,0098* |
| Error | 18 | 0,041 | 0,002 | | |

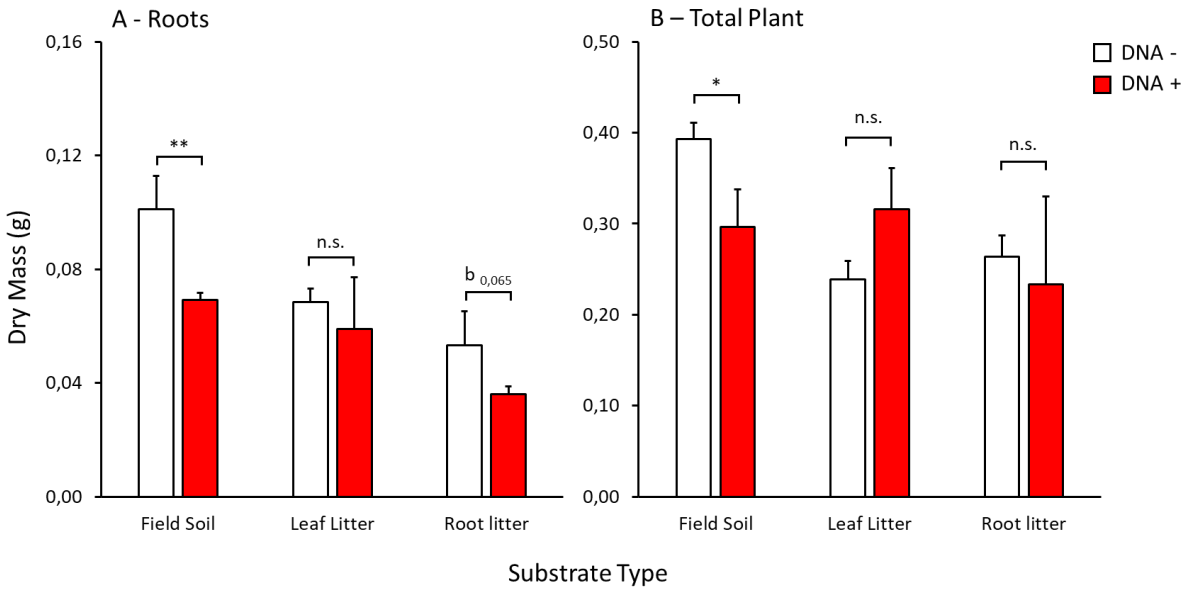


**Fig. 4.5:** Interactive effects of DNA with three different substrate type (Field soil, Leaf Litter and Root Litter) on (**A**) root and (**B**) whole plant dry mass of beech seedling (*F. sylvatica*) after 30 days of growth. Data are marked with asterisks within each substrate type to indicating significant differences (***, P < 0.001; **, P < 0.01; *, P < 0.05; b = Borderline; n.s. > 0.05; post hoc Duncan tests from a two way Anova in Table 4.4).

### High-order factorial effects

The fully factorial ANOVA model jointly considering all the experimental combinations of substrate type, treatment and DNA addition produced several significant effects, intrinsically difficult to disentangle, especially in the case of 3$^{rd}$ and 4$^{th}$ order effects. Then, the results are presented in Table 4.5 and 4.6, but will be analysed in-depth elsewhere or in future reports.

**Table 4.5**. Results of four-ways ANOVA testing for the effects of Substrate Type, DNA, AC, and ST on root and total plant mass. Significant P-values are marked in italic font.

| Effect | DoF | SS | MS | F | P |
|---|---|---|---|---|---|
| *Root mass* | | | | | |
| Substrate Type (T) | 2 | 3.939 | 1.969 | 5.955 | *0.0045* |
| DNA (D) | 1 | 10.080 | 10.080 | 30.475 | *< 0.0001* |
| AC (A) | 1 | 21.679 | 21.679 | 65.545 | *< 0.0001* |
| ST (S) | 1 | 0.140 | 0.140 | 0.422 | 0.5186 |
| T × D | 2 | 0.971 | 0.486 | 1.468 | 0.2391 |
| T × A | 2 | 4.827 | 2.414 | 7.298 | *0.0015* |
| D × A | 1 | 1.087 | 1.087 | 3.287 | 0.0752 |
| T × S | 2 | 6.235 | 3.117 | 9.425 | *0.0003* |
| D × S | 1 | 0.044 | 0.044 | 0.134 | 0.7153 |
| AC × ST | 1 | 0.481 | 0.481 | 1.455 | 0.2327 |
| T x D x A | 2 | 7.253 | 3.627 | 10.965 | *< 0.0001* |
| T x D x S | 2 | 4.011 | 2.005 | 6.063 | *0.0041* |
| T x A x S | 2 | 17.139 | 8.570 | 25.910 | *< 0.0001* |
| D x A x S | 1 | 0.634 | 0.634 | 1.918 | 0.1716 |
| T x D x A x S | 2 | 0.130 | 0.065 | 0.197 | 0.8221 |
| Error | 56 | 18.522 | 0.331 | | |

**Table 4.6**. Results of four-ways ANOVA testing for the effects of Substrate Type, DNA, AC, and ST on total plant mass. Significant P-values are marked in italic font.

| Effect | DoF | SS | MS | F | P |
|---|---|---|---|---|---|
| *Total plant mass* | | | | | |
| Substrate Type (T) | 2 | 36,246 | 18,123 | 5,995 | *0,0041* |
| DNA (D) | 1 | 14,924 | 14,924 | 4,937 | *< 0.0001* |
| AC (A) | 1 | 73,269 | 73,269 | 24,239 | *< 0.0001* |
| ST (S) | 1 | 0,480 | 0,480 | 0,159 | 0,6917 |
| T × D | 2 | 8,969 | 4,485 | 1,484 | 0,2346 |
| T × A | 2 | 51,288 | 25,644 | 8,484 | *0,0005* |
| D × A | 1 | 0,051 | 0,051 | 0,017 | 0,8971 |
| T × S | 2 | 34,307 | 17,154 | 5,675 | *0,0054* |
| D × S | 1 | 11,290 | 11,290 | 3,735 | 0,0578 |
| AC × ST | 1 | 13,650 | 13,650 | 4,516 | 0,0375 |
| T x D x A | 2 | 60,111 | 30,056 | 9,943 | *< 0.0001* |
| T x D x S | 2 | 26,396 | 13,198 | 4,366 | *0,0168* |
| T x A x S | 2 | 76,727 | 38,363 | 12,692 | *< 0.0001* |
| D x A x S | 1 | 2,693 | 2,693 | 0,891 | 0,3488 |
| T x D x A x S | 2 | 6,197 | 3,098 | 1,025 | 0,3647 |
| Error | 63 | 190,433 | 3,023 | | |

## 4.5. Discussion

This work demonstrated how the growth of beech seedlings is strongly influenced by the type of growing substrate and that the putative causal agents for the substrate–dependent effects differ for different substrates. Considering the response to different substrates, a mechanistic hypothesis might be provided by nitrogen immobilization in aged litter (Michelsen et al., 1995). Indeed, high C-to-N ratio (>30) in the substrate, as that we observed in root litter, can correspond to low N availability, bearing a negative effect on seedling performance (Hodge et al., 2000, Bonanomi et al., 2010). Then, N immobilization could apparently explain our result in the case of seedlings grown on decomposed root litter vs. home soil, but not for seedlings grown on aged leaf litter, which showed the same growth inhibition, with C-to-N not differing from that of the home soil. However, in both treatments litter was added to a N-rich mixing pot, purposely used to avoid nutrient shortage during the growing period. This, in addition to a relatively short growing phase, allows to exclude an effect of N immobilization on the growth response to substrate type. Therefore, further processes should be taken into account.

The different treatments considered in this study (self-DNA addition, sterilization or activated carbon addition) strongly changed the seedling fitness in terms of biomass production over the growing period. The higher seedling performance in the home field soil as compared to that observed in leaf and root litters could be ascribable to an inhibitory effect of litter chemicals (Inderjit, 1996), as being neutralized by the active carbon addition, rather than on biological effects mediated by the decomposer community, as it is not released by substrate sterilization. In parallel, the remarkable negative effect observed in sterilized home field soil, due to the removal of the home filed microbial community, clearly confirms that inhibition by conspecific litter is not related to the litter microbiome, while the same result demonstrates a clear beneficial effect of the home soil microbiome on the seedling performance, possibly related to HFA effects. Consistently, in a

study of soil feedbacks on seedling growing, Wurst et al. (2015) showed that beech recruitment is advantaged by home field, suggesting that the species may be less sensitive to soil-borne pathogens. The ability of a plant to shape the decomposer community (Austin et al., 2014) creating specific microbiomes (Keiser et al., 2011; Lin et al., 2019) and resulting in a plant-specific patterns in nutrient release (Perez et al., 2013) can contribute to HFA (Veen et al., 2018). The sterilization, with the associated alteration of the beneficial microbiome (Austin et al., 2014) may have resulted in a diminished capability to acquire nutrients from the substrate, explaining the reduced grown. Differently, the sterilization of the litter substrate produced an increase of seedling growth, especially observable over leaf litter. When leaf litter falls on forest ground the composition of the litter microbiome may be determined by the order in which species immigrate and colonize the litter (Fukami, 2015). Different studies demonstrated that microbiome can differ between plant species (Persoh, 2013) and genotypes (Wagner et al., 2016) and can depend on plant functional traits (Kembel et al., 2014) or even environmental conditions (Kraft et al., 2015).

The addition of a great quantity of self exDNA produced a remarkable negative impact on the seedlings grown over the home field soil compared to that in leaves and root litter. These results could appear surprising, as at first glance not completely supporting the occurrence of inhibition by self-DNA in the tested system. However, it is of primary relevance the fact that we observed self-DNA inhibition in the combination of experimental conditions closest to the real system, in terms of substrate molecular and biological composition, where the microbiome was unaltered, and the DNA addition reliably mimicked conspecific DNA accumulation after release by repeated litterfall yearly cycles.

On the other hand, the absence of remarkable self-DNA inhibition by both litter substrate types could be related to the different dosage, in relative terms, of the DNA addition in litter DNA+ treatments as compared to soil DNA+ treatment that produced the inhibition. Indeed, the same

quantity of self-DNA was added to all the substrates, but it is clear that the DNA- field soil could have contained at most negligible beech DNA amounts, in comparison to the DNA- litters. On the other hand, the decomposed litter materials contained an amount of self-DNA comparable to that then added in DNA+ mesocosms. In particular, each DNA- seedling grown over conspecific litter was in facts exposed to self-DNA, with a dose of approximately 200 μg per seedling (corresponding to 10 g of litter multiplied by the self-DNA content estimated by the extraction yield reported in Table 3.2.1, possibly slightly variable between leaf and root materials) and such materials produced an inhibitory effect. The inhibition removal by AC treatment indicates that such inhibition could be unrelated to self-DNA. Then, in litter DNA+ treatments, the addition of 200 μg of self-DNA did not exacerbated the inhibitory effect, which of course does not imply that self-DNA had not an effect.

Most of the available evidence on the self-DNA inhibition relies on *in vitro* tests in which, in the case of plants, seeds are exposed to DNA solutions over filter papers in Petri dishes, therefore in absence of a true substrate where all the DNA molecule sin the solution are biologically available. Therefore, a possible confounding effect of self-DNA bioavailability in our mesocosms cannot be completely excluded. The only previous evidence of self-DNA inhibition carried out on plants in mesocosms in presence of an organic substrate, was reported by Mazzoleni et al. (2015a), who tested the effect of self-DNA on *Medicago sativa* grown in a glasshouse experiment at different treatment concentrations. In suich test, remarkably higher dosages were applied as compared to our bioassay, making speculative any effect cpomparison. In any case, it is undoubtfully interesting to further test the reliability of self-DNA inhibition in fielde conditions in beech-spruce forest, as it could provide a new explanation underlying tree species substitution dynamics (Fox, 1977; Oliver & Larson, 1990; Del Favero, 1998). Accordingly, the starting phase of the substituiion would take place after the persistence in soil of exDNA from either species that, locally accumulating by litter decay, would prevent a proper development of conspecific recruitment, hence favouring that of other

species. In order to explore such fascinating hypothesis, further studies should be carred out, firstly addressing self-DNA bioavailability in field conditions, in the soil layers where seedling roots develop, and then testing the species-specificity of the inhibitory effect in mesocosms representative of the actual field conditions. In then latter case, cross-factorial tests (e.g. exposing beech seedlings to spruce DNA and viceversa) should be considering, extending the conditions explored in our study.

## 4.6. Conclusions

The results of this study allowed to clearly disentangle the effects of the home field soil microbiome to those of litter chemicals on *Fagus sylvatica* seedling growth. In particular, the home-field advantage holds for beech seedlings as compared to litter-conditioned substrates, due to two main drivers which act in parallel. On one hand, the inhibitory effect of litter chemicals, more pronounced for root litter as compared to leaf litter, which was clearly removed by treating the litter substrate with active carbon. On the other hand, we clearly demonstrated a positive enhancing effect of the home soil microbiome, which is distinctively removed by sterilizing the home soil.

Interestingly we indirectly demonstrate the wide range of effects produced by different substrate microbiomes, which can be clearly positive, as in the HFA effect of the home soil, but also clearly negative, as observed for leaf litter substrate, or even neutral as in the case of root litter microbiome, at least as resulting from the sterilization of the substrates carried out in our bioassay, and certainly limited to the tested experimental conditions. The very interesting and previously reported functional role of self-DNA as a causal agent of species-specific plant-soil negative feedback was confirmed by our findings in the mesocosms more representative of the real environmental conditions. In the other tested conditions, in the conspecific litter substrates, the result is only apparently inconsistent with the previous findings, likely due to a problematic

evaluation of the dosage effect in exposed vs. unexposed experimental groups, as well as concerning the self-DNA bioavailability. Therefore, further investigation of the self-DNA biological and ecological functions is certainly needed, testing more and more species and substrates, under realistic experimental set ups, representative of the real field conditions.

# Chapter 5

# General conclusions

The aims of this PhD thesis were multiple and interconnected. First of all, we wanted to deepening the knowledge of exDNA distribution in natural ecosystems. Fascinated by the discovery of the self-DNA inhibition principle by Mazzoleni et al., we selected a beech-spruce forest of Fusine Lakes (UD) in NE Italy and we focused on the two target species (*F. sylvatica* and *P. abies*) that are dominant and subjected to still unclear species alternation mechanism, to test both the reliability of the functional role of extracellular self-DNA based on the actual quantitate distributional pattern, and its biological effects on the target species seedlings. To the first aim, being able to separately extract iDNA and exDNA from the field soil samples was providential and opened the possibility of a further assessment on the more labile exDNA fraction with the metabarcoding approach. The observation of a direct correlation between the abundance aboveground of the two target species and the abundance in the soil of the species-specific exDNA provided new information on the functioning of the exanimated system, showing that plant exDNA accumulates in soil not only in relation to the more easily detectable input of plant material aboveground by litterfall, but also, more intriguingly, that the contribution of belowground processes such as root turnover, which occur at different soil depth for different species, must be taken into account. Also considering the idea that exDNA in soil can be protected from degradation, and persist for a long period of time, our findings lead to the new conclusion that exDNA follows a species-specific differential distribution along the soil profile. Accordingly, the report presented in Chapter 2 of this thesis has been recently submitted to international journal *Soil Biology and Biochemistry*.

The second aim of this thesis was to test the possibility to produce large amounts of plant DNA labelled with stable isotopes in such a way to making possible further experimental field studies exploring DNA fate in real field conditions. As the dynamics of plant tissue isotopic labelling during vegetative growth were known, but much less was clarified about the same dynamics concerning plant nucleic acids, a simple preliminary test to explore plant DNA labelling with $^{15}N$, oriented to

maximize the extract quality while minimizing the production costs, was tested in controlled condition in a greenhouse experiment on *Brassica napus*. Furthermore, the possible isotopic fractionation effect during plant vegetative growth was investigated. We clarified the dynamics of $^{15}N$ incorporation in plant DNA during vegetative growth as a function of the total N availability in the pot solution, for different labelled chemical species of the fertilizer. A such, the results presented in the first section of Chapter 3 were published in the international journal *PLoS ONE*. Moreover, we found negligible effects of enzymatic $^{15}N$ discrimination, and a high level of DNA labelling by heavily $^{15}N$ enriched NH4 fertilization, which both opened the possibility to cultivate labelled plant for subsequent $^{15}N$ DNA extraction. This possibility was tested setting up a semi-automated fertirrigation system on 56 juveniles of our target tree species (*Fagus sylvatica*), showing positive results in terms of the level of isotopic signature of both the leaf material and the DNA thereof purified, especially considering the remarkable level of $^{15}N$ content in the DNA, which shall be used in further studies about exDNA tracking in natural field conditions.

The third study of this PhD thesis was linked to the recent developments in the field of plant-soil negative feedback research, in particular about the proposed causal role of plant self-DNA in species-specific NF, and the main aim was to provide a first contribution from beech forest systems, testing the occurrence of rhizosphere-and litter-mediated effects on beech seedling, and their interplay with self-DNA, under more realistic experimental conditions as compared to previous studies about the self-DNA inhibition. Our bioassay on *F. sylvatica* cross-factorially testing the effect of substrate type, its interaction with the known causal agents of species-specific NF (substrate microbiome and chemicals) and their interplay with self-DNA addition provided promising results. In particular, a beneficial effect of the home soil microbiome and a detrimental effect of litter chemicals we confirmed for the first time on beech seedlings. The principle of self-DNA inhibition holds also when tested under realistic experimental conditions. However, clarifying the functional

role of self-DNA within the interplay between litter- and rhizosphere-mediated effects requires further investigation, mostly due to a problematic assessment of self-DNA bioavailability in the complex matrix of soil amendments. Besides its relevance for pure knowledge in natural ecosystems, such issue is of foremost importance in the view of self-DNA applications in agro-ecosystems, for the species-specific control of weeds and pathogens.

# References


Abramo E, Michelutti G (1998). Guida ai suoli forestali nella regione Friuli-Venezia Giulia. Regione Autonoma Friuli-Venezia Giulia, Direzione Regionale delle Foreste servizio della selvicoltura.

Agnelli A, Ascher J, Corti G, Ceccherini MT, Nannipieri P, and Pietramellara G (2004). Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DGGE of total and extracellular DNA. *Soil Biology and Biochemistry*, 36: 859-868.

Agnelli A, Ascher J, Corti G, Ceccherini MT, Pietramellara G, and Nannipieri P (2007). Purification and isotopic signatures ($\delta^{13}$ C, $\delta^{15}$ N, $\Delta^{14}$ C) of soil extracellular DNA. *Biology and Fertility of Soils*, 44: 353-361.

Albers D, Migge S, Schaefer M and Scheu S (2004). Decomposition of beech leaves (*Fagus sylvatica*) and spruce needles (*Picea abies*) in pure and mixed stands of beech and spruce. *Soil Biology and Biochemistry,* 36: 155-164.

Alberti G, Boscutti F, Pirotti F, Bertacco C, De Simon G, Sigura M, Cazorzi F and Bonfanti P (2013). A LiDAR-based approach for a multi-purpose characterization of Alpine forests: An Italian case study. *iForest-Biogeosciences and Forestry*, 6: 156.

An M, Pratley JE, and Haig T (2001). Phytotoxicity of vulpia residues: IV. Dynamics of allelochemicals during decomposition of vulpia residues and their corresponding phytotoxicity. *Journal of Chemical Ecology*, 27: 395-409.

Anderson MA, Cleland WW, Huang DT, Chan C, Shojaei M, Christopherson RI (2006). $^{13}$C and $^{15}$N isotope effects for conversion of l-Dihydroorotate to N-Carbamyl-l-aspartate using dihydroorotase from hamster and bacillus caldolyticus. *Biochemistry*.; 45: 7132–7139.

Andersson M, Kjøller A and Struwe S (2004). Microbial enzyme activities in leaf litter, humus and mineral soil layers of European forests. *Soil Biology and Biochemistry*, 36: 1527-1537.

Ascher J, Ceccherini MT, Pantani OL, Agnelli A, Borgogni F, Guerri G, Nannipieri P and Pietramellara G (2009). Sequential extraction and genetic fingerprinting of a forest soil metagenome. *Applied Soil Ecology*, 42: 176-181.

Asplund J, Hustoft E, Nybakken L, Ohlson M and Lie MH (2018). Litter impair spruce seedling emergence in beech forests: a litter manipulation experiment. *Scandinavian journal of forest research*, 33: 332-337.

Attiwill PM, Adams MA (1993) Nutrient cycling in forests. *New Phytologist*, 124:561–582

Austin AT, Vivanco L, González-Arzac A and Pérez LI (2014). There's no place like home? An exploration of the mechanisms behind plant litter–decomposer affinity in terrestrial ecosystems. *New Phytologist*. 204: 307–314.

Baker RT (1977). Humic acid-associated organic phosphate. *New Zealand Journal of Science* 20: 439–441.

Barbero F, Guglielmotto M, Capuzzo A and Maffei ME (2016). Extracellular self-DNA (esDNA), but not heterologous plant or insect DNA (etDNA), induces plasma membrane depolarization and calcium signaling in lima bean (*Phaseolus lunatus*) and maize (*Zea mays*). *International Journal of Molecular Sciences*, 17: 1659.

Bell KL, Loeffler VM and Brosi BJ (2017). An rbcL reference library to aid in the identification of plant species mixtures by DNA metabarcoding. *Applications in plant sciences*, 5.

Bell T, Freckleton RP and Lewis OT (2006). Plant pathogens drive density-dependent seedling mortality in a tropical tree. *Ecology letters*, 9: 569-574.

Bennett JA, Maherali H, Reinhart KO, Lekberg Y, Hart MM and Klironomos J (2017). Plant-soil feedbacks and mycorrhizal type influence temperate forest population dynamics. *Science*, 355: 181-184.

Berg B and Staaf H (1981). Leaching, accumulation and release of nitrogen in decomposing forest litter. *Ecological Bulletins*, 163-178.

Berger TW and Berger P (2012). Greater accumulation of litter in spruce (*Picea abies*) compared to beech (*Fagus sylvatica*) stands is not a consequence of the inherent recalcitrance of needles. *Plant and soil*, 358: 349-369.

Berger TW and Berger P (2014). Does mixing of beech (*Fagus sylvatica*) and spruce (*Picea abies*) litter hasten decomposition? *Plant and soil*, 377: 217-234.

Bergersen FJ, Peoples MB, Turner GL (1988). Isotopic discriminations during the accumulation of nitrogen by soybeans. *Plant Physiologist*, 15: 407–420.

Bever JD (2003). Soil community feedback and the coexistence of competitors: conceptual frameworks and empirical tests. *New phytologist*, 157: 465-473.

Bever JD, Platt TG and Morton ER (2012). Microbial population and community dynamics on plant roots and their feedbacks on plant communities. *Annual review of microbiology*, 66: 265-283.

Bever JD, Westover KM and Antonovics J (1997). Incorporating the soil community into plant population dynamics: the utility of the feedback approach. *Journal of Ecology*, 561-573.

Bhat A, Ryu CM (2016). Plant Perceptions of Extracellular DNA and RNA. *Molecular plant* 9: 956–958.

Bohmann K, Evans A, Gilbert MTP, Carvalho GR, Creer S, Knapp M, Yu DW and de Bruyn M (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution*, 29: 358–367.

Bonanomi G, Antignani V, Barile E, Lanzotti V and Scala F (2011). Decomposition of Medicago sativa residues affects phytotoxicity, fungal growth and soil-borne pathogen diseases. *Journal of Plant Pathology*, 57-69.

Bonanomi G, Cesarano G, Lombardi N, Motti R, Scala F, Mazzoleni S and Incerti G (2017). Litter chemistry explains contrasting feeding preferences of bacteria, fungi, and higher plants. *Scientific reports*, 7: 1-13.

Bonanomi G, Giannino F and Mazzoleni S (2005). Negative plant–soil feedback and species coexistence. *Oikos*, 111: 311-321.

Bonanomi G, Incerti G, Antignani V, Capodilupo M, Mazzoleni S (2010). Decomposition and nutrient dynamics in mixed litter of Mediterranean species. *Plant and Soil*, 331: 481–496.

Bonanomi G, Incerti G, Barile E, Capodilupo M, Antignani V, Mingo A, Lanzotti V, Scala F and Mazzoleni S (2011). Phytotoxicity, not nitrogen immobilization, explains plant litter inhibitory effects: evidence from solid-state 13C NMR spectroscopy. *New Phytologist*, 191: 1018-1030.

Bonanomi G, Incerti G, Giannino F, Mingo A, Lanzotti V, Mazzoleni S (2013). Litter quality assessed by solid state 13C NMR spectroscopy predicts decay rate better than C/N and lignin/N ratios. Soil Biological and Biochemistry, 56:40–48

Borgogni F, Lavecchia A, Mastrolonardo G, Certini G, Ceccherini MT and Pietramellara G (2019). Immediate-and Short-term Wildfire Impact on Soil Microbial Diversity and Activity in a Mediterranean Forest Soil. *Soil Science*, 184: 35-42.

Boto L, Pineda M and Pineda R (2019). Potential impacts of horizontal gene transfer on human health and physiology and how anthropogenic activity can affect it. *The FEBS journal*, 286: 3959-3967.

Bowman GM and Hutka J (2002) Particle Size Analysis. In Soil Physical Measurement and Interpretation for Land Evaluation (Eds N McKenzie, K Coughlan, H Cresswell) pp 224-239. CSIRO Publishing: Victoria

Bradford MA, Berg B, Maynard DS, Wieder WR, Wood SA (2016). Understanding the dominant controls on litter decomposition. *Journal of Ecology*, 104:229–238

Brand WA (2011). New reporting guidelines for stable isotopes – an announcement to isotope users. *Isotopes in environmental and health studies*, 47: 535–536.

Brand WA and Coplen TB (2012). Stable isotope deltas: tiny, yet robust signatures in nature. *Isotopes in environmental and health studies*, 48: 393-409.

Brinson MM, Bradshaw HD, Holmes RN and Elkins Jr JB (1980). Litterfall, stemflow, and throughfall nutrient fluxes in an alluvial swamp forest. *Ecology*, 61: 827-835.

Bromand S, Whalen JK, Janzen HH, Schjoerring JK and Ellert BH (2001). A pulse-labelling method to generate 13C-enriched plant materials. *Plant and Soil*, 235: 253-257.

Cadisch G, Espana M, Causey R, Richter M, Shaw E, Morgan JAW, Rahn C and Bending GD (2005). Technical considerations for the use of 15N-DNA stable-isotope probing for functional microbial activity in soils. Rapid Communications in *Mass Spectrometry: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry*, 19: 1424-1428.

Carini P, Marsden PJ, Leff JW, Morgan EE, Strickland MS, Fierer N (2016) Relic DNA is abundant in soil and obscures estimates of soil microbial diversity. *Nature microbiology*, 2: 1-6.

Carlson C, Pierson LS, Rosen JJ, Ingraham JL (1983). *Pseudomonas stutzeri* and related species undergo natural transformation. *Journal of Bacteriology* 153: 93–99.

Carlson C, Steenbergen SA, Ingraham JL (1984). Natural transformation of Pseudomonas stutzeri by plasmids that contain cloned fragments of chromosomal deoxyribonucleic acid. *Archives of Microbiology* 40: 134–138.

Cartenì F, Bonanomi G, Giannino F, Incerti G, Vincenot CE, Chiusano ML and Mazzoleni S (2016). Self-DNA inhibitory effects: underlying mechanisms and ecological implications. *Plant signaling & behavior*, 11: e1158381.

Cartenì F, Marasco A, Bonanomi G, Mazzoleni S, Rietkerk M and Giannino F (2012). Negative plant soil feedback explaining ring formation in clonal plants. *Journal of theoretical biology*, 313: 153-161.

CBol Group, Hollingsworth PM, Forrest LL, Spouge JL, Hajibabaei M, Ratnasingham S et al (2009). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences*, 106: 12794-12797.

Ceccherini M, Pote´ J, Kay E, Van VT, Marechal J, Pietramellara G, Nannipieri P, Vogel TM, Simonet P (2003). Degradation and transformability of DNA from transgenic leaves. *Applied and Environmental Microbiology*, 69: 673–678.

Ceccherini MT, Ascher J, Pietramellara G, Vogel TM and Nannipieri P (2007). Vertical advection of extracellular DNA by water capillarity in soil columns. *Soil Biology and Biochemistry*, 39: 158-163.

Ćeranić A, Doppler M, Büschl C, Parich A, Xu K, Koutnik, A and Schuhmacher R (2020). Preparation of uniformly labelled 13 C-and 15 N-plants using customised growth chambers. *Plant methods*, 16: 1-15.

Cesarano G, Zotti M, Antignani V, Marra R, Scala F, Bonanomi G. (2017). Soil sickness and negative plant-soil feedback: A reappraisal of hypotheses. *Journal of Plant Pathology*, 99: 545-570.

Cheng T, Xu C, Lei L, Li C, Zhang Y and Zhou S (2016). Barcoding the kingdom Plantae: new PCR primers for ITS regions of plants with improved universality and specificity. *Molecular ecology resources* 16: 138-149.

Chomel M, Guittonny-Larchevêque M, Fernandez C, Gallet C, DesRochers A, Paré D., Jakson JB and Baldy V (2016). Plant secondary metabolites: a key driver of litter decomposition and soil nutrient cycling. *Jurnal of Ecology*, 104: 1527–1541.

Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A, Vermat T et al (2007). Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic Acids Research* 3.

Collins RA, Wangensteen OS, O'Gorman EJ, Mariani S, Sims DW and Genner MJ (2018). Persistence of environmental DNA in marine systems. *Communications Biology*, 1: 1-11.

Connell JH (1971). On the role of natural enemies in preventing competitive exclusion in some marine animals and in rain forest trees. *Dynamics of populations*, 298, 312.

Coplen TB. (2011) Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. Rapid Communications in Mass Spectrometry; 25: 2538–2560.

Craine JM, Brookshire ENJ, Cramer MD, Hasselquist NJ, Koba K, Marin-Spiotta E and Wang L (2015). Ecological interpretations of nitrogen isotope ratios of terrestrial plants and soils. *Plant and Soil*, 396: 1-26.

Crecchio C, Ruggiero P, Curci M, Colombo C, Palumbo G and Stotzky G (2005). Binding of DNA from Bacillus subtilis on montmorillonite–humic acids–aluminum or iron hydroxypolymers: effects on transformation and protection against DNase. *Soil Science Society of America Journal*, 69: 834-841.

Crecchio, C, Stotzky, G, 1998. Binding of DNA on humic acids: effect on transformation of Bacillus subtilis and resistance to DNase. Soil Biology & Biochemistry 30, 1060–1067

Cui J, Lamade E, Fourel F, Tcherkez G. (2020) δ15N values in plants are determined by both nitrate assimilation and circulation. *New Phytology*, 226: 1696-1707.

De Nobili M and Petrussi F (1988). Humification index (HI) as evaluation of the stabilization degree during composting. *Journal of fermentation technology*, 66: 577-583.

De Vries J and Wackernagel W (2005). Microbial horizontal gene transfer and the DNA release from transgenic crop plants. *Plant and Soil*, 266: 91-104.

De Vries J, Heine M, Harms K, Wackernagel W (2003). Spread of recombinant DNA by roots and pollen of transgenic potato plants, identified by highly specific biomonitoring using natural transformation of an *Acinetobacter sp*. *Applied and Environmental Microbiology* 69: 4455–4462.

Deininger PL (1983). Random subcloning of sonicated DNA: application to shotgun DNA sequence analysis. *Analytical biochemistry*, 129: 216-223.

Del Fabbro C, Scalabrin S, Morgante M, Giorgi, FM (2013). An extensive evaluation of read trimming effects on Illumina NGS data analysis. *PloS one*, 8: e85024.

Del Favero R (2004) I boschi delle regioni alpine italiane. CLEUP, Padova, 595 pp.

Del Favero R, Poldini L and Bortoli PL (1998). La vegetazione forestale e la selvicoltura nella regione Friuli-Venezia Giulia. Regione Autonoma Friuli-Venezia Giulia, Direzione Regionale delle Foreste servizio della selvicoltura.

Demaneche S, Jocteur-Monrozier L, Quiquampoix H, Simonet P (2001). Evaluation of biological and physical protection against nuclease degradation of clay-bound plasmid DNA. *Applied and Environmental Microbiology* 67, 293–299.

Dias T, Dukes A, Antunes PM (2015). Accounting for soil biotic effects on soil health and crop productivity in the design of crop rotations. *Journal of the Science of Food and Agriculture*, 95: 447–454

Diepenbrock W (2000). Yield analysis of winter oilseed rape (*Brassica napus L*.): a review. *Field Crops Research,* 67: 35-49.

Dong W, Xu C, Cheng T, Lin K and Zhou S (2013). Sequencing angiosperm plastid genomes made easy: a complete set of universal primers and a case study on the phylogeny of Saxifragales. *Genome Biology and Evolution*, 5: 989-997.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bullettin*, 19: 11–15.

Duran-Flores D and Heil M (2015). Growth inhibition by self-DNA: a phenomenon and its multiple explanations. *New Phytologist*, 207: 482-485.

Duran-Flores D and Heil M (2016). Sources of specificity in plant damaged-self recognition. *Current Opinion in Plant Biology*, 32: 77-87.

Duran-Flores D and Heil M (2018). Extracellular self-DNA as a damage-associated molecular pattern (DAMP) that triggers self-specific immunity induction in plants. *Brain, Behavior, and Immunity*, 72: 78-88.

Edgar RC (2010). Usearch. Lawrence Berkeley National Lab.(LBNL), Berkeley, CA (United States), https://www.drive5.com/usearch/

Edgar RC, Haas BJ, Clemente JC, Quince C and Knight R (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, 27: 2194-2200.

Ehrenfeld JG, Ravit B and Elgersma K (2005). Feedback in the plant-soil system. Annual Review of Environment and Resources, 30: 75-115.

Elgersma KJ, Yu S, Vor T and Ehrenfeld JG (2012). Microbial-mediated feedbacks of leaf litter on invasive plant growth and interspecific competition. *Plant and Soil* 356: 341–355.

England LS and Trevors JT (2003). The microbial DNA cycle in soil. *Biology Forum* 96, 317–326.

Epp L (2012). Environmental DNA barcoding to track ancient Arctic ecosystem changes. *Quaternary International,* 279-280: 132-133

Ettema CH and Wardle DA (2002). Spatial soil ecology. *Trends in ecology & evolution*, 17: 177-183.

Evans RD (2001). Physiological mechanisms influencing plant nitrogen isotope composition. *Trends in Plant Science*, 6: 121–126.

Evans RD, Bloom AJ, Sukrapanna SS, Ehleringer JR. (1996). Nitrogen isotope composition of tomato (*Lycopersicon esculentum* Mill. Cv. T-5) grown under ammonium or nitrate nutrition. *Plant, Cell and Environment*, 19: 1317–1323.

Eveleigh ES, McCann KS, McCarthy PC, Pollock SJ, Lucarotti CJ, Morin B, McDougall GA, Strongman DB, Huber JT, Umbanhowar J and Faria, L. D. (2007). Fluctuations in density of an outbreak species drive diversity cascades in food webs. *Proceedings of the National Academy of Sciences*, 104: 16976-16981.

Facelli JM and Pickett ST (1991). Plant litter: its dynamics and effects on plant community structure. *The botanical review*, 57: 1-32.

Fahner NA, Shokralla S, Baird DJ and Hajibabaei M (2016). Large-scale monitoring of plants through environmental DNA metabarcoding of soil: recovery, resolution, and annotation of four DNA markers. *PloS one*, 11: e0157505.

Felton A, Lindbladh M, Brunet J and Fritz Ö (2010). Replacing coniferous monocultures with mixed-species production stands: an assessment of the potential benefits for forest biodiversity in northern Europe. *Forest ecology and management,* 260: 939-947.

Felton A, Nilsson U, Sonesson J, Felton AM, Roberge JM, Ranius T et al (2016). Replacing monocultures with mixed-species stands: Ecosystem service implications of two production forest alternatives in Sweden. *Ambio*, 45: 124-139.

Ficetola GF, Miaud C, Pompanon F and Taberlet P (2008). Species detection using environmental DNA from water samples. *Biology letters*, 4: 423-425.

Fitter A (2003). Making allelopathy respectable. *Science*, 301: 1337-1338.

Folloni S, Kagkli DM, Rajcevic B, Guimarães NC, Van Droogenbroeck B, Valicente FH, Eede GVD and Van den Bulcke M (2012). Detection of airborne genetically modified maize pollen by real-time PCR. *Molecular Ecology Resources*, 12: 810-821.

Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MO and Dantas G (2012). The shared antibiotic resistome of soil bacteria and human pathogens. *science*, 337: 1107-1111.

Foscari A, Leonarduzzi G and Incerti G (2021). N uptake, assimilation and isotopic fractioning control δ 15N dynamics in plant DNA: A heavy labelling experiment on Brassica napus L. *PloS one*, 16: e0247842.

Fox JF, (1977). Alternation and coexistence of tree species. *The American Naturalist*, 111(977), 69-89.

Freund DM, Hegeman AD (2017). Recent advances in stable isotope-enabled mass spectrometry-based plant metabolomics. *Current Opinion in Biotechnology,* 43: 41-48.

Fukami T (2015). Historical contingency in community assembly: Integrating niches, species pools, and priority effects. *Annual Review of Ecology, Evolution, and Systematics*, 46: 1–23

Gauthier PP, Lamothe M, Mahé A, Molero G, Nogués S, Hodges M and Tcherkez G (2013). Metabolic origin of δ15N values in nitrogenous compounds from Brassica napus L. leaves. *Plant, cell & environment*, 36: 128-137

Giacobbe A (1929) Arch Bot, 4.

Givnish TJ (1999). On the causes of gradients in tropical tree diversity. *Journal of Ecology*; 87:193-210

Glavac V and Jochheim H (1993). A contribution to understanding the internal nitrogen budget of beech (*Fagus sylvatica L*.). *Trees*, 7: 237-241.

Harper JL (1977). Population biology of plants. *Population biology of plants*.

Hart TB, Hart JA, Murphy PG (1989). Monodominant and species-rich forests of the humid tropics: causes for their co-occurrence. *The American Naturalist* 133: 613-633.

Hattenschwiler S and Gasse P (2005). Soil animals alter plant litter diversity effects on decomposition. *Proceedings of the National Academy of Sciences*, 102, 1519–1524.

Hättenschwiler S, Tiunov AV and Scheu S (2005). Biodiversity and litter decomposition in terrestrial ecosystems. *Annual Review of Ecology, Evolution, and Systematics,* 36: 191-218.

Hawes MC (1990). Living plant cells released from the root cap: A regulator of microbial populations in the rhizosphere? *Plant and Soil*, 129: 19-27.

Hawes MC, Curlango-Rivera G, Wen F, White GJ, VanEtten HD, Xiong ZG (2011) Extracellular DNA: the tip of root defenses? *Plant Science*, 180:741–745.

Hawes MC, Curlango-Rivera G, Xiong ZG, Kessler JO (2012). Roles of root border cells in plant defense and regulation of rhizosphere microbial populations by extracellular DNA 'trapping'. *Plant and Soil,* 355:1–16.

Hayes JM (2004). An introduction to isotopic calculations. Woods Hole, MA, USA: Woods Hole Oceanogaphic Institution;

Haynes RJ (1986). Uptake and assimilation of mineral nitrogen by plants. In: Kozlowski TT, editor. Mineral nitrogen in the plant-soil system. Orlando: Academic Press, pp. 303–358.

Hebert PD, Cywinska A, Ball SL and Dewaard JR (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: *Biological Sciences*, 270: 313-321.

Hille M, den Ouden J. (2005). Charcoal and activated carbon as adsorbate of phytotoxic compounds – a comparative study. *Oikos* 108: 202–207.

Hobbie EA and Högberg P (2012). Nitrogen isotopes link mycorrhizal fungi and plants to nitrogen dynamics. *New phytologist*, 196: 367-382.

Hobbie SE, Oleksyn J, Eissenstat DM and Reich PB (2010). Fine root decomposition rates do not mirror those of leaf litter among temperate tree species. *Oecologia* 162: 505–513.

Hodge A (2004). The plastic plant: root responses to heterogeneous supplies of nutrients. *New phytologist*, 162: 9-24.

Hodge A, Robinson D and Fitter A (2000). Are microorganisms more effective than plants at competing for nitrogen? *Trends in plant science*, 5: 304-308.

Hodge A, Robinson D, Fitter AH. (2000). Are microorganisms more effective than plants at competing for nitrogen? *Trends in Plant Science*, 5: 304–308.

Högberg P (1999). Nitrogen isotope fractionation during nitrogen uptake by ectomycorrhizal and non-mycorrhizal Pinus sylvestris. *New Phytologist*, 142: 569–576.

Hollingsworth PM (2011). Refining the DNA barcode for land plants. *Proceedings of the National Academy of Sciences,* 108: 19451-19452.

Hollingsworth PM, Graham SW and Little DP (2011). Choosing and using a plant DNA barcode. *PloS one*, 6: e19254.

Huang LF, Song LX, Xia XJ, Mao WH, Shi K, Zhou YH and Yu JQ (2013). Plant-soil feedbacks and soil sickness: from mechanisms to application in agriculture. *Journal of chemical ecology*, 39: 232-242.

Hugenholtz P, Goebel BM and Pace NR (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of bacteriology*, 180: 4765-4774.

Iijima M, Morita S and Barlow PW (2008). Structure and function of the root cap. *Plant production science*, 11: 17-27.

Illuminati A, López-Angulo J, de la Cruz M, Chacón-Labella J, Pescador DS, Pías B, Sánchez AM, Escudero A and Matesanz S. (2021). Larger aboveground neighbourhood scales maximise similarity but do not eliminate discrepancies with belowground plant diversity in a Mediterranean shrubland. *Plant and Soil*, 1-13.

Incerti G, Bonanomi G, Giannino F, Cartenì F, Spaccini R, Mazzei P, Piccolo A and Mazzoleni S (2017). OMDY: a new model of organic matter decomposition based on biomolecular content as assessed by 13 C-CPMAS-NMR. *Plant and Soil*, 411(1-2), 377-394.

Incerti G, Bonanomi G, Giannino F, Piermatteo D, Castaldi S, Fioretto A, Papa S, De Marco A, Fierro A, Maggi O, Persiani AM, Rutigliano FA, Feoli E, Virzo De Santo A, Mazzoleni S (2011) Litter decomposition in Mediterranean ecosystems: modelling the controlling role of climatic conditions and litter quality. *Applied Soil Ecology*, 49:148–157

Inderjit (1996). Plant phenolics in allelopathy. *The Botanical Review*, 62:186–202.

INFC (2006). *Inventario Nazionale delle Foreste e dei Serbatoi Forestali di Carbonio INFC. Procedure di posizionamento e di rilievo degli attributi di terza fase*. CRA-Istituto Sperimentale per l'Assestamento Forestale e per l'Alpicoltura, Trento, 292 pp.

Ingraham JL, Maaloe O, Neidhardt FC (1983). Growth of the Bacterial Cell. Sinauer, Sunderland, MA, 435pp

IUSS Working Group WRB (2006). *World reference base for soil resources 2006*. World Soil Resources Reports No. 103. FAO, Rome.

Janzen DH (1970). Herbivores and the number of tree species in tropical forests. *The American Naturalist*, 104: 501-528.

Johnson JE, Berry JA (2013). The influence of leaf-atmosphere NH3 (g) exchange on the isotopic composition of nitrogen in plants and the atmosphere. *Plant, Cell and Environment*, 36: 1783-1801.

Joly FX, Coq S, Coulis M, Nahmani J and Hättenschwiler S (2018). Litter conversion into detritivore faeces reshuffles the quality control over C and N dynamics during decomposition. *Functional Ecology*, 32: 2605–2614.

Jones C, Brown BD, Engel R, Horneck D and Olson-Rutz K (2013). Nitrogen fertilizer volatilization. Montana State University Extension, EBO208.

Jørgensen T, Kjaer KH, Haile J, Rasmussen M, Boessenkool S, Andersen K et al. (2012). Islands in the ice: detecting past vegetation on Greenlandic nunataks using historical records and sedimentary ancient DNA Meta-barcoding. *Molecular Ecology*, 21: 1980-1988.

Kalcsits LA, Buschhaus HA, Guy RD (2014). Nitrogen isotope discrimination as an integrated measure of nitrogen fluxes, assimilation and allocation in plants. *Plant Physiology*, 151: 293–304.

Kardol P, Cornips NJ, van Kempen MM, Bakx-Schotman JT and van der Putten WH (2007). Microbe-mediated plant–soil feedback causes historical contingency effects in plant community assembly. *Ecological monographs*, 77: 147-162.

Kardol P, Veen GF, Teste FP and Perring MP (2015). Peeking into the black box: a trait-based approach to predicting plant-soil feedback. *New Phytologist*, 206: 1-4.

Katahira R, Ashihara H (2002). Profiles of pyrimidine biosynthesis, salvage and degradation in disks of potato (*Solanum tuberosum L*.) tubers. *Planta* 215: 821–828.

Kay E, Vogel TM, Bertolla F, Nalin R, Simonet P (2002). In situ transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. Applied and Environmental *Microbiology,* 68: 3345–3351.

Keese P (2008). Risks from GMOs due to horizontal gene transfer. Environmental biosafety research, 7: 123-149.

Keiser AD, Strickland MS, Fierer N and Bradford MA (2011). The effect of resource history on the functioning of soil microbial communities is maintained across time. *Biogeosciences,* 8: 1477–1486.

Keller A, Danner N, Grimmer G, Ankenbrand MVD, Von Der Ohe K, Von Der Ohe, W, Rost S, Härtel S, Steffan-Dewenter I (2015). Evaluating multiplexed next-generation sequencing as a method in palynology for mixed pollen samples. *Plant Biology*, 17: 558-566.

Kembel SW, O'Connor TK, Arnold HK, Hubbell SP, Wright SJ and Green JL (2014). Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. *Proceedings of the National Academy of Sciences of the United States of America*, 111: 13715–13720.

Klironomos JN (2002). Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature*, 417: 67-70.

Kohl DH, Shearer G (1980). Isotopic fractionation associated with symbiotic N2 fixation and uptake of NO3- by plants. *Plant Physiology,* 66: 51–56.

Kolb KJ, Evans RD (2003). Influence of nitrogen source and concentration on nitrogen isotopic discrimination in two barley genotypes (*Hordeum vulgare* L.). *Plant and Cell Environonment*, 26: 1431–1440.

Kraft NJB, Adler PB, Godoy O, James EC, Fuller S and Levine JM (2015). Community assembly, coexistence and the environmental filtering metaphor. *Functional Ecology*, 29: 592–599

Kraus TE, Dahlgren RA, Zasoski RJ. (2003). Tannins in nutrient dynamics of forest ecosystems - a review. *Plant and Soil,* 256: 41-66.

Kuiters AT (1991). Phenolic substances in forest leaf litter and their impact on plant growth in forest vegetations. In Ecological responses to environmental stresses (pp. 252-261). Springer, Dordrecht.

Kulmatiski A, Beard KH, Stevens JR and Cobbold SM (2008). Plant–soil feedbacks: a meta-analytical review. *Ecology letters*, 11: 980-992.

Lamb PD, Hunter E, Pinnegar JK, Creer S, Davies RG and Taylor MI (2019). How quantitative is metabarcoding: A meta-analytical approach. *Molecular Ecology*, 28: 420-430.

Laskowski R and Berg B (2006). Litter decomposition: guide to carbon and nutrient turnover. Elsevier, Amsterdam, 428 pp.

Lebret M, Nys C and Forgeard F (2001). Litter production in an Atlantic beech (*Fagus sylvatica L.*) time sequence. *Annals of forest science*, 58: 755-768.

Levy-Booth DJ, Campbell RG, Gulden RH, Hart MM, Powell JR, Klironomos JN, Pauls KP, Swanton CJ, Trevors JT and Dunfield KE (2007). Cycling of extracellular DNA in the soil environment. *Soil Biology and Biochemistry,* 39: 2977-2991.

Li Z-H, Wang Q, Ruan X, Pan C-D, Jiang D-A (2010). Phenolics and plant allelopathy. *Molecules*, 15: 8933-8952.

Lia D-Z, Gaoa L-M, Lia H-T, Wanga H, Gea X-J, Liua J-Q, Chena Z-D et al. (2011) Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. *Proceedings of the National Academy of Sciences,* 108:19641–19646.

Liang J, Crowther TW, Picard N, Wiser S, Zhou M, Alberti G, et al. (2016). Positive biodiversity-productivity relationship predominant in global forests. *Science*, 354.

Lin D, Pang M, Fanin N, Wang H, Qian S, Zhao L, Yang Y, Mi X and Ma K (2019). Fungi participate in driving home-field advantage of litter decomposition in a subtropical forest. *Plant and Soil*, 434: 467–480.

Liski J, Nissinen ARI, Erhard M and Taskinen O (2003). Climatic effects on litter decomposition from arctic tundra to tropical rainforest. *Global change biology*, 9: 575-584.

Logan J, Logan JM, Edwards KJ and Saunders NA (2009). Real-time PCR: current technology and applications. Horizon Scientific Press.

López-Angulo J, de la Cruz M, Chacón-Labella J, Illuminati A, Matesanz S, Pescador DS, Pías B, Sánchez AM and Escudero A (2020). The role of root community attributes in predicting soil fungal and bacterial community patterns. *New Phytologist*, 228: 1070-1082.

Lorenz MG, Wackernagel W (1987). Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. *Applied and Environmental Microbiology* 53: 2948–2952.

Lukac M (2012). Fine root turnover. In Measuring roots (pp. 363-373). Springer, Berlin, Heidelberg.

Macfadyen LP, Chen D, Vo HC, Liao D, Sinotte R, Redfield RJ (2001). Competence development by Haemophilus influenzae in regulated by the availability of nucleic acid precursors. *Molecular Microbiology,* 40: 700–707.

Magurran AE (2005). Biological diversity. *Current Biology*, 15: R116-R118.

Makarov MI (2019). The role of mycorrhiza in transformation of nitrogen compounds in soil and nitrogen nutrition of plants: a review. *Eurasian Soil Science*, 52: 193-205.

Mangan SA, Schnitzer SA, Herre EA, Mack KM, Valencia MC, Sanchez EI and Bever JD (2010). Negative plant–soil feedback predicts tree-species relative abundance in a tropical forest. *Nature*, 466:, 752-755.

Marinče L, Poldini L and Zupančič M (1990). Beitrag zur Kenntniss der Gesellschaft Anemono-Fagetum.

Mariotti A, Mariotti F, Champigny M, Amarger N, Moyse A (1982). Nitrogen isotope fractionation associated with nitrate reductase activity and uptake of NO3- by Pearl Millet. *Plant Physiology*, 69: 880–884.

Martin M (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal*, 17: 10-12.

Martin-Laurent F, Philippot L, Hallet S, Chaussod R, Germon JC, Soulas G and Catroux G (2001). DNA extraction from soils: old bias for new microbial diversity analysis methods. *Applied and environmental microbiology*, 67: 2354-2359.

Matesanz S, Pescador DS, Pías B, Sánchez AM, Chacón-Labella J, Illuminati A, de la Cruz M, López-Angulo J, Marí-Mena N, Vizcaíno A and Escudero A (2019). Estimating belowground plant abundance with DNA metabarcoding. *Molecular ecology resources*, 19: 1265-1277.

Mazzoleni S, Bonanomi G, Giannino F, Incerti G, Dekker SC and Rietkerk M (2010). Modelling the effects of litter decomposition on tree diversity patterns. *Ecological modelling*, 221: 2784-2792.

Mazzoleni S, Bonanomi G, Giannino F, Rietkerk M, Dekker S and Zucconi F (2007). Is plant biodiversity driven by decomposition processes? An emerging new theory on plant diversity. *Community Ecology*, 8: 103-109.

Mazzoleni S, Bonanomi G, Incerti G, Chiusano ML, Termolino P, Mingo A, Senatore M, Giannino F, Cartenì F, Rietkerk M, Lanzotti V (2015a). Inhibitory and toxic effects of extracellular self-DNA in litter: a mechanism for negative plant–soil feedbacks? *New Phytologist*, 205:1195–1210.

Mazzoleni S, Cartenì F, Bonanomi G, Incerti G, Chiusano ML, Termolino P, Migliozzi A, Senatore M, Giannino F, Rietkerk M, Risitano A and Lanzotti V (2014). New perspectives on the use of

nucleic acids in pharmacological applications: inhibitory action of extracellular self-DNA in biological systems. *Phytochemistry reviews*, 13(4), 937-946.

Mazzoleni S, Cartenì F, Bonanomi G, Senatore M, Termolino P, Giannino F, Incerti G, Rietkerk M, Lanzotti V and Chiusano ML (2015b). Inhibitory effects of extracellular self-DNA: a general biological process? *New Phytologist*, 206: 127-132.

McColl JG (1966). Accession and decomposition of litter in spotted gum forests. *Australian Forestry,* 30: 191-198.

McDonald BA and Stukenbrock EH (2016). Rapid emergence of pathogens in agro-ecosystems: global threats to agricultural sustainability and food security. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371: 20160026.

McLean EO (1982). Soil pH and lime requirement. In Page, A. L, R. H. Miller and D. R. Keeney (eds.) Methods of soil analysis. Part 2 - Chemical and microbiological properties. (2nd Ed.). Agronomy 9:199-223.

McNaughton KG and Jarvis PG (1984). Using the Penman-Monteith equation predictively. *Agricultural Water Management*, 8: 263-278.

Meisinger JJ and Delgado JA (2002). Principles for managing nitrogen leaching. *Journal of soil and water conservation*, 57: 485-498.

Michelsen A, Schmidt IK, Jonasson S, Dighton J, Jones HE, Callaghan TV (1995). Inhibition of growth, and effects on nutrient uptake of artic graminoids leaf extracts – allelopathy or resource competition between plants and microbes? *Oecologia* 103: 407–418.

Miller HG, Cooper JM, Miller JD and Pauline OJ (1979). Nutrient cycles in pine and their adaptation to poor soils. *Canadian Journal of Forest Research*, 9: 19-26.

Moll RH, Kamprath E, Jackson WA (1982). Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. *Agronomy Journal*, 74: 562-564

Morrissey EM, McHugh TA, Preteska L, Hayer M, Dijkstra P, Hungate BA, Schwartz E (2015) Dynamics of extracellular DNA decomposition and bacterial community composition in soil. *Soil Biology and Biochemistry*, 86:42–49.

Mucina L (1997). Conspectus of classes of European vegetation. *Folia Geobotanica*, 32: 117-172.

Muller CH (1966). The role of chemical inhibition (allelopathy) in vegetational composition. *Bulletin of the Torrey Botanical Club,* 93: 332–351.

Nadrowski K, Wirth C and Scherer-Lorenzen M (2010). Is forest diversity driving ecosystem function and service? *Current Opinion in Environmental Sustainability*, 2: 75-79.

Nagler M, Insam H, Pietramellara G and Ascher-Jenull J (2018). Extracellular DNA in natural environments: features, relevance and applications. *Applied microbiology and biotechnology*, 102: 6343-6356.

Nelissen FH, Tessari M, Wijmenga SS and Heus HA (2016). Stable isotope labeling methods for DNA. *Progress in nuclear magnetic resonance spectroscopy*, 96: 89-108.

Nielsen KM, Bones AM, Smalla K, van Elsas JD (1998) Horizontal gene transfer from transgenic plants to terrestrial bacteria—a rare event? *FEMS Microbiology Reviews*, 22:79–103.

Nielsen KM, Johnsen PJ, Bensasson D and Daffonchio D (2007). Release and persistence of extracellular DNA in the environment. *Environmental biosafety research*, 6: 37-53.

Nieuwenhuize J, Maas YEM, Middleburg JJ (1994). Rapid analysis of organic carbon and nitrogen in particulate materials. *Marine Chemistry*, 45: 217–224

Nimis P, Martellos S(2006). Guide alla flora II. Pordenone (PN), Risorgive di Flambro (UD), Laghi di Fusine (UD), M. Matajur (UD) (pp. 1-371). Edizioni Goliardiche.

Ogram A, Sayler GS, Barkay T (1987). The extraction and purification of microbial DNA from sediments. *Journal of Microbiological Methods*, 7: 57–66.

Oliver C, Larson B (1990) Forest stand dynamics. McGraw-Hill, 467 pp.

Olson BE and Wallander RT (2002). Effects of invasive forb litter on seed germination, seedling growth and survival. *Basic and Applied Ecology*, 3: 309-317.

Ostle NJ, Bol R, Petzke KJ, Jarvis SC (1999). Compound specific $\delta^{15}$N‰ values: amino acids in grassland and arable soils. *Soil Biology and Biochemistry*, 31: 1751-1755.

Packer A and Clay K (2000). Soil pathogens and spatial patterns of seedling mortality in a temperate tree. *Nature*, 404: 278-281.

Packham JR, Thomas PA, Atkinson MD and Degen T (2012). Biological F lora of the British Isles: *Fagus sylvatica*. *Journal of ecology*, 100(6), 1557-1608.

Pajtík J, Konôpka B and Lukac M (2011). Individual biomass factors for beech, oak and pine in Slovakia: a comparative study in young naturally regenerated stands. *Trees*, 25: 277-288.

Pansu J, De Danieli S, Puissant J, Gonzalez JM, Gielly L, Cordonnier T, Zinger L, Brun JJ, Choler P, Taberlet P and Cécillon L (2015). Landscape-scale distribution patterns of earthworms inferred from soil DNA. *Soil Biology and Biochemistry*, 83: 100-105.

Pathan SI, Arfaioli P, Ceccherini MT, Ascher-Jenull J and Pietramellara G (2020). Preliminary evidences of the presence of extracellular DNA single stranded forms in soil. *PloS one*, 15: e0227296.

Paungfoo-Lonhienne C, Lonhienne TG and Schmidt S (2010). DNA uptake by Arabidopsis induces changes in the expression of CLE peptides which control root morphology. *Plant signaling & behavior*, 5: 1112-1114.

Pedersen LB and Bille-Hansen J (1999). A comparison of litterfall and element fluxes in even aged Norway spruce, sitka spruce and beech stands in Denmark. *Forest ecology and management*, 114: 55-70.

Perez G, Aubert M, Decaens T, Trap J and Chauvat M (2013). Home-field advantage: a matter of interaction between litter biochemistry and decomposer biota. *Soil Biology and Biochemistry*, 67: 245–254.

Persoh D (2013). Factors shaping community structure of endophytic fungi-evidence from the Pinus-Viscum-system. *Fungal Diversity*, 60: 55–69.

Piccolo A (2002). The supramolecular structure of humic substances: a novel understanding of humus chemistry and implications in soil science. *Advances in Agronomy*, 75:57-134

Pietramellara G, Ascher J, Borgogni F, Ceccherini MT, Guerri G and Nannipieri P (2009). Extracellular DNA in soil and sediment: fate and ecological relevance. *Biology and Fertility of Soils*, 45: 219–235.

Pietramellara G, Ascher J, Borgogni F, Ceccherini MT, Guerri G and Nannipieri P (2009). Extracellular DNA in soil and sediment: fate and ecological relevance. *Biology and Fertility of Soils*, 45: 219-235.

Pietramellara G, Ascher J, Ceccherini MT, Nannipieri P, Wenderoth D (2007). Adsorption of pure and dirty bacterial DNA on clay minerals and their transformation frequency. *Biology and Fertility of Soils,* 43:731–739.

Poldini L. (1991). Atlante corologico delle piante vascolari nel Friuli-Venezia Giulia. Regione autonoma Friuli-Venezia Giulia Direzione regionale delle foreste e dei parchi & Università degli studi di Trieste, Dipartimento di biologia, Udine.

Polverari A, Buonaurio R, Guiderdone S, Pezatti M, Marte M (2000). Ultrastructural observations and DNA degradation analysis of pepper leaves undergoing a hypersensitive reaction to *Xanthomonas campestris p.v. vesicatoria*. *European Journal of Plant Pathology,* 106: 423–431

Pompanon F, Deagle BE, Symondson WO, Brown DS, Jarman SN And Taberlet P (2012). Who is eating what: diet assessment using next generation sequencing. *Molecular ecology*, 21: 1931-1950.

Poté J, Ceccherini MT, Rosselli W, Wildi W, Simonet P and Vogel TM (2010). Leaching and transformability of transgenic DNA in unsaturated soil columns. *Ecotoxicology and environmental safety*, 73: 67-72.

Poté J, Ceccherini MT, Van VT, Rosselli W, Wildi W, Simonet P, Vogel TM (2003) Fate and transport of antibiotic resistance genes in saturated soil columns. *European Journal of Soil Biology*, 39: 65–71.

Poté J, Rossé P, Rosselli W and Wildi W (2005). Kinetics of mass and DNA decomposition in tomato leaves. *Chemosphere*, 61: 677-684.

Poté J, Rossé P, Rosselli W and Wildi W (2007). Release and leaching of plant DNA in unsaturated soil column. *Ecotoxicology and environmental safety*, 68: 293-298.

Radajewski S, Ineson P, Parekh NR andMurrell JC (2000). Stable-isotope probing as a tool in microbial ecology. *Nature*, 403: 646-649.

Ranjard L, Richaume A (2001). Quantitative and qualitative microscale distribution of bacteria in soil. *Research in microbiology*, 152: 707-716.

Reigosa MJ, et al. (2006) Allelopathy. Dordrecht, the Netherlands: Springer.

Reynolds HL, PackerA, Bever JD and Clay K (2003). Grassroots ecology: plant–microbe–soil interactions as drivers of plant community structure and dynamics. *Ecology*, 84: 2281-2291

Riaz T, Shehzad W, Viari A, Pompanon F, Taberlet P and Coissac E (2011). ecoPrimers: inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic acids research*, 39: e145-e145.

Rice EL (1984) Allelophathy. London, UK: Academic Press.

Richardson RT, Lin CH, Sponsler DB, Quijia JO, Goodell K and Johnson RM (2015). Application of ITS2 metabarcoding to determine the provenance of pollen collected by honey bees in an agroecosystem. *Applications in Plant Sciences*, 3: 1400066.

Rishavy MA, Cleland WW, Lusty CJ (2000). $^{15}$N Isotope effects in glutamine hydrolysis catalyzed by carbamyl phosphate synthetase: evidence for a tetrahedral intermediate in the mechanism. *Biochemistry*, 39: 7309–7315.

Rivas-Martínez S, Penas A and Diaz TE (2004). Biogeographic map of Europe. Cartographic Service, University of Léon, Spain.

Robinson L, Handley LL, Scrimgeour CM (1998). A theory for 15N/14N fractionation in nitrate-grown vascular plants. *Planta*, 205: 397-406.

Rossato L, Lainé P, Ourry A (2001). Nitrogen storage and remobilization in Brassica napus L. during the growth cycle: nitrogen fluxes within the plant and changes in soluble protein patterns. *Journal of Experimental Bothany,* 52: 1655-1663.

Schall P, Lödige C, Beck M and Ammer C (2012). Biomass allocation to roots and shoots is more sensitive to shade and drought in European beech than in Norway spruce seedlings. *Forest Ecology and Management*, 266: 246-253.

Schjoerring JK, Mattson M (2001). Quantification of ammonia exchange between agricultural cropland and the atmosphere: Measurements over two complete growth cycles of oilseed rape, wheat, barley and pea. *Plant and Soil*, 228: 105–115.

Schütz JP (1999). Close-to-nature silviculture: is this concept compatible with species diversity? *Forestry*, 72: 359-366.

Siegrist JA, McCulley RL, Bush LP and Phillips TD (2010). Alkaloids may not be responsible for endophyte-associated reductions in tall fescue decomposition rates. *Functional Ecology*, 24: 460–468.

Simon C and Daniel R (2011). Metagenomic analyses: past and future trends. *Applied and environmental microbiology*, 77: 1153-1161.

Singh H, Batish DR, Kohli R (1999). Autotoxicity: concept, organisms, and ecological significance. *Critical Reviews in Plant Sciences,* 18: 757-772.

Singh HP, Batish DR and Kohli RK (1999). Autotoxicity: concept, organisms, and ecological significance. *Critical Reviews in Plant Sciences*, 18: 757-772.

Sirois SH andBuckley DH (2019). Factors governing extracellular DNA degradation dynamics in soil. *Environmental microbiology reports*, 11: 173-184.

Šnajdr J, Valášková V, Merhautová V, Herinková J, Cajthaml T and Baldrian P (2008). Spatial variability of enzyme activities and microbial biomass in the upper layers of *Quercus petraea* forest soil. *Soil Biology and Biochemistry*, 40: 2068-2075.

Spangenberg JE, Schweizer M, Zufferey V (2020). Shifts in carbon and nitrogen stable isotope composition and epicuticular lipids in leaves reflect early water-stress in vineyards. *Science of the Total Environment*, 739: 140343.

Spangenberg JE, Zufferey V (2018). Changes in soil water availability in vineyards can be traced by the carbon and nitrogen isotope composition of dried wines. *Science of the Total Environment*, 635: 178-187.

Staaf H (1980). Release of plant nutrients from decomposing leaf litter in a South Swedish beech forest. *Ecography*, 3: 129-136.

Staaf H and Stjernquist I (1986). Seasonal dynamics, especially autumnal retranslocation, of nitrogen and phosphorus in foliage of dominant and suppressed trees of beech, *Fagus sylvatica*. *Scandinavian Journal of Forest Research,* 1: 333-342.

Staaf H and Stjernquist I (1986). Seasonal dynamics, especially autumnal retranslocation, of nitrogen and phosphorus in foliage of dominant and suppressed trees of beech, Fagus sylvatica. *Scandinavian Journal of Forest Research*, 1: 333-342.

Stohlgren TJ (2007). Measuring plant diversity: lessons from the field. OUP USA.

Susmel L (1951) - Struttura, rinnovazione e trattamento delle abetine del Comelico. L'Italia Forestale e Montana, 6: 177-199.

Swamy V and Terborgh JW (2010). Distance-responsive natural enemies strongly influence seedling establishment patterns of multiple species in an Amazonian rain forest. *Journal of Ecology*, 98: 1096-1107.

Syvanen M and Kado CI (2001). Horizontal gene transfer. Academic Press, New York, 445 pp.

Taberlet P, Bonin A, Zinger L and Coissac E (2018). Environmental DNA: For biodiversity research and monitoring. Oxford University Press.

Taberlet P, Coissac E, Pompanon F, Brochmann C and Willerslev E (2012b). Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular ecology*, 21: 2045-2050.

Taberlet P, Prud'homme SM, Campione E, Roy J, Miquel C, Shehzad W, Gielly L, Rioux D, Choler P, Clément J-C, Melodelima C, Pompanon F and Coissac E (2012a). Soil sampling and isolation of extracellular DNA from large amount of starting material suitable for metabarcoding studies. *Molecular Ecology*, 21: 1816–1820.

Tcherkez G, Farquhar GD (2006). Isotopic fractionation by plant nitrate reductase, twenty years later. *Functional Plant Biology*, 33: 531–537.

Tea I, Tcherkez G (2017). Natural isotope abundance in metabolites: techniques and kinetic isotope effect measurement in plant, animal and human tissues. *Methods in Enzymology*, 596: 113-147.

Teste FP, Kardol P, Turner BL, Wardle DA, Zemunik G, Renton M and Laliberté E (2017). Plant-soil feedback and the maintenance of diversity in Mediterranean-climate shrublands. *Science*, 355:, 173-176.

Thomas CM, Nielsen KM (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Reviews Microbiology,* 3:711– 721.

Tribouillois H, Fort F, Cruz P, Charles R, Flores O, Garnier E and Justes E (2015). A functional characterization of a wide range of cover crops species: growth and nitrogen acquisition rates, leaf traits and ecological strategies. *PLoS ONE*, 10: e0122156

Trifonova R, Postma J, Verstappen FW, Bouwmeester HJ, Ketelaars JJ and van Elsas JD (2008). Removal of phytotoxic compounds from torrefied grass fibres by plant-beneficial microorganisms. *FEMS microbiology ecology*, 66: 158-166.

Uribelarrea M, Carcova J, Otegui ME, Westgate ME (2002). Pollen production, pollination dynamics, and kernel set in maize. *Crop Science,* 42: 1910–1918.

Vacek Z, Prokůpková A, Vacek S, Bulušek D, Šimůnek V, Hájek V and Králíček I (2021). Mixed vs. monospecific mountain forests in response to climate change: structural and growth perspectives of Norway spruce and European beech. *Forest Ecology and Management*, 488: 119019.

Vahdat E, Nourbakhsh F and Basiri M (2011). Lignin content of range plant residues controls N mineralization in soil. *European Journal of Biology*, 47: 243–246.

Van der Putten WH (2003). Plant defense belowground and spatiotemporal processes in natural vegetation. *Ecology*, 84: 2269-2280.

Van der Putten WH, Bardgett RD, Bever JD, Bezemer TM, Casper BB, Fukami T, Kardol P, Klironomos JN, Kulmatiski A, Schweitzer JA, Suding KN, Van de Voorde TFJ and Wardle DA (2013). Plant–soil feedbacks: the past, the present and future challenges. *Journal of Ecology*, 101: 265-276.

Van der Putten WH, Bradford MA, Brinkman PE, van de Voorde TF and Veen GF (2016). Where, when and how plant–soil feedback matters in a changing world. *Functional Ecology*, 30: 1109-1121.

Van der Putten WH, Van Dijk C and Peters BAM (1993). Plant-specific soil-borne diseases contribute to succession in foredune vegetation. *Nature*, 362: 53-56.

Van der Putten WH, Van Dijk C and Troelstra SR (1988). Biotic soil factors affecting the growth and development of *Ammophila arenaria. Oecologia*, 76: 313-320.

Veen GF, Fry EL, ten Hooven FC, Kardol P, Morriën E and De Long JR (2019a). The role of plant litter in driving plant-soil feedbacks. *Frontiers in Environmental Science*, 7: 168.

Veen GF, Keiser AD, van der Putten WH and Wardle DA (2018). Variation in home-field advantage and ability in leaf litter decomposition across successional gradients. *Functional Ecology*, 32: 1563–1574.

Veen GF, Snoek BL, Bakx-Schotman T, Wardle DA and van der Putten WH (2019b). Relationships between fungal community composition in decomposing leaf litter and home-field advantage effects. *Functional Ecology*, 33: 1524–1535.

Veresoglou SD, Aguilar-Trigueros CA, Mansour I and Rillig MC (2015). Self-DNA: a blessing in disguise? *New Phytologist*, 207: 488-490.

Vitousek PM and Sanford Jr RL (1986). Nutrient cycling in moist tropical forest. *Annual review of Ecology and Systematics*, 17: 137-167.

Von Lioba P and Schiitt P (1987). Auswaschung phytotoxischer Substanzen aus Blattern kranker und gesunder Buchen - Schaden an der Bodenflora. *European Journal of Plant Pathology,* 17: 356-362.

Wagner MR, Lundberg DS, del Rio TG, Tringe SG, Dangl JL and Mitchell-Olds T (2016). Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nature Communications*, 7: 15.

Waldrop GL, Urbauer JL, Cleland WW (1992). Nitrogen-15 isotope effects on nonenzymic and aspartate transcarbamylase catalyzed reactions of carbamyl phosphate. *Journal of the American Chemical Society,* 114: 5941–5945.

Walsh RPD and Voigt PJ (1977). Vegetation litter: an underestimated variable in hydrology and geomorphology. *Journal of Biogeography*, 253-274.

Wardle DA, Bardgett RD, Klironomos JN, Setälä H, Van Der Putten WH and Wall DH (2004). Ecological linkages between aboveground and belowground biota. *Science*, 304: 1629-1633.

Wen F, White GJ, VanEtten HD, Xiong Z and Hawes MC (2009). Extracellular DNA is required for root tip resistance to fungal infection. *Plant physiology,* 151: 820-829.

Wen FS, Curlango-Rivera G, Huskey DA, Xiong ZG, Hawes MC (2017) Visualization of extracellular DNA released during border cell separation from the root cap. *American Journal of Botany*, 104: 970–978

Werner RA, Schmidt HL (2002). The in vivo nitrogen isotope discrimination among organic plant compounds. *Phytochemistry,* 61: 465-484.

White TJ, Arnheim N and Erlich HA (1989). The polymerase chain reaction. *Trends in genetics*, 5: 185-189.

Widmer F, Seidler RJ, Donegan KK, Reed GL (1997). Quantification of transgenic plant marker gene persistence in the field. *Molecular Ecology*, 6: 1–7.

Willerslev E, Hansen AJ, Binladen J, Brand TB, Gilbert MTP, Shapiro B, Bunce M, Wiuf C, Gilichinsky DA and Cooper A (2003). Diverse plant and animal genetic records from Holocene and Pleistocene sediments. *Science*, 300: 791-795.

Williams DE (1948). A rapid manometer method for the determination of carbonate in soils. *Soil Science Society of America, Proceedings*, 13:127–129

Williams JD, McCool DK, Reardon CL, Douglas CL, Albrecht SL, Rickman RW (2013). Root: shoot ratios and belowground biomass distribution for Pacific Northwest dryland crops. *Journal of Soil and Water Conservation,* 68: 349-360.

Wilson E (1999) The diversity of life. Harvard University Press.

Wright JS (2002). Plant diversity in tropical forests: a review of mechanisms of species coexistence. *Oecologia*, 130: 1-14.

Wurst S, Kaiser N, Nitzsche S, Haase J, Auge H, Rillig MC and Powell JR (2015). Tree diversity modifies distance-dependent effects on seedling emergence but not plant–soil feedbacks of temperate trees. *Ecology*, 96: 1529-1539.

Yang C, Wang X, Miller JA, de Blécourt M, Ji Y, Yang C, Harrisone RH and Douglas WY (2014). Using metabarcoding to ask if easily collected soil and leaf-litter samples can be used as a general biodiversity indicator. *Ecological Indicators*, 46: 379-389.

Yang Y, Wang H, Tang J and Chen X (2007). Effects of weed management practices on orchard soil biological and fertility properties in southeastern China. *Soil and Tillage Research*, 93: 179-185.

Yoccoz NG (2012). The future of environmental DNA in ecology. *Molecular ecology*, 21: 2031-2038.

Yoccoz, N. G, Bråthen, K. A, Gielly, L, Haile, J, Edwards, M. E, Goslar, T, Stedingk VH, Brysting AK, Coissac E, Pompanon F, Sønstebø JH, Miquel C, Valentini A, De Bello F, Chave J, Thuiller W, Wincker P, Cruaud C, Gavory F, Rasmussen M, Gilbert MTP, Orlando L, Brochmann C, Willerslev E and Sønstebø JH (2012). DNA from soil mirrors plant taxonomic and growth form diversity. Molecular *Ecology*, 21: 3647-3655.

Yoneyama T, Fujiwara H, Wilson JM (1998). Variations in fractionation of carbon and nitrogen isotopes in higher plants: N metabolism and partitioning in phloem and xylem. In: Griffiths H, editor. Stable Isotopes: integration of biological, ecological and geochemical processes. Oxford: BIOS Scientific Publishers; pp. 99–110.

Yoneyama T, Ito O, Engelaar WMHG (2003). Uptake, metabolism and distribution of nitrogen in crop plants traced by enriched and natural 15N: Progress over the last 30 years. *Phytochemical Review*, 2: 121–132.

Yoneyama T, Kamachi K, Yamaya T, Mae T (1993). Fractionation of nitrogen isotopes by glutamine synthetase isolated from spinach leaves. *Plant and Cell Physiology,* 34: 489–491.

Yoneyama T, Kaneko A (1989). Variations in the natural abundance of 15N in nitrogenous fractions of Komatsuna plants supplied with nitrate. *Plant and Cell Physiology,* 30: 957–962.

Yoneyama T, Matsumaru T, Usui K, Engelaar WMH (2001). Discrimination of nitrogen isotopes during absorption of ammonium and nitrate at different nitrogen concentrations by rice (Oryza sativa L.) pants. *Plant, Cell and Environment*,24: 133–139.

Yoneyama T, Omata T, Nakata S, Yazaki J (1991). Fractionation of nitrogen isotopes during the uptake and assimilation of ammonia by plants. *Plant and Cell Physiology*, 32: 1211–1217.

Yoneyama T, Suzuki A (2019). Exploration of nitrate-to-glutamate assimilation in non-photosynthetic roots of higher plants by studies of 15N-tracing enzymes involved, reductant supply, and nitrate signaling: A review and synthesis. *Plant Physiology and Biochemistry*, 136: 245-254.

Yoneyama T, Tanno F, Tatsumi J, Mae T (2016). Whole-plant dynamic system of nitrogen use for vegetative growth and grain filling in rice plants (*Oryza sativa L*.) as revealed through the production of 350 grains from a germinated seed over 150 days: A review and synthesis. *Frontiers in Plant Science*, 7: 1151.

Yu J, Xue JH and Zhou SL (2011). New universal matK primers for DNA barcoding angiosperms. *Journal of Systematics and Evolution*, 49: 176-181.

Zanin L, Zamboni A, Monte R, Tomasi N, Varanini Z, Cesco S and Pinton R (2015). Transcriptomic Analysis Highlights Reciprocal Interactions of Urea and Nitrate for Nitrogen Acquisition by Maize Roots. *Plant and Cell Physiology,* 56: 532-548.

Zeller B, Colin-Belgrand M, Dambrine É and Martin F (1998). 15N partitioning and production of 15N-labelled litter in beech trees following [15N] urea spray. *Annales des sciences forestières*, 55: 375-383).

Zeller, B., Colin-Belgrand, M., Dambrine, E., Martin, F., & Bottner, P. (2000). Decomposition of 15 N-labelled beech litter and fate of nitrogen derived from litter in a beech forest. Oecologia, 123(4), 550-559.

Zhang N, Van der Putten WH and Veen GF (2016b). Effects of root decomposition on plant–soil feedback of early- and mid-successional plant species. *New Phytology,* 212: 220–231

Zhang Y, Luo M, Schramm VL (2009). Transition states of plasmodium falciparum and human orotate Phosphoribosyltransferases. *Journal of the American Chemical Society,* 131: 4685–4694.

Zhang Z, Xiao H, Zheng N, Gao N, Zhu R (2016a). Compound-specific isotope analysis of amino acids labelling with stable isotope nitrogen ($^{15}$N) in higher plants. *Chromatographia*, 79: 1197–1205.

Zinger L, Taberlet P, Schimann H, Bonin A, Boyer F, De Barba M, Gaucher P, Gielly L, Giguet-Covex C, Iribar A, Réjou-Méchain M, Rayé G, Rioux D, Schilling V, Tymen B, Viers J, Zouiten C, Thuiller W, Coissac E and Chave J (2019). Body size determines soil community assembly in a tropical forest. *Molecular Ecology*, 28: 528-543.

# Acknowledgement

Make a PhD is not easy, especially far from home and with a world global pandemic crisis in the middle. It has been an intense and long formative journey and in the end i can say “I survived”. This would not have been possible only with my strength, so it is my duty to thank all the people who have helped and supported me. First i would like to thank *Prof.* ***Stefano Mazzoleni*** that introduced me to the fascinating world of plant soil negative feedbackby self-DNA. Obviously, I thank very much my supervisor *Prof.* ***Guido Incerti*****,** for all the passion shown in ours work during my PhD and for having trained me in the best possible way. I thank *Prof.* **Giorgio Alberti** for his invaluable help in the technical set up of the semi-automated fertirrigation system; *Prof.* **Alessandro Peressotti** and *dott.* **Gemini Delle Vedove** for all the theoretical and technical advice given me. A super thanks for the Genetic research group composed by *Dott.* **Emanuele De Paoli,** *dott.ssa* **Giusy Zaina** and **Nicoletta Felice** for all the help they gave me to understand the “strange world of genetics” and for all the patience shown in training me in the laboratory. Thanks at **Diego Chiabà** for all the help provided on the hard fieldwork and for introducing me to local food and wine and **Andrea Fabris** for his “explosive” training on the use of CHN-IRMS analyser. A special thanks at others collaborators and friends, **Giulia Leonarduzzi, Maurizio Zotti, Daniel Lizzi, Chiara Palandrani, Federico Toffoletti, Marco Pontello, Jessica De Marco** and **Nicole Salvatori**, that helped and supported me in every possible (and crazy) way. The most loving thanks go to **Giulia Patricelli** for everything that has happened during this long journey and for what has yet to happen. Finally, I would thank my Family for all the love and support they gave me.