



N fertilization in a Mediterranean ecosystem alters N and P turnover in soil, roots and the ectomycorrhizal community



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ABSTRACT

Increased anthropogenic nitrogen (N) deposition is a major contributor to alteration of soil nutrient cycles particularly in nutrient poor ecosystems, such as the Mediterranean basin, where co-limitation of N and phosphorus (P) occurs and N addition might thus lead to an exacerbation of P limitation. Here, we measured the effect of medium term (6 years) N fertilization in different forms and doses (40 kg N ha⁻¹ yr⁻¹ as 1:1 NH₄Cl and (NH₄)₂SO₄; 40 and 80 kg N ha⁻¹ yr⁻¹ as NH₄NO₃) on nutrient stoichiometry, potential turnover rates and abundance of roots, ectomycorrhizal (ECM) root tips and adjacent soil in *Cistus ladanifer* L. In order to assess the impact of N addition at its most extreme point, we sampled roots and topsoil (10 cm) with and without plant influence in the summer months. We analysed N and P concentrations in soil and roots and determined the abundance of the most dominant mycorrhizal root tip morphotypes. We also assessed nutrient turnover in soil, roots and mycorrhizal root tips by measuring their N and C related enzyme activities (EAs) as well as acid phosphatase (AP) activity.

Results: showed decreased soil P_{inorg} and increased soil N:P_{inorg} in the treatment plots. Also, a decline in *Cenococcum geophilum* in N addition plots was found and a general reduction in ECM colonization in the treatment receiving ammonium without nitrate. We also detected a decrease of *C. geophilum* absolute EA and AP, as well as N related EA in the whole soil compartment. Furthermore, we observed lower root AP activity and found a loss of correlation between N related EA and AP activity in all treatments, while a high correlation between N related EA and C related EA persisted in all plots. EA was also generally negatively related with root P/soil P, which we used as a measure for plant P status.

The negative effect of ammonium on the ECM community of *C. ladanifer* and a putative loss of short distance exploration morphotypes, such as *C. geophilum*, together with decreased AP activity in the plant roots, might be connected with low P_{inorg} availability in soil with plant influence, thus being in line with the hypothesis of P depletion due to N addition. Furthermore, the decrease of N related EA in the soil compartment, as well as the decoupling of N and P cycles, might be signs of altered soil microbial communities. This decoupling, together with the strong dependence of EA on plant P status, could point to a shift from N and P scavenging ECM communities to more copiotrophic saprophytic fungi that rely on C and N acquisition from soil organic matter rather than plant C inputs. We posit that a decline in root colonization by ECM fungi and changes in N:P cycling could be detrimental to ecosystem development, as *C. ladanifer* is a common ECM species in early successional stages, providing a host for ECM fungi that also colonize late-successional plants.

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1. Introduction

The increase in reactive nitrogen (N) availability due to anthropogenic activities has surpassed the input from natural biotic

processes and is predicted to further increase twofold until 2050 (Peñuelas et al., 2012), which has poorly understood effects on the biosphere, particularly concerning belowground microbial responses (Ramirez et al., 2012). One proposed consequence of increased N availability could be an increased terrestrial phosphorus (P) limitation (Vitousek et al., 2010) as the rate of anthropogenic P fertilization is far smaller than that of N fertilization (Peñuelas et al., 2012). Microbial growth is highly dependent on

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both soil N and P availabilities, which together determine the rate of carbon (C) turnover in the soil (Cleveland and Liptzin, 2007). While nutrient turnover is essential for microbial life in the soil, it also drives primary production of biomass, as especially in nutrient-poor ecosystems plants heavily depend on microbial associations for nutrient acquisition (Van Der Heijden et al., 2008). Plants that do not form arbuscular mycorrhizal (AM) symbiosis under these conditions, often have ectomycorrhizal (ECM) fungi as the most important partners for the acquisition of both N and P. ECM fungi are a significant component of ecosystem functioning in forest systems of boreal, temperate and Mediterranean climate zones (Courty et al., 2010). Mediterranean forests, woodlands and scrubs are global biodiversity hotspots, which show the second highest mean plant species richness of all biomes worldwide (Kier et al., 2005) and also host very diverse ECM communities (Azul et al., 2014). However, despite their importance for biodiversity, the response of Mediterranean ecosystems to intense increased N availability remains largely unknown (Dias et al., 2014).

The global trend of plant root colonization by ECM with increasing N addition was found to be negative (Treseder, 2004; Li et al., 2015) and long term responses of ECM fungi to increased N availability can be substantial, including diversity loss, changes in community composition and declines in ECM root tips (Cox et al., 2010). The decline in ECM fungi is most probably connected to a lower plant C allocation belowground (Treseder, 2004) as alleviation of N limitation diminishes the dependence on symbiosis for plant growth. However, in an N and P co-limited system such as the Mediterranean maquis (Dias et al., 2012), this does not necessarily apply, as root C allocation will be driven by the limitation of both nutrients (Ågren et al., 2012). Direct responses of N addition on ECM fungal species frequency and abundance were found to vary by genera and to depend on fungal functional type (Lilleskov et al., 2011). It was thus suggested that the addition of N would favour ECM types that are focusing on the uptake of labile N in contrast to ECM types capable of accessing complex organic N sources (Lilleskov et al., 2011). The mobilization of organic N sources by ECM is dependent on fungal enzyme activities (EA), which are also crucial for the functioning of ECM nutrient supply to the plant (Pritsch and Garbaye, 2011). Nutrient turnover rates in the soil follow a strongly constrained stoichiometry between C, N and P, as the degradation of organic molecules by EA is required to obtain both N and P (Sinsabaugh et al., 2009). The addition of N should thus lead to an increased investment in P cycling through the production of phosphatases in order to delay P limitation for plant productivity (Marklein and Houlton, 2012). While in most systems, this co-limitation in N and P is simultaneous, the Mediterranean system also has a temporal discrepancy between N availability and growth period. While in the mild, more humid Mediterranean spring most of the available N will be taken up and stored in the biotic compartment, N will be released during the late summer due to leaf shedding by summer deciduous species, such as *Cistus ladanifer* L. (Dias et al., 2012). Summer deciduous species, such as *Cistus* spp. are replaced in the post fire succession by evergreen sclerophyllous shrub and tree species, a development accompanied by a shift in soil inorganic N from a NO_3^- to a more NH_4^+ dominated state (Cruz et al., 2003). *C. ladanifer* is a pyrophytic shrub that serves as a bridge for post-fire re-colonization by ECM fungi, which are associated with both shrub and late successional tree species (Martín-Pinto et al., 2006), so induced succession and decline in *Cistus* spp. by anthropogenic N - addition might have indirect negative effects on ECM fungi. Indeed, while there are indications of initial plant biodiversity increase after N addition in Mediterranean scrubs undergoing post-fire succession (Dias et al., 2011), medium term (5 years) changes in plant richness were found to depend on the N form applied: the availability of NO_x is in general

increasing aboveground biomass, whereas the availability of NH_4^+ is decreasing the abundance of summer deciduous N-affected plants, for example *Cistus ladanifer* (Dias et al., 2014). This species belongs to the *Cistus* genus, which is comprised of plants that are highly mycorrhizal, as is clear from their potential association with 200 fungal species belonging to 40 genera (Comandini et al., 2006) of which several have economical value (Mediavilla et al., 2016). Thus, we hypothesized that *C. ladanifer* could be a promising candidate to monitor the effects of anthropogenic N addition on ECM fungi and that the negative growth response to ammonium observed in this plant species will be directly related with a decrease in ECM colonization. In contrast, we supposed that NO_x is connected to increased plant growth, which would shift N and P pools from the soil towards the biotic compartment with effects on soil EA and ECM fungal community, postulating that increased N availability in the biotic compartment will lead to an alteration in EA towards P acquisition. These hypotheses were tested by assessing the ECM fungal community structure in mycorrhizal root tips and determining the EA of the bulk soil, the roots and the most common morphotypes of mycorrhizal root tips in order to relate these values with soil nutrient and plant tissue stoichiometry.

2. Materials and methods

2.1. Study site

This study was conducted in a Natura 2000 site located south of Lisbon, Portugal (PTCON0010 Arrábida/Espichel) in Arrábida Natural Park. The plots ($38^\circ 29'N$, $9^\circ 01'W$) are situated on a southeast-facing slope (5%) at 130 m a.s.l. Soil is skeletal (15–20 cm deep) and consists of 57% silt, 28% clay and 15% sand (silt-sand-loam – Correia, 1988). The site was burned in a fire event in 2003, after which the vegetation regenerated into a dense Mediterranean maquis (Eunis class F5.2 - <http://eunis.eea.europa.eu/>). In 2007, a reactive nitrogen (N) addition experiment was started, using 3 N - treatments: 40 and 80 kg N $\text{ha}^{-1} \text{yr}^{-1}$ of ammonium nitrate NH_4NO_3 (termed “40AN” and “80AN” respectively) and 40 kg N $\text{ha}^{-1} \text{yr}^{-1}$ of ammonium in 1:1 mix of NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$ (termed “40A”). Background nitrogen deposition is 5.2 kg N $\text{ha}^{-1} \text{yr}^{-1}$ (2.9 kg NO_x + 2.3 kg NH_4^+ - http://webdab.emep.int/Unified_Model_Results/AN/). Each treatment and the control have 3 replicate plots in squares of 400 m^2 , but in order to avoid boundary effects sampling was performed in a central 100 m^2 square. The summer deciduous *Cistus ladanifer* was used in this study as it is the dominating shrub species and known to form ectomycorrhiza.

2.2. Soil sampling, sample collection and preparation

Sampling took place on the 3rd of July 2013. At each plot, topsoil (10 cm) was sampled at 5 random spots without vegetation cover (termed “soil without plant influence”). Also, 3 *C. ladanifer* plants of ca. 50 cm height were chosen and the bulk soil-root agglomerate around the stem sampled into plastic bags. Samples were stored as bulk in the dark at room temperature (20 °C) until usage within the next 7 days. To calculate dry weight contribution of each mycorrhizal root tip, soil and total roots (Fig. 1), 100 g agglomerate were weighed and then separated into roots and soil (“soil with plant influence”), taking soil subsamples and finishing the separation by carefully rinsing the roots with tap water. Roots were further separated into 5 fractions using a Binocular (Zeiss, Oberkochen, Germany): roots without visible hyphae or mycorrhizal root tip mantle (termed “roots”), highly pigmented root tips that were completely black in appearance (termed “black”), highly branched root tips with a wrinkled surface (termed “coral”), non-pigmented root tips that were translucent (termed “white”) and root tips with

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