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Mixing of Aleppo pine and Holm oak litter increases biochemical diversity and alleviates N limitations of microbial activity

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Soil N availability is a primary limitation for plants and microbes in Mediterranean ecosystems. Few studies have examined the role of secondary compounds (e.g. polyphenols and volatile organic compounds) in mixed litter in Mediterranean ecosystems. Here, we ask whether the natural mixing of Aleppo pine and Holm oak litter decreases the inhibitory effect of secondary compounds and increases microbial activities involved in C cycling. *In situ* N fertilisation was used to demonstrate the limiting role of N to soil microbial activities, particularly in pure pine stands, and we hypothesised that these inhibitory effects would be alleviated in mixed litter. We examined the concentrations, structure and diversity of biochemical compounds in litter from single-species or mixed stands of pine and oak, along with associated microbial activities (i.e. net ammonification and nitrification, cellulase, fluorescein diacetate hydrolase (FDAse) and phenol oxidase activities). We tested the relationships between biochemical compounds and microbial activities with co-inertia analyses. Pine litter clearly shaped the biochemical composition of mixed litter in the organic litter horizon, and this mixed litter was dominated by high molecular weight compounds (total phenols, sesqui- and diterpenes). In contrast, oak litter was dominated by small compounds such as monoterpenes and hydro-soluble phenols. Pine litter clearly affected microbial activities (i.e. antagonistic effect) in unfertilised mixed litter. We demonstrated by co-inertia analysis that this antagonistic effect was related to N availability. Mixed litter had the highest VOC (volatile organic compounds) richness and evenness, along with the highest enzyme activities (i.e. synergistic effect), and N availability did not constrain microbial activities. However, an apparent nitrification limitation suggested greater microbial specialisation and efficiency of N recovery in mixed litter. Our results highlight how mixing two recalcitrant litters can alleviate N limitation and could explain transition in Mediterranean forest secondary succession.

1. Introduction

Since the late 19th century, silvicultural and postfire reforestation practices in the Northern Mediterranean Basin have promoted monospecific stands of Aleppo pine (*Pinus halepensis* Mill.), a common pioneer and highly competitive species (Vilà et al., 2003). The potential ecosystem consequences of the resulting loss in tree species richness include a decrease in nutrient cycling (Parrotta, 1999) and a decline in community stability (i.e. resistance and resilience) in response to climatic stresses (Royer-Tardif et al., 2010). Additionally, plant productivity of these monospecific stands can be lower than that of more diverse forest stands. Vilà et al. (2007) reported that Mediterranean mixed forest stands produced 30% more wood than monospecific stands of Aleppo pine or Holm oak (*Quercus ilex* L.), the most common evergreen that naturally co-occurs in Aleppo pine forests in the Mediterranean basin of southern France and Spain (Pausas et al., 2004; Sheffer, 2012).

Plant litter decomposition, mediated largely by soil microbes, is a major ecosystem process linking plant productivity to nutrient

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cycling (Hobbie, 1992; Chapman and Koch, 2007). In the context of monospecific versus mixed forest stands, the influence of single-species versus mixed (composed of foliage of 2 or more species) litter could have significant consequences on nutrient cycling. Compared to single-species litter, decomposition (i.e. mass loss) rates of mixed litter can be additive (i.e. predictable based on the mass loss dynamics of each species when decomposed alone) or non-additive (differing from that expected based on single-species mass loss dynamics) (Gartner and Cardon, 2004). In the case of the latter non-additive responses, decomposition rates of mixed litter can be enhanced or faster than expected (referred to as a synergistic response) or slower than expected (an antagonistic response) (Gartner and Cardon, 2004). We hypothesised that litter in mixed Aleppo pine- Holm oak stands would have greater microbial activity and decomposition rates, leading to greater nutrient availability that could in turn, explain the greater wood productivity observed in mixed forests (Vilà et al., 2007). Two mechanisms could explain this synergistic response: facilitation among microbial communities (e.g. a 'priming' or 'fertilisation' effect) (Chapman et al., 1988) or resource partitioning among microbial communities, known as the niche complementarity effect (Paquette and Messier, 2011). Because the niche complementarity effect can be more important in less productive, harsher environments (Paquette and Messier, 2011), and has a major effect on litter decomposition and nutrient availability in temperate forests (Vos et al., 2013), we suspected this mechanism might be dominant in our system.

The influence of mixed litter on decomposition rates has been assessed in dozens of studies (e.g. Wardle et al., 2006; Lecerf et al., 2011; Chapman et al., 2013; Chomel et al., 2015) and was recently reviewed by Song et al. (2010). Most experiments have included at least one litter species of high quality (i.e. high N and/or low concentrations of lignin, polyphenols or tannins) (Chapman and Koch, 2007). Litters of pine and oak are both considered recalcitrant, having low N concentrations (Yuste et al., 2012) and high concentrations of secondary compounds such as polyphenols, tannins or terpenes (Fernandez et al., 2013; Sheffer et al., 2015). These biochemical compounds make them relatively unfavourable for microbes (Fioretto et al., 1998; White, 1988; 1994), and in turn can slow the turnover of organic matter and nutrient cycling (Smolander et al., 2006; Chomel et al., 2014). On the other hand, polyphenols can have a positive influence on litter decomposition since they can serve as a C substrate, particularly for fungi (Castells et al., 2004). The decomposition of polyphenols would involve microbial specialisation such as the ability to produce phenol oxidase (Hättenschwiler and Vitousek, 2000).

Net N mineralisation, a major soil process controlling N availability, can be limited in early stages of litter decomposition in an evergreen forest, as a result of polyphenols, possibly because of their toxicity to microbes (Fierer et al., 2001). Aleppo pine is a terpene-storing species and needles typically contain relatively high concentrations of monoterpenes which can inhibit N mineralisation and nitrification (White, 1994). In contrast, Holm oak does not store terpene but its foliage does contain many other polyphenols that are also recalcitrant to decomposers (Brossa et al., 2009; Sheffer et al., 2015).

Most studies that have addressed litter mixtures have been conducted in boreal, temperate or tropical ecosystems. Several studies have addressed litter decomposition in mixed Mediterranean stands (De Oliveira et al., 2010; De Marco et al., 2011; Bonanomi et al., 2010; Maisto et al., 2011; Aponte et al., 2012; Bonanomi et al., 2014; Santonja et al., 2015; Sheffer et al., 2015) but few of these focused on the microbial functions involved in litter decomposition. While Bonanomi et al. (2010) found that mixed litter decomposed faster than single-species litter, it is unclear how mixtures of litters with high concentrations of secondary compounds might accelerate decomposition (Bonanomi et al., 2010; Chapman et al., 2013; Sheffer et al., 2015). To our knowledge, this study is the first to examine how litter mixing influences microbial functions in a Mediterranean ecosystem and could provide an explanation for how mixed litter alleviates N limitation in forest secondary succession.

The main objectives of this study were 1) to examine the secondary compounds in litter from single-species or mixed stands of Aleppo pine and Holm oak, 2) to assess the effect of mixing litter on microbial activities and 3) to examine the relationship between microbial and biochemical properties and whether these relationships are affected by N availability. We expected that mixed litter would have a greater richness and evenness of secondary compounds and that their concentrations would be diluted, thereby reducing their inhibitory effects. Thus, a niche complementary effect would improve microbial activities by increasing nutrient mineralisation and availability. We also used *in situ* inorganic N fertilisation to alleviate N limitations on soil microbial activity. We expected greater N availability would increase microbial activity in early stage of litter decomposition, particularly in monospecific pine litter.

2. Material and methods

2.1. Study site and experimental design

The study sites were in southeastern France (Provence Alpes Côte d'Azur region) within 50 km of Marseille. Climate is Mediterranean with dry, hot summers and wet, temperate winters, with precipitation occurring primarily during 4 months (September-November and April). Mean annual temperature and precipitation are 13 °C and 650 mm, respectively (meteorological data of the PACA region, 2000–2007, MétéoFrance).

Three typical Mediterranean forest types were studied: monospecific stands of Aleppo pine, monospecific stands of Holm oak, and mixed stands, almost at equal dominance between pine and oak cover density (i.e. Pine 52% ± 3% and Oak 48% ± 3%). In each stand type, we chose 3 study sites (10,000 m^2). All study sites were similar in terms of age of dominant trees (30–35 years old), canopy density (70–90%), slope (15–20%), aspect (NW-W) and soils (Calcaric Cambisol of 50 cm depth (World Reference Base)).

At each study site, two 16 m^2 plots, 15 m apart, were delineated. One plot ('fertilised') received 450 kg N ha^{-1}, applied as ammonium sulphate $(NH_4)_2SO_4$ (granules <0.2 mm diameter) in January 2006. We selected this high level of N fertilisation to maximise *in situ* N effects. The second plot ('non-fertilised') served as a control. Overall, the experiment consisted of 3 stand types, 3 sites within each stand type, and 2 plots (fertilised and non-fertilised) within each site.

Four months after the application of the fertiliser (May 2006), two layers of the soil organic horizon (OL: organic litter and OF-H: organic fermentative and humic) comparable in terms of depth among forest types were sampled. The OL horizon was the upper (0–3 cm depth), less fragmented litter layer (easily identifiable to species) while the OF-H horizon (3–7 cm depth) was fragmented, humified, oxidized organic matter, and not identifiable as to litter species. In each plot (both fertilised and unfertilised), we collected 20 randomly located samples from each layer and these were pooled to obtain a composite sample. No contributions from plant species other than Aleppo pine or Holm oak were found in the upper layer which infers that both litter layers were likely comprised of nearly exclusively these 2 species. Samples were kept at 4 °C until analyses (within 1 week for microbial analyses). Briefly, litter organic matter concentration (%) was obtained by loss on ignition in a muffle furnace (16 h, 550 °C) of 10 g subsamples for