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The effects of mineral nitrogen limitation, competition, arbuscular mycorrhiza, and their respective interactions, on morphological and chemical plant traits of *Plantago lanceolata*

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ABSTRACT

Plants are sessile organisms that suffer from a multitude of challenges such as abiotic stress or the interactions with competitors, antagonists and symbionts, which influence their performance as well as their eco-physiological and biochemical responses in complex ways. In particular, the combination of different stressors and their impact on plant biomass production and the plant's ability to metabolically adjust to these challenges are less well understood. To study the effects of mineral nitrogen (N) availability, interspecific competition and the association with arbuscular mycorrhizal fungi (AMF) on biomass production, biomass allocation patterns (root/shoot ratio, specific leaf area) and metabolic responses, we chose the model organism *Plantago lanceolata* L. (Plantaginaceae). Plants were grown in a full factorial experiment. Biomass production and its allocation patterns were assessed at harvest, and the influence of the different treatments and their interactions on the plant metabolome were analysed using a metabolic fingerprinting approach with ultra-high performance liquid chromatography coupled with time-of-flight-mass spectrometry. Limited supply of mineral N caused the most pronounced changes with respect to plant biomass and biomass allocation patterns, and altered the concentrations of more than one third of the polar plant metabolome. Competition also impaired plant biomass production, yet affected the plant metabolome to a much lesser extent than limited mineral N supply. The interaction of competition and limited mineral N supply often caused additive changes on several traits. The association with AMF did not enhance biomass production, but altered biomass allocation patterns such as the root/shoot ratio and the specific leaf area. Interestingly, we did not find significant changes in the plant metabolome caused by AMF. A targeted analysis revealed that only limited mineral N supply reduced the concentrations of one of the main target defence compounds of *P. lanceolata*, the iridoid glycoside catalpol. In general, the interaction of competition and limited mineral N supply led to additive changes, while the association with AMF in any case alleviated the observed stress responses. Our results show that the joint analysis of biomass/allocation patterns and metabolic traits allows a more comprehensive interpretation of plant responses to different biotic and abiotic challenges; specifically, when multiple stresses interact.

1. Introduction

Plants are sessile organisms that suffer from a multitude of different stressors during their life span. Besides abiotic conditions, biotic interactions with competitors, antagonists and symbionts influence their performance and fitness in complex ways. Specifically during succession, when plants colonise ruderal sites after human disturbance, the particular interplay between these factors will determine the successful establishment of individuals. In disturbed or nutrient-poor habitats, plants may experience less competition than in established plant communities, but the general availability of macronutrients might be limited. Furthermore, arbuscular mycorrhizal fungi (AMF), which improve nutrient acquisition in a multitude of plant species (Adler et al., 1995; Smith and Read, 2008), may be sparsely and patchily distributed during early succession stages (Hart et al., 2003). Yet, even though plant characteristics that enhance plant fitness during different stages of succession have been studied intensely, much less is known about the interactive effects of nutrient limitation, interspecific competition and the association with AMF on eco-physiological and biochemical plant responses.

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Nitrogen (N) is an essential plant macronutrient that influences plant growth, consequently biomass production and ontogenetic development, and thus has a huge impact on plant performance, plant physiology and resource allocation constraints (Alvarez et al., 2012; Kraiser et al., 2011). Plants assimilate nutrients such as N from the soil, after microbial mineralisation from organic forms (e.g. proteins, amino acids, chitins and urea) to mineral forms [nitrate (NO_3^-) and ammonium (NH_4^+)] has taken place. Limited availability of mineral N in the form of nitrate and ammonium in the soil decreases plant performance, can change levels in gene expression (Lillo et al., 2008), and therefore can induce severe alterations of the plant proteome (Møller et al., 2011). Further downstream, a limited availability of mineral N can cause pronounced qualitative and quantitative changes in primary plant metabolites such as amino acids (Tschoep et al., 2009) and organic acids (Tschoep et al., 2009), but also in secondary plant metabolites such as flavonoids (Lillo et al., 2008) and terpenoids (Kleine and Müller, 2013). Nutrient-related changes of the plant metabolome can be critical to the outcome of multitrophic interactions between plants and other organisms (Kutyniok and Müller, 2013; Staley et al., 2011). Intra- and interspecific competition can also lead to the depletion of essential macronutrients such as N, phosphate and potassium in the soil, and at the same time, might reduce photosynthesis and carbon (C) assimilation rates of the target plants due to shading. These effects might cause alterations in transcript levels and potentially downstream changes of the plant metabolome. The association with AMF may increase photosynthetic rates (Jakobsen et al., 1991; Smith and Read, 2008), but also may interfere with the C pools of a plant by draining photoassimilates (Jakobsen et al., 1991; Parniske, 2008). These opposing effects could change metabolite fluxes without necessarily changing metabolite pool sizes. Moreover, the association with AMF can cause specific alterations of the plant metabolome leading to concentration changes of primary and secondary chemical defence compounds (Vannette et al., 2013; Schweiger et al., 2014). As physiological and chemical changes in response to AMF might be rather specific and can additionally depend on abiotic conditions or might be modified by biotic interactions such as competition, the particular consequences of an association with AMF on the plant metabolome are difficult to predict.

To study the effects of nutrient availability, competition and the association with AMF alone and in combination, we chose *Plantago lanceolata* L. (Plantaginaceae), which is a well-established model organism in ecological (Bowers et al., 1992; Marak et al., 2003; Quintero et al., 2014) and metabolomic research (Pankoke et al., 2013; Sutter and Müller, 2011). This plant species is a typical 'generalist' growing in nutrient-rich habitats with a high availability of N and other macronutrients, but which also occurs in ruderal sites and nutrient-poor grasslands, in which macronutrients are more limited. *P. lanceolata* produces specific terpenoid defence compounds, the iridoid glycosides, which mediate biotic interactions such as plant–insect and plant–pathogen interactions (Marak et al., 2002a; Dobler et al., 2011; Pankoke and Dobler, 2015), and which are inducible to some extent (Marak et al., 2002b; Pankoke and Müller, 2013; Quintero and Bowers, 2011). Challenge-specific responses of iridoid glycosides to various individual environmental factors such as nutrition, competition, or the association with AMF have been studied thoroughly (Barton and Bowers, 2006; Bennett et al., 2009; Gange and West, 1994; Marak et al., 2003; Wurst et al., 2004), revealing a high plasticity to the respective challenges. However, the impact of these environmental factors on the overall polar plant metabolome is less explored. In particular, plant responses to interactions of these challenges in different combinations have been rarely considered.

To study the discrete and interactive effects of abiotic and biotic challenges on plant biomass production, biomass allocation patterns and metabolic responses of *P. lanceolata*, we performed a full factorial experiment with limited mineral N supply, in which the two common mineral forms of N, nitrate and ammonium, were reduced by 80%, interspecific competition with *Hieracium pilosella*, and the association with AMF as treatments. We examined morphological and allocation-related traits, as well as the chemical composition using an untargeted metabolic fingerprinting approach combined with the analysis of specific target defence compounds such as iridoid glycosides and phenolic compounds. We expected limited mineral N supply to reduce biomass production and alter biomass allocation patterns, and to additionally cause significant changes of the plant metabolome. *H. pilosella*, which in nutrient poor grasslands is a natural competitor of *P. lanceolata* (H. Pankoke & I. Höpfner, personal observation), was expected to more uniformly reduce the availability of macro- and micronutrients in comparison to the N limitation treatment. We also expected interspecific competition to negatively affect biomass production, yet to cause less pronounced changes in the plant metabolome. The factorial combination of limited mineral N supply and interspecific competition was expected to additively affect plant growth and the plant metabolome, and thus to cause the most severe stress for the plants. As the effects of the association with AMF on the host depend on the nutrient availability, we expected positive effects of mycorrhiza on biomass production under mineral N limitation and competition, but less pronounced effects under full nutrient availability. In line with the growth-differentiation hypothesis (Herms and Mattson, 1992; Stamp, 2003), we expected nutritional constraints such as those caused by severe limitation of mineral N supply and by competition to decrease concentrations of the carbon-based target defence compounds such as the iridoid glycosides and various phenolic compounds. As phenolic compounds are produced in the shikimic acid pathway *via* metabolisation of the amino acids phenylalanine or tyrosine, we expected N limitation to decrease the concentrations of the most abundant phenolic compounds more than competition or mycorrhization.

2. Results

2.1. Effects of limited mineral N supply, competition and the association with AMF on biomass production and biomass allocation

To study the effects of different challenges such as abiotic stress (80% reduced nitrate and ammonium supply), biotic stress (competition with *H. pilosella*), the association with AMF and the interactions between these factors, we analysed growth and biomass allocation of *P. lanceolata* plants in a balanced full-factorial multivariate design. A permutational multivariate analysis of variance (perMANOVA) on different biomass and allocation parameters showed that limited mineral N supply ($F_{1,32} = 22.84$, $P < 0.001$, $R^2 = 0.21$), competition with *H. pilosella* ($F_{1,32} = 19.08$, $P < 0.001$, $R^2 = 0.18$), and presence of AMF ($F_{1,32} = 24.72$, $P < 0.001$, $R^2 = 0.231$), as well as the interaction between competition and the presence of AMF ($F_{1,32} = 6.36$, $P < 0.005$, $R^2 = 0.06$) explained 68.5% and thus most of the variation in the growth-related data, while the other interaction terms had no significant effect on biomass production of *P. lanceolata* ($P > 0.05$). The joint analysis of growth and biomass allocation parameters *via* principal component analysis (PCA) also showed that mineral N fertilisation (Mann–Whitney-*U*-Test, $W = 86$, $P = 0.002$) and interspecific competition ($W = 340$, $P < 0.001$) explained the main proportion of the total variation (equaling 65.6%) on PC1 with the highest loadings being total dry mass (−0.50), shoot dry mass (−0.50), root dry mass (−0.48), and leaf area (−0.45) (Fig. 1). Limited mineral N ($W = 349$, $P < 0.001$) and the association with AMF ($W = 291$,

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