RESEARCH ARTICLE

Positive intercropping effects on biomass production are species-specific and involve rhizosphere enzyme activities: Evidence from a field study

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HIGHLIGHTS

GRAPHICAL ABSTRACT

• Intercropping effects on yield advantages are crop species specific.

• We measured kinetic parameters of three important enzymes in the rhizospheres of individual crop species in both mono and mixed cultures.

• In moderately nitrogen enriched soils, phosphorus becomes important nutrient element, involved in nutrient facilitation.

• Positive relative interaction index for faba bean when intercropped with either lupine or maize showed net facilitative interactions.



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ABSTRACT

Less attention has been given to soil enzymes that contribute to beneficial rhizosphere interactions in intercropping systems. Therefore, we performed a field experiment by growing faba bean, lupine, and maize in mono and mixed cultures in a moderately fertile soil. We measured shoot biomass and the kinetic parameters (maximal velocity (V_{max}) and Michaelis-constant (K_m)) of three key enzymes in the rhizosphere: Leucine-aminopeptidase (LAP), β -1,4-N-acetylglucosaminidase (NAG), and phosphomonoesterase (PHO). Faba bean benefitted in mixed cultures by greater shoot biomass production with both maize and lupine compared to its expected biomass in monoculture. Next, LAP and NAG kinetic parameters were less responsive to mono and mixed cultures across the crop species. In contrast, both the V_{max} and K_m values of PHO increased in the faba bean rhizosphere when grown in mixed cultures with maize and lupine. A positive relative interaction index for shoot P and N uptake for faba bean showed its net facilitative interactions in the mixed cultures. Overall, these results suggest that over-productivity in intercropping is crop-specific and the positive intercorpping effects could be modulated by P availability. We argue that the enzyme activities involved in nutrient cycling should be incorporated in further research.

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1 Introduction

Intercropping and mixed cultures (hereafter intercropping) are

increasingly seen as a viable prospect for a more sustainable, regenerative and less intensive agriculture, that reduces environmental impacts such as soil degradation, biodiversity loss, and nutrient leaching (Ehrmann and Ritz, 2013; Wezel et al., 2014). Yield or biomass increasing effects of intercropping rely on trait complementarity of the interacting crop species such that the total resource use is effective while creating the facilitative effects of one species on the other. The classic example is the "Three Sisters" combination of maize, squash, and bean: the bean fixes atmospheric nitrogen (N) and its neighbors profit from it. Beans use the maize as growth support whereas the squash covers the ground, thus reducing water loss from the soil (Lewandowski, 1987). Even the root systems of these three species show complementarity in their root architecture and thus nutrient uptake in a modeling study (Postma and Lynch, 2012).

Root-root interactions contribute to plant nutrient uptake in intercropping systems. For instance, it is well known that growing legumes together with cereals leads to efficient N uptake of cereals. In line with the stress gradient hypothesis (Bertness and Callaway, 1994), the potential for strong facilitation effects during intercropping is expected to be highest when abiotic conditions are relatively extreme (Holmgren and Scheffer, 2010). For intercropping, this means when soils are relatively nutrient-poor. There is now abundant evidence for facilitation occurring in nutrient-poor sites between crop species from different plant functional groups (e.g., as in the Three Sisters example). There is however potential to increase overall nutrient use efficiency in moderately fertile to fertile cropping sites by fostering facilitative interactions while reducing or even avoiding mineral fertilization. Addition of fertilizers is known to suppress biological N fixation in Fabaceae species (Voisin et al., 2002; Li et al., 2009), but this is not always the case (Nabel et al., 2016). Therefore, one could envisage a combination of fostering facilitation through the selection of ideal cropping combinations with or without additional fertilization within regenerative agriculture.

Improved N uptake in intercropping between legumes and their neighbors occurs mainly through two processes: 1) N sparing and 2) N transfer. In intercropping systems, sparing of N is a phenomenon, in which, legumes meet their N demand mainly from fixing atmospheric N through biological fixation. As a result, cereals can access the soil N spared by legumes. Transfer of N from legumes to neighboring cereals both by roots and mycorrhizal networks can also lead to greater N uptake in cereals (Meng et al., 2015; Wahbi et al., 2016). It is also hypothesized that cereals can quickly exhaust the soil N pool which increases the biological N fixation by the legume and ultimately increasing soil N pools (Xiao et al., 2004).

It is reasonably accepted that in N poor soils, yield advantages in intercropping systems are mainly driven by N complementarity, but we know less about the overall effects of phosphorus (P) in mediating such effects on yield when N is not limiting plant growth. Unlike N, which involves characteristic N sparing (a unique characteristic of legumes), intercropped species rely on a given amount of P present in soil, which may lead to strong competition for P. Higher P demand for biological N fixation may further exacerbate P requirements for legumes. To maximize their P uptake, intercropped species can differently regulate exudation of organic acids to mobilize the otherwise plant unavailable P (Li et al., 2010), change their root architecture and attract mycorrhizal fungi to efficiently explore soil volumes (Wen et al., 2017; Kumar et al., 2019), and upregulate the production of P mobilizing enzymes (phosphomonoesterases; PHO) from their roots in the rhizosphere to transform organically bound P (Olander and Vitousek, 2000). Moreover, root-derived microbial activation can also trigger microbial production of PHO enzymes in the rhizosphere to mine for P from soil organic matter (SOM) and plants can benefit from this by acquiring the available P. A strong plant regulation on activities of different enzymes was demonstrated in maize rhizosphere under field conditions with downregulation of activities of N cycling enzymes under N fertilization (Kumar et al., 2018). Similarly, P fertilization resulted in the downregulation of PHO activity in the maize rhizosphere (Kumar et al., 2019). These findings hint at preferential uptake of N and P derived from fertilizers by microbes and plants, as well as a reduction of their respective enzymes synthesis related to N and P cycling. These results also indicate the suitability of nutrient cycling enzymes as sensitive indicators of C, N, and P cycling in the rhizosphere, even though it is not possible to separate their sources (microbial versus plants) with enzyme kinetics assays. Nonetheless, enzyme activities in the rhizosphere are believed to be the fingerprint of interactions between plants and microbes (Gianfreda, 2015).

Despite it being clear that inter-specific facilitation and spatio-temporal resource partitioning are among the main drivers of yield advantage in intercropping systems (Li et al., 2014), many studies have ignored the processes of an altered plant nutrition mediated by enzyme-driven nutrient mobilization in soils. For example, the breakdown of amide-linked polypeptides (the primary form of organic N in soil) is associated with L-leucine aminopeptidase (LAP) activity contributing predominantly to N cycling (Finzi et al., 2015) but it also contribute to C cycling. Phosphomonoesterase (PHO) activity is linked to the breakdown of the ester-linked P contained in organic compounds (Sinsabaugh and Shah, 2011), whereas β-1,4-N-acetylglucosaminidase (NAG) targets the breakdown of chitin and peptidoglycans from microbial cell wall components and therefore, can also serve as a proxy for microbial turnover (Beier and Bertilsson, 2013; Tischer et al., 2015). As different crops have a distinct impact on rhizosphere microbial communities (Marschner et al., 2004), changes in a suite of kinetic parameters of different enzymes in their rhizosphere are most likely to occur by intercropping compared to the monocultures. Determination of the changes in enzyme kinetics could provide a promising novel avenue to explore intercropping, going beyond more classical niche complementarity concept. Considering shifts in enzyme kinetic parameters might be useful to identify

optimal species combinations when evaluating the potential for particularly sustainable and effective intercropping strategies. Therefore, the overall aim of our study was to test whether crop species (maize, faba bean, and lupine) influence kinetic parameters of three key enzymes (PHO, LAP, and NAG) in their rhizosphere differently in mixed cultures (a combination of two crop species) as compared to their respective monocultures. To investigate this, we set up a field intercropping experiment in relatively fertile soil in relation to N (0.15%–0.2% total N) but with low P availability (0.1% total P).

We hypothesized:

1. Greater activities of PHO, LAP, and NAG in rhizosphere than bare-fallow soil as a net effect root-mediated microbial activation.

2. Higher activities of N cycling enzymes (LAP and NAG) in maize than in legume (both faba bean and lupine) rhizo-spheres due to contrasting N acquisition strategies.

3. Legumes will fix more N when intercropped with nonlegume (here maize) and hence be less dependent on soil N. Therefore, LAP and NAG activities will decrease and PHO activity increase in the legume rhizosphere under intercropping to sustain the higher N fixation rates.

2 Materials and methods

2.1 Experimental design and management

The field experiment was conducted in an arable field in Rettmer, Lüneburg, Germany in 2019 (53°12'N and 10°22'E). Soil is classified as Cambisol with coarse sand (>100 µm), fine sand (<100 µm), silt, and clay of 87.76%, 6.21%, 5.95%, and 0.07%, respectively and contains approximately 2.1% total carbon, 0.2% total nitrogen, 0.1% total phosphorus, and a pH of 6 (measured in distilled water). The climatic conditions were typical of temperate regions with mild summers during experimental period. The daily mean precipitation and temperature are provided in supplementary data (Supplementary Fig. 1). Weather data was acquired from the nearest weather station (https://www.dwd.de/DE/klimaumwelt/cdc/ cdc node.html). The experimental field was under conventional agricultural practices where 800 kg ha⁻¹ chalk lime, Caralonkali containing 30% K, 12% P, 6% Mg, and 4% S, and 470 HAS solution containing 6% S and 20% N were applied for summer barley a year ago. The field experiment consisted of a block design (covering variation across the field) with randomized plots within each block. In total, there were five blocks and 7 plots were randomly arranged within each block giving a total of 35 plots. The 2 m × 2 m plots were separated from each other with 1 m wide strips to avoid edge effects. Each plot consisted of maize (Zea mays L. cv. Colisee), faba bean (Vicia faba L. cv. Tiffany), white lupine (Lupinus albus L. cv. Energy) either in mono (Maize-Maize: M-M; Faba bean-Faba bean: Fb-Fb; and Lupine-Lupine: L-L) or mixed cultures

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(a combination of two crops; Maize + Faba bean (M-Fb), Maize + Lupine (M-L), and Faba bean + Lupine (Fb-L)). One plot within each block was kept without vegetation (bare fallow) throughout the experimental duration by regular weeding. Crops were grown in rows (with alternating rows of each crop species in mixtures). Maize, faba bean, and lupine monocultures were sown at planting densities of 12, 42, and 42 plants m⁻², respectively. Inter row and inter-plant distance within a row were 30, 15, and 15 cm for maize, faba bean, and lupine, respectively. When grown in mixed cultures, the planting density was reduced to half for individual crops (6, 21, and 21 plants m^{-2} for maize, faba bean, and lupine, respectively). Inter row and inter-plant distance remained similar for faba bean and lupine mixed cultures as these crops were sown at the same planting density. But when either of these crops (faba bean or lupine) was grown in mixed culture with maize, the inter-row (between two rows) distance was 20 cm whereas the inter-plant distance (within a row) for either faba bean or lupine was 11.25 cm and inter-plant distance (within a row) for maize was 40 cm. Seeds were received from Feldsaaten Freudenberger without artificial fungicide and pesticide coating. Seeds of all three crops were sown simultaneously from 9th to 10th May 2019. After the seedling emergence, each plot was manually irrigated to avoid any water stress during their establishment. We did not apply any fertilizer, pesticides, or insecticides throughout the experimental duration.

2.2 Plant and soil sampling

Two months after sowing, two plants of each crop species were destructively harvested from each plot. Shoot biomass was cut at the soil surface and dried at 60°C until constant weight. The total aboveground biomass in intercrops for each species was calculated as difference between observed and expected biomass values in mixed cultures compared to their respective monocultures. Rhizosphere soil of two plants of each crop species was pooled together to make one composite sample and stored at 4°C for less than a week before analyses. Rhizosphere soil was defined here as the soil attached to roots after vigorous shaking and was collected with tweezers.

2.3 Enzyme assays

Rhizosphere enzyme activity and kinetic parameters of phosphomonoesterase (PHO) and β -1,4-N-acetylglucosaminidase (NAG) were measured with 4-Methylumbelliferone (MUB)-based artificial substrates (Marx et al., 2001; Kumar et al., 2019). The activity of L-leucine aminopeptidase (LAP) was measured with fluorogenic 7-amino-4-methycoumarin (AMC)-based artificial substrate (Marx et al., 2001). For this, soil suspension was prepared by dissolving 1g fresh soil in 50 mL distilled water and shaken for 1 h. Then 50 µL of soil suspension and 50 µL of either MES (for MUB based

substrates) or TRIZMA (for AMC based substrate) buffers were added into each well of a black 96-well microplate (PureGrade[™], KG, Wertheim, Germany). Afterward, 100 µL of MUB-based substrate 4-Methylumbelliferyl-phosphate and 4-Methy-lumbelliferyl-N-acetyl-β-D-glucosaminide was added to each well for PHO and NAG, respectively. For LAP, 100 µL of L-Leucine-7-amido-4-methylcoumarine hydrochloride was added as an AMC-based substrate. For each enzyme, we measured the activity in a range of substrate concentrations 0, 10, 50, 100, 200, 300, 400, and 500 μM for both MUB and AMC based substrates. Microplates were gently shaken immediately after substrate addition and fluorogenic measurements were taken using a fluorometric plate reader (Victor3 1420-050 Multi-label Counter, PerkinElmer, USA) with excitation and emission wavelengths of 360 nm and 450 nm, respectively. Fluorescence values were then converted to the amount of MUB or AMC cleaved using specific standards. Enzyme activities were expressed as the amount of MUB or AMC cleaved in nanomoles per gram soil per hour (nmol MUB/AMC cleaved g^{-1} soil h^{-1}). For each enzyme, kinetic parameters, i.e., maximal velocity (V_{max}) and Michaelis-constant (K_m) were calculated using the Michaelis-Menten equation as follows:

$$v = \frac{V_{\max} \times [S]}{K_m + [S]} \tag{1}$$

where v is the rate of reaction, [S] is the substrate concentration, K_m is the substrate concentration at which the reaction rate is half of its maximum.

To evaluate the level of crop interactions, we also calculated the relative interaction index (*RII*) for shoot P (*RII*_P) and shoot N (*RII*_N) uptake (in leaves) as follow (Armas et al., 2004):

$$RII_{P(or N)} = [Total P(or N) uptake in mixed culture$$

- Total P(or N) uptake in monoculture]

 \div [Total P(or N) uptake in monoculture

+ Total P(or N) uptake in mixed culture] (2)

Positive values indicate facilitation whereas negative values indicate competition between interacting plant species.

2.4 Statistics

All statistical analyses were performed within R 3.6.3 environment (R Core Team, 2020) and graphs were prepared with the 'ggplot2' (Wickham, 2016) and ggpubr (Kassambara, 2019) libraries. Due to recent discussion on the significance and null hypothesis testing using $\alpha = 0.05$, we instead assessed the measured variables by comparing effect sizes (absolute differences) and their confidence intervals (Cls) of 95% computed by using non-parametric bootstrap resampling with 10 000 iterations (Amrhein et al., 2019; Rillig et al., 2019).

3 Results

3.1 Rhizosphere pH and shoot biomass

On average, pH was similar in lupine and maize rhizosphere but decreased slightly in the faba bean rhizosphere but this was not modulated by growth in mono or mixed culture (Supplementary Fig. 2). Shoot biomass was affected by the specific crop species combinations grown, with faba bean benefitting from facilitative interactions for shoot biomass, whereas maize and lupine did not (Supplementary Table 1). Compared to its monoculture (Fb-Fb), the expected shoot biomass of faba bean increased with both lupine (Fb-L) and maize (M-Fb). On the other hand, the expected shoot biomass of lupine and maize was similar in their respective mono and mixed cultures (Fig. 1).



Fig. 1 Effects sizes of the impact of intercropping on shoot biomass (kg m⁻²) production. Dashed lines indicate biomass of corresponding monocultures. Effect sizes were calculated as the absolute differences between mixed and monocultures for each crop species. Values above and below zero represent higher and lower shoot biomass compared to that in respective monocultures. Values are the means and 95% confidence intervals using non-parametric bootstrapping with 10000 iterations. M-L: maize + lupine mixed culture, M-Fb: maize + faba beam mixed culture, Fb-L: faba bean + lupine mixed culture.

3.2 Crop specific effects on enzyme kinetic parameters

The maximal reaction rates (V_{max}) of PHO, LAP, and NAG increased in the rhizosphere compared to bare-fallow soil except that PHO activity remained similar between bare fallow soil and faba bean rhizosphere in its monoculture (Fig. 2). However, the effects of species-specific rhizospheres on V_{max} in mono and mixed cultures were depended on specific enzymes. For instance, in the rhizosphere of faba bean, the V_{max} of PHO was higher in M-Fb and Fb-L mixed cultures compared to Fb-Fb monoculture. On the other hand, in the rhizosphere of lupine, V_{max} of PHO was lower in M-L mixed culture relative to L-L monoculture. In the maize rhizosphere, PHO activity remained similar under both mono and mixed cultures (Fig. 2A). Relative to Fb-Fb monoculture, the K_m value of PHO was increased in the rhizosphere of faba bean when grown in mixed cultures (Fig. 2B).

 V_{max} of LAP remained similar in the rhizosphere of faba bean and maize both in mono and mixed cultures whereas it



Fig. 2 Effect sizes of the impact of rhizosphere on (A), (C), (E) maximum potential activity (V_{max} ; nmol MUB/AMC cleaved g⁻¹ soil h⁻¹) and (B), (D), (F) Michaelis constant (K_m ; µmol g⁻¹ soil) of PHO, LAP, and NAG under mixed and monocultures. Effect sizes were calculated as the absolute differences between mixed and monocultures for each crop species. Dashed lines indicate the V_{max} and K_m values in bare fallow soil. Values are the means and 95% confidence intervals using non-parametric bootstrapping with 10 000 iterations. Fb-Fb: faba bean monoculture, L-L: lupine monoculture, M-M: maize monoculture, M-L: maize + lupine mixed culture, M-Fb: maize + faba beam mixed culture, Fb-L: faba bean + lupine mixed culture.

decreased in the lupine rhizosphere when grown in mixed culture with maize (M-L). The K_m value of LAP remained similar across the rhizospheres of both mono and mixed cultures and did not differ to that of bare fallow except a slightly lower K_m value in faba bean rhizosphere when grown in mixed culture with lupine (Fig. 2D).

 V_{max} of NAG was similar in the rhizosphere of faba bean and maize both in mono and mixed cultures. However, in the rhizosphere of lupine, V_{max} of NAG was higher in mixed culture with faba bean (Fb-L) compared to lupine monoculture (L-L) (Fig. 2E). Further, K_m of NAG remained similar across the different rhizospheres and in both mono and mixed cultures and was higher than that of bare fallow (Fig. 2F).

3.3 Relative interaction index for shoot P and N uptake

The RII values for P (RII_P) were on average 0.16 and 0.1

greater for faba bean when grown in mixed culture with lupine and maize, respectively. On the other hand, RII_P values for lupine and maize remained similar in both mono and mixed cultures (Fig. 3A). Interestingly, RII_N values showed a similar pattern as RII_P except that RII_N value decreased for maize, when grown in mixed culture with faba bean compared to lupine (Fig. 3B). For absolute values, refer to supplementary Table 1.

4 Discussion

4.1 Crop-specific effects of intercropping on biomass production

We showed that positive intercropping effects on shoot biomass production were crop-specific. Shoot biomass of



Fig. 3 Impact of intercropping on relative interaction index (*RII*) for (A) phosphorus (*RII*_P) and (B) nitrogen (*RII*_N) uptake for each crop species. Positive values indicate facilitative whereas negative values indicate competitive plant-plant interactions. Presented are the means and 95% confidence intervals using nonparametric bootstrapping with 10 000 iterations. M-L: maize + lupine mixed culture, M-Fb: maize + faba beam mixed culture, Fb-L: faba bean + lupine mixed culture.

faba bean was higher in mixed cultures relative to its monoculture counterpart. Surprisingly, we found the strongest positive intercropping effect in Fb-L mixed culture and not in maize intercropped with legumes as generally found in previous studies (Li et al., 2007; Yu et al., 2010). Also, the shoot biomass of lupine was similar in its corresponding mono and mixed cultures. There was a tendency for higher biomass production for maize in mixed cultures which is similar to other intercropping findings, even if weaker (Zhang et al., 2010; Dissanayaka et al., 2015; Li et al., 2016). Our study confirms that even in moderately fertile soils, facilitation can play an important role in higher biomass production (Holmgren and Scheffer, 2010). Even though that faba bean biomass increased both with lupine and maize, the underlying mechanisms in mixed culture with maize might be different from that in a mixed culture of lupine considering their corresponding rhizosphere properties.

Increased faba bean biomass in mixed culture with maize has most likely been driven by inter-specific rhizospheric interactions. Root exudates not only act as a microbial energy source but also as signaling molecules to interact with other organisms in the rhizosphere (Dakora and Phillips, 2002). For example, maize root exudates can induce root nodulation as well as an increased rate of biological N fixation in faba bean. Hence, greater faba bean productivity in mixed culture with maize can be expected as was also shown by Li et al. (2016). This also suggests that maize exudate-driven signaling molecules enhance N availability to the neighboring faba bean indirectly through atmospheric N fixation. Next, the temporal and spatial segregation in nutrient demand between maize and faba bean may also have contributed to increased productivity of faba bean without affecting that of maize (Li et al., 2003; Zhang et al., 2017). In the mixed culture with maize, planting density was reduced to half resulting in an overall lower number of plants per unit area (21 faba bean + 6 maize = 27 plants m⁻² in mixed culture compared to 42 plants m⁻² in faba bean mono culture) and therefore, in lower competition between maize and faba bean. Moreover, faba bean was in the flowering stage at which P demand is the highest (Peng et al., 2016), whereas maize was still in the vegetative phase leading to temporal segregation of P requirement.

Temporal segregation of nutrient demand as potential underlying mechanisms of increased biomass production of faba bean is less likely to be the case in faba bean and lupine mixed culture. This is because both faba bean and lupine have comparable growth rates and mature relatively at the same time indicating strong competition for resource availability. Although the planting density of lupine and faba bean was half than their respective monocultures, the overall planting density per unit area remained similar (21 faba bean + 21 lupine = 42 plants m^{-2}). Yet, lupine and faba bean positively interacted in the mixed culture. One possible explanation could be that lupine increased in intercropping its secretion of organic acids to mobilize P compared to the monoculture. This has previously been observed in many studies (Gardner and Boundy, 1983; Li et al., 2014, Wen et al., 2019). This is unlikely to explain the higher shoot biomass in intercropped faba bean with lupine in the present study because pH in lupine rhizosphere remained similar both in mono and mixed cultures with faba bean and lupine. No change in pH in lupine rhizosphere is similar to recent findings from Ma et al. (2019) which showed that P mobilization strategy of lupine is phenology dependent and after the cluster root formation, PHO activity contributes higher in P mobilization that the rhizosphere acidification.

4.2 Root-mediated microbial activation increased rhizosphere enzyme activities

We showed that the presence of plants increased the enzyme activities compared to bare fallow soil (except for faba bean monoculture). This is in support of our first hypothesis confirming root-mediated microbial activation in the rhizosphere, in which, root-derived substrates stimulate microbial metabolic activity (Cheng and Kuzyakov, 2005; Nannipieri et al., 2012). 'Microbial triggering' by root-derived substrates in the rhizosphere may also have contributed to increased enzyme activities by accelerating their metabolism and energy state in expectation of 'food event' (De Nobili et al., 2001; Blagodatskaya and Kuzyakov, 2008; Mason-Jones and Kuzyakov, 2017). To fulfill their nutrient demands, metabolically active microorganisms produce and release enzymes in the soil to mobilize the nutrients from SOM via decomposition a phenomenon generally termed as rhizosphere priming effects on SOM decomposition (Kuzyakov, 2002; Hamer and Marschner, 2005; Fontaine et al., 2011).

4.3 Nitrogen cycling enzyme parameters remained similar in maize and legume rhizospheres

On the rhizosphere level, we observed comparable effects of maize, lupine, and faba bean rhizosphere on LAP and NAG activities but there was a tendency of higher NAG and LAP potential activities in the lupine rhizosphere (except for LAP in lupine rhizosphere in mixed culture with maize). This is in contrast to our second hypothesis where we expected higher LAP and NAG activities in maize than in legume rhizospheres due to the legumes' N fixing capacity by the symbiosis with rhizobia. Negative feedback of nutrient availability on enzyme activities is consistent and has been observed in previous studies (Olander and Vitousek, 2000; Dilly and Nannipieri, 2001; Ai et al., 2012). Moderately N fertile soils might have resulted in such observed effects in the maize rhizosphere. When a particular nutrient element is not the most limiting factor, plants and microbes downregulate their production of enzymes involved in its mobilization to avoid resource tradeoffs (Fontaine et al., 2011; Kumar et al., 2019). This suggests another element besides N is restricting maize growth predominantly.

4.4 Activity of LAP was lower in lupine rhizosphere in mixed culture with maize whereas PHO activity was higher in faba bean rhizosphere in mixed culture with lupine and maize

Maximum potential activities and K_m values of N cycling enzymes (LAP and NAG) did not change in faba bean rhizosphere if grown in mixed cultures with both lupine and maize (M-Fb and Fb-L) compared to monoculture (Fig. 2). This lack of shift in LAP and NAG kinetic parameters (both V_{max} and K_m) in faba bean rhizosphere support the notion of its lower dependence on soil N pools (Temperton et al., 2007). However, LAP activity was markedly lower in the lupine rhizosphere in mixed culture with maize (M-L). On the other hand, LAP activity was also lower in maize rhizosphere when grown in mixed culture with lupine. This joint reduction in LAP activity suggests reduced competition for N between maize and lupine in mixed cultures. Overall, these results suggest toward reduced soil N mediated effects on enzyme activities and shoot biomass more likely due to pool substitution in which legumes acquire N predominantly from the atmosphere through biological fixation and maize can access the N spared by legumes. Interestingly, we found a tendency of enhanced NAG activity, one of the key enzymes involved in microbial turnover, in the lupine rhizosphere when grown in mixed cultures (M-L and Fb-L). This might suggest one complex strategy of lupine to hydrolyze microbial cell wall components to avoid nutrient 'stealing' from the biologically fixed atmosphere-derived N. Increased secretion of NAG by lupine roots followed by higher citrate exudation to avoid P 'stealing' has previously been shown (Weisskopf et al., 2006). In general, rhizosphere enzyme activities in the lupine rhizosphere are dominated by its root exudates and less by a contribution from microbes (Wasaki et al., 2005). Altered maximum activities (V_{max}) of LAP and NAG (also PHO activity, Fig. 2) in maize and lupine rhizosphere without affecting the K_m values hints that their secretion from roots (and hence the root activity) have been modulated in mono and mixed cultures.

With respect to our third hypothesis, we did not observe an overall higher PHO activity in the legume rhizospheres (for both faba bean and lupine) than that of maize. This is in contrast to the findings from Li et al., (2004) where they showed that PHO activity was higher in legume (chickpea) rhizosphere than that of maize irrespective of P availability. No remarkable change in PHO activity in maize and legume rhizospheres could be due to spatial segregation for P requirements or as a result of different crop species. Especially leguminous crops are shown to have different P uptake strategies depending on their development stage by altering root physiologic traits. For instance, distinct strategies to maximize P uptake were developed by lupine (Lupinus albus L.) depending on plant phenology (Ma et al., 2019). Before cluster root formation by lupine, strong acidification of rhizosphere and increased PHO activity contributed to rhizosphere broadening and P mobilization around the tap root systems, whereas, after the formation of cluster roots, PHO decreased around the tap roots and increased in close proximity with cluster roots (Ma et al., 2019). Further, increased rhizosphere acidification has been linked to P mobilization without affecting PHO activity (Li et al., 2014). We further showed that the maximal PHO activity and K_m values were increased in faba bean rhizosphere in mixed culture with both maize and lupine (M-Fb and Fb-L) compared to its monoculture (Fig. 2A and 2B). Thus, faba bean seems to show the strongest interactive effects when grown in mixed cultures, at least regarding the P cycle. Planting diversity has been shown to impact microbial activity and especially that of decomposers activity via more diverse substrate inputs in the soil which may feedback to plant growth (Chung et al., 2007; Cong et al., 2015). More specifically, higher K_m values for PHO in faba bean rhizosphere in mixed cultures (both in Fb-L and M-Fb) indicate a shift in rhizosphere microbial community toward more copiotrophs which are characterized by higher growth rate but by low affinity to the substrate (Killham and Prosser, 2015; Tischer et al., 2015; 2019). Changes in the rhizosphere microbial community of faba bean in mixed cultures have previously been reported in acidic soils (Li et al., 2010). To support the fast growth rate of faba bean, P demand is higher than of many other plants, especially those used in this study. This has been translated in higher PHO activities (V_{max}) in faba bean rhizosphere when grown in mixed cultures (Fig. 2). This finding demonstrates that not only faba bean root nodulation is induced when grown in mixed cultures (Li et al., 2016) but that the rhizosphere P cycling is also accelerated to meet the P requirement feeding the high ATP demand of the N

fixation. We believe that the responsiveness of PHO kinetic parameters in the faba bean rhizosphere and the corresponding greater shoot biomass of faba bean in mixed cultures are supporting the interpretation of P facilitation in faba bean intercropping. Positive RII_P values for faba bean when grown in mixed cultures with both lupine and maize demonstrate such facilitative interactions (Fig. 3). This supports the more general idea that P facilitation is a mechanism underlying the positive biodiversity effects (Yu et al., 2020). Interestingly, RII_N values for faba bean showed a similar pattern to that of RII_P except for a lower RII_N value for maize when grown in mixed culture with faba bean. This decrease in RII_N values for maize, however, did not translate into maize biomass reduction further suggesting that N was not the most limiting factor for plant biomass production in our study.

5 Conclusions

We demonstrated positive inter-specific facilitation on shoot biomass production in an intercropping system and highlighted that such effects were crop-specific. Shifts in enzyme functioning in the rhizosphere in mixed cultures relative to the corresponding monocultures were enzyme-specific depending on the plant's nutrient demand. Either no change or even decrease in LAP and NAG activities without affecting their K_m values in mono and mixed cultures across the individual rhizospheres suggest that the observed overyielding effects were less mediated by the N availability. Greater PHO activity and K_m values in faba bean rhizosphere in mixed cultures (M-Fb and Fb-L) highlight that microbial communities and enzyme functioning were altered (evident from altered K_m values of PHO enzymes) with positive feedback effects on plant performance. Further, positive interaction indices in relation to both P and N (RII_P and RII_N) for faba bean in mixed cultures showed facilitative interactions which resulted in greater shoot biomass. Overall, we show that in relatively N rich but P limited soils, P facilitation could significantly contribute to increased productivity in mixed cultures and this occurred via changes in rhizosphere P cycling.

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Electronic supplementary material

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