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ORIGINAL PAPER

Can Organic P Inputs Alleviate P Limitation Effects on Nutrient Uptake and Biological N_2-Fixing Capacity of Hairy Vetch (*Vicia villosa*)?

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Abstract
Phosphorus (P) is a limiting nutrient in many agroecosystems and, apart from affecting plant growth, can also limit biological N_2 fixation (BNF) by leguminous plants. Thus, increasing P supply can have a positive effect on BNF particularly in P-deficient soils. Here, we provide new insights into the response of hairy vetch (*Vicia villosa*), widely adopted as a legume cover crop, to P limitations, by comparing the effects of inorganic (Pi) and organic (Po) P supply on plant growth and BNF capacity. This was achieved by means of a greenhouse experiment in which rhizobia-inoculated hairy vetch was grown in a P-limited agricultural soil and changes in plant growth, nitrogen (N) and P uptake, BNF capacity, and soil phosphatases activities were evaluated as a function of Pi and Po inputs, in the form of orthophosphate or phytic acid, respectively. When compared to P-deficient conditions where BNF was primarily limited by plant growth rather than directly due to the high P costs of symbiotic N fixation, Pi addition substantially enhanced plant growth (threefold), nodule formation (16-fold), P acquisition (sixfold), and BNF efficiency (sevenfold). In contrast, even with the addition of the highest dose of Po, the increase in plant growth, nodule formation, P acquisition, and BNF capacity (1.7, 3.5, 2.4 and 2.1-fold, respectively) was much less expressed, indicating that hairy vetch could only minimally access Po sources over the growth period in order to alleviate the P limitation effect on N_2 fixation in under P-deficient conditions. These findings suggest that hairy vetch will not be able to provide sufficient BNF for improving soil N inputs in low-fertility cropping systems that rely on organic inputs.

Keywords Legume cover crop · Phosphorus deficiency · Rhizobia · Phytic acid · Stable isotope dilution · Phosphatase activity

1 Introduction

Legume cover crops are often included in agricultural cropping systems for enhancing soil nitrogen (N) availability and improving the productivity and sustainability of succeeding cash crops under low-input systems (Torbert et al. 1996). Apart from increasing inputs of organic matter to the soil, growing legumes as winter cover crops may increase the net N inputs through biological N_2 fixation (BNF), a process that involves the establishment of a symbiotic relationship between plants and rhizobia N-fixers hosted in their roots (Fageria et al. 2005).

The BNF capacity of a leguminous crop, expressed as the proportion of N derived from the atmosphere (%Ndfa) by symbiotic association, is known to greatly depend on soil fertility (Romanyà and Casals, 2020). Low and high N availability can reduce or even suppress BNF either by limiting plant photosynthetic capacity (low N; Moreau et al. 2008; Vitousek et al. 2013) or by reducing %Ndfa due to the higher energetic cost of symbiotic fixation compared to soil N acquisition (high N; Rastetter et al. 2001; Vitousek et al. 2002; Walley et al. 2011). Adequate phosphorus (P) nutrition is also an important component of legume production systems due to their greater P requirements than cereals (Pang et al. 2018). The dependence of symbiotic performance on P availability was reported to depend on the overall nutritional status of the soil, with a stronger effect of

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inorganic P (Pi) addition on BNF in soils with a low P status where rhizobia compete with plants for available P, but not in P-rich soils (Raji et al. 2019). Soil P availability can affect BNF capacity directly by modulating nodule growth, formation, and functioning as a result of the high P costs of symbiotic N fixation (Divito and Sadras, 2014; Raven 2012) or indirectly by affecting plant growth and allocation of photosynthetically assimilated C to the symbionts (Püschel et al. 2017; Walley et al. 2011).

Nonetheless, legumes are also known to hold an advantage in P acquisition due to their capability to mobilize sparingly soluble P in the soil through a variety of root mechanisms in order to provide for the great P demand necessary to maintain the rhizobial symbiosis (Jakobsen, 1985). Under P-limited conditions, leguminous plants have been reported to respond by acidification of the rhizosphere, increasing the exudation of organic acids, up-regulating the production of extracellular phosphatase enzymes, and favoring symbiotic mycorrhizal associations (Hinsinger, 2001; Houlton et al. 2008; Nasto et al. 2014). In particular, considering that organic P (Po) may account for 30–80% of the total P in arable soils primarily in the form of inositol phosphates and other phosphate monoesters (Turner et al. 2002), the greater investment in phosphatase enzymes with respect to non-N_2-fixing species could facilitate P acquisition by catalyzing the hydrolysis of organic P esters releasing Pi for uptake by plant roots (Olde Venterink, 2011; Tarafdar and Claassen 1988). However, Png et al. (2017) have recently evidenced that the greater root phosphatase activity of legumes, particularly at low soil P availability, was likely a phylogenetic trait of rhizobial legumes rather than being directly related to their N_2-fixing capacity. On the other hand, enzymatic activity may be hampered by the great chemical stabilization of phosphate monoesters, especially inositol phosphates, by interaction with mineral surfaces (Celi et al. 2020; Giaveno et al. 2010). In this regard, although the specific capability of legumes to mobilize sparingly accessible P may represent a further advantage for Po recycling compared to other species, this has received scant attention.

Various studies have evidenced a positive effect of P supply on BNF by N_2-fixing plant species (e.g. Isaac et al., 2011), with increasing P supply improving nodule number, nodule biomass, and BNF rates (Chekanai et al. 2018; Olivera et al. 2004). In particular, Bukovsky-Reyes et al. (2019) reported a positive relationship between soil-available P and vetch root N content when vetch was P-limited at soil-available P contents below 70 mg kg^{-1}. Hairy vetch (*Vicia villosa* Roth) is widely used in agroecosystems as a winter legume cover crop and green manure contributing between 100 and 230 kg N ha^{-1} with biomass incorporation and having a BNF capacity over the plant growth period, ranging between 60 and 100%Ndfa (Parr et al. 2011; Wagger 1989). It has been shown to perform better than other vetch species in terms of aboveground biomass production, root morphological characteristics, P and K uptake, and N_2-fixing activity (Solangi et al. 2019), with an appropriate adaptability to both P-limiting and non-limiting conditions (Anugroho et al. 2010). However, little is known on the responses of *Vicia villosa* to P limitations, the consequences for N_2 fixation rates, and adaptive plant strategies for coping with nutrient deficiencies in low fertility soils. Moreover, whereas most studies focused on understanding the effects of Pi availability on the mechanisms controlling P acquisition and implications on BNF rates under low P supply, there still remains a lack of evidence on role of Po sources in controlling BNF in leguminous plants. These Po sources may contribute to partially offset the dependence on labile Pi to satisfy plant P requirements by leguminous plants (Turner 2008).

Based on these considerations, we hypothesized that under severe P limitation, (a) improving P supply through Pi inputs can favor plant growth and C allocation to the root nodules and consequently enhance the BNF capacity of hairy vetch plants, while (b) increasing Po inputs can only partially alleviate the P limitation effect on N_2 fixation, because the ability of vetch plants to access organic P sources is limited by strong Po fixation processes. We tested these hypotheses by growing rhizobia-inoculated hairy vetch plants in a P-limited soil and evaluating plant growth, N and P uptake, BNF capacity (by isotope dilution), and soil enzyme activities related to P cycling, as a function of Pi and Po inputs in the form of orthophosphate or inositol hexaphosphate, respectively.

2 Materials and Methods

2.1 Experimental Design

The effects of P availability on nutrient uptake and BNF capacity of *Vicia villosa* Roth were evaluated by means of a greenhouse pot experiment in which hairy vetch was planted in a P-poor agricultural soil (Olsen P < 3 mg kg^{-1}) amended with two levels of added P in the form of mineral (Pi) and organic P (Po), as well as an unfertilized control (CNT). The experiment was conducted in a fully factorial experimental design with three sampling times over the plant growth period (seeding to flowering stage) and five biological replicates per treatment, and thus comprised a total of 75 pots. The plants were seeded in mid-April and were grown for 10 weeks in 1.2-l pots (containing 800 g of soil) positioned randomly in a greenhouse under natural light conditions. The duration of the greenhouse experiment covered the same plant growth stages as those observed in the field when cover crop termination is generally carried out around the flowering stage. The soil used was collected from the topsoil (0–30 cm) of an acidic, sandy loam agricultural soil having a

pH of 5.0; a clay and sand content of 8.1 and 56.4%, respectively; an available P content (Olsen P) of 2.94 mg kg^{-1}; 5.1 and 12.3 mg N kg^{-1} of available ammonium and nitrate–N, respectively; an organic C content of 6.02 g kg^{-1}; and a C/N ratio of 11.8. Prior to use, the soil was air-dried and passed through a 2-mm sieve.

A gradient of P supply was obtained by applying 0 (CNT), 40 (PiL and PoL) or 120 mg P kg^{-1} (PiH and PoH) in the form of inorganic (KH_2PO_4) or organic P (potassium *myo*-inositol hexaphosphate, < 2% hydrolyzed P) prior to planting. These amounts of added P, chosen on the basis of adsorption and desorption isotherms of Pi and Po onto the agricultural soil used, did not exceed 20 and 2% of the maximum sorption capacity for Pi (Langmuir coefficients: $Q_{max} = 655$ mg P kg^{-1}; $K_L = 0.52$ L μg^{-1}) and Po (Langmuir coefficients: $Q_{max} = 6084$ mg P kg^{-1}; $K_L = 0.34$ L μg^{-1}), respectively, and were therefore completely adsorbed. The doses applied were thus intended to obtain soils with a range of P nutritional statuses representing the different scenarios of available P in agricultural soils. These amounts were 4- to tenfold greater that the annual application doses of P with mineral or organic (e.g., bovine manure) fertilization generally applied in conventional cropping systems. The amount of Olsen-extractable P in the soil receiving 40 mg P kg^{-1} was equivalent to 12 and 8 mg P kg^{-1} for PiL and PoL treatments, respectively, and 62 and 10 mg P kg^{-1} for PiH and PoH soils receiving 120 mg P kg^{-1}.

Ten seeds of hairy vetch were planted in each pot, and after 10 d these were thinned to 5 plants per pot. All plants were inoculated at sowing with a commercial rhizobial inoculant (ALOSCA® group F; strain WSM1455) that included *Rhizobium leguminosarum* bv. viciae previously tested to be compatible with the host plant species. During the growing period, a nutrient solution (50 ml) containing 235 mg L^{-1} K, 200 mg L^{-1} Ca, 64 mg L^{-1} S, 50 mg L^{-1} Mg, 0.5 mg L^{-1} B and Mn, and 0.05 mg L^{-1} of Zn and Mo was applied weekly, while the soil moisture was regularly checked gravimetrically and adjusted to around 60% of the water-holding capacity by adding water to compensate for losses by evapotranspiration.

In order to distinguish N uptake by plant via root and BNF pathways, and to calculate the BNF capacity of hairy vetch as a function of P availability by isotope dilution, a relatively small amount of isotopically labelled N fertilizer was applied to each pot as $K^{15}NO_3$ (10 at% ^{15}N; 10 mg N kg^{-1}) 20 days after seeding (DAS) to prevent potential suppression of nodulation at the early stages of plant development.

2.2 Plant and Soil Sampling and Analyses

Five pots per treatment were destructively sampled 30, 50, and 70 DAS. During harvesting, hairy vetch plants were removed without damaging roots and biomass. The shoots were cut at the soil surface, while the roots were carefully removed from pots, gently shaken to remove most of the soil and subsequently carefully washed in water to remove adhered soil particles. Visible nodules growing on the roots were collected aseptically and pooled. Fresh samples were dried at 65 °C until constant mass, weighed, and then milled for subsequent analysis. The soil in the pot was collected after plant harvesting, air-dried, and ground (2-mm sieve) prior to analyses.

Total N contents and the N isotopic ratio in plant tissues were measured by high-temperature combustion using an elemental analyzer (Vario Isotope Select, Elementar Analysensysteme GmbH, Hanau, Germany) coupled to an isotope ratio mass spectrometer (IsoPrime 100, Elementar Analysensysteme GmbH). Total P concentrations in the plant shoots, roots, and nodules were determined by sulfuric-perchloric sample digestion followed by spectrophotometric analysis using the malachite green method (Ohno and Zibilske, 1991). Phosphorus-acquisition efficiency (PAE) was calculated as the ratio of total plant P uptake to soil available P (P Olsen), while P-utilization efficiency (PUE) was calculated as the ratio of dry biomass to P content in the plant tissues (Neto et al. 2016).

Soil P fractions were determined by extraction with 0.5 M $NaHCO_3$ (P Olsen) and 10 mM citrate (P Citrate) to represent plant-available and labile Pi (Kirk 1999; Olsen et al. 1954), while 0.1 M NaOH + 1 M NaCl (1:1; P NaOH) was used to extract less labile Pi sorbed on Fe and Al mineral surfaces (Buehler et al. 2002; Cross and Schlesinger 1995). The molybdate-reactive phosphate content in the extracts was determined spectrophotometrically (Murphy and Riley 1962). Nitrate and ammonium concentrations in the soil were determined by extraction in 1 M KCl followed by spectrophotometric quantification using modified Greiss and Berthelot methods, respectively, as described by Cucu et al. (2014). Isotopic enrichment of the extracted mineral N pool was determined by a combination of micro-diffusion and ^{15}N stable isotope analysis (Vario Isotope Select and IsoPrime 100) as described by Schleppi et al. (2006), and subsequently used to calculate biological N fixation by the plants.

2.3 Soil Enzyme Activities

Acid (acP, EC.3.1.3.2) and alkaline (alkP, EC.3.1.3.1) phosphomonoesterase activities as well as inositol-P phosphatase activity (inositP, EC 3.1.3.25) were determined in soil extracts by using fluorogenic substrates, according to Cowie et al. (2013). Extracts were obtained with a bead-beating procedure using 300 mg of dry soil in 3% lysozyme buffer (pH 6.0) as desorbant. After centrifugation, enzyme activities in the supernatants were assayed

fluorometrically in microplates using 4-methyl-umbelliferyl based substrates in appropriate buffers, and expressed as nmol g^{-1} h^{-1}.

2.4 Calculation of BNF

Biological N_2 fixation (BNF) was calculated as the product of plant N biomass and the proportion of N derived from the atmosphere (%Ndfa). The %Ndfa was calculated by isotope dilution (Chalk and Craswell, 2018; Unkovich et al., 2008), which compares the isotopic signature of the leguminous plant biomass with that of the isotopically labelled plant-available soil mineral N pool, according to the equation:

$$\%\text{Ndfa} = \left(1 - \frac{\text{atom \% } ^{15}\text{N excess}_{\text{legume}}}{E*}\right) \times 100$$

where $E*$ is the time-integrated pool enrichment of the soil mineral N available for plant uptake and that takes into account the exponential decline in the ^{15}N enrichment (atom % excess) of the mineral N pool over the plant growth period due to the supply of unlabeled N through the mineralization of soil organic N. The initial soil mineral N isotopic enrichment (E_0) and the first-order rate constant (k in d^{-1}) for the decline in the ^{15}N enrichment of the soil mineral N pool over the growth period were estimated by fitting the change in ^{15}N enrichment (atom% excess) over time (t) into the exponential equation:

$$E_t = E_0 e^{-kt}$$

$E*$ over a specific time interval (i.e., over 30, 50, and 70 DAS) was obtained by mathematical integration of the exponential equation using the formula:

$$E^* = \frac{E_0(e^{-kt_1} - e^{-kt_2})}{k(t_1 - t_2)}$$

2.5 Statistical Analysis

Prior to analysis of variance (ANOVA), the data sets were tested for normality and homogeneity of variance by Shapiro–Wilk ($p > 0.05$) and Levene test ($p > 0.05$), respectively. Any data that were not fit for normal distribution were log-transformed. One-way ANOVA was used to assess the effects P forms and doses on all measured parameters separately for each sampling time. Significant ($p < 0.05$) differences between means were identified using the post hoc Tukey HSD test. All ANOVA analyses were performed using SPSS version 19.0 (SPSS Inc., USA).

3 Results

3.1 Soil P Fractions and Available N

Plant-available P during the growth period generally reflected the application of P with the different treatments, although P availability at 30 DAS was already much lower than initial availabilities. Lowest Olsen and citrate-extractable P were obtained for the untreated control, while highest contents were observed for the Pi-treated soils, with PiH showing significantly higher values than PiL ($p < 0.05$; Table 1). Irrespective of the dose, application of Po did not result in significantly different Olsen P contents when compared to CNT ($p > 0.05$), while citrate-extractable P was slightly higher than CNT ($p < 0.05$) but not different between PoL and PoH treatments ($p > 0.05$; Table 1). These differences were consistent over the entire growth period. Most of the Pi applied to the soils was recovered in the NaOH-extractable fraction resulting in proportionally higher P contents in the PiL and PiH treatments than CNT ($p < 0.05$). On the other hand, NaOH-extractable P in soils treated with PoL and PoH was not significantly different from CNT ($p > 0.05$; Table 1).

Soil mineral N contents (sum of ammonium and nitrate N) were generally relatively low (< 5 ppm) with little or no differences between treatments throughout the growth period expect for the earliest sampling time (30 DAS; data not shown). At this time, mineral N concentrations were generally > 5 ppm with highest concentrations of 18.2 mg N kg^{-1} measured for PoH, which were slightly but significantly higher than that obtained for CNT (13.2 mg N kg^{-1}; $p < 0.05$).

3.2 Plant Growth and Nodulation

Aboveground biomass was significantly influenced by P application over the whole growth period ($p < 0.001$). Application of Pi strongly enhanced shoot growth when compared to the control even when applied at low doses, and at 70 DAS greatest shoot biomass was observed for PiH followed by PiL (Fig. 1a). On the other hand, application of Po did not result in significant differences in shoot growth ($p > 0.05$), even though shoot biomass for PoH at 70 DAS was slightly greater than CNT ($p = 0.084$; Fig. 1a). Root growth showed a different response to P addition when compared to shoot growth. After 50 DAS, only PoH showed a significantly greater root biomass with respect to all the other treatments including the control ($p < 0.05$), resulting in the highest root:shoot (R/S) ratio of 1.5 (Fig. 1a, b). However, these differences were no longer observed at 70 DAS when both root biomass and

Table 1 Soil mineral N and soil P fractions as a function of different forms (Pi, inorganic P; Po, organic P) and amounts (CNT, no P; L, low P; H, high P) of applied P after 30, 50, and 70 days after seeding (DAS). Values represent the mean ± standard error ($n = 5$), while different letters indicate significant differences between treatments within each sampling date ($p < 0.05$)

Treatment	P Olsen (mg P kg^{-1})	P Citrate (mg P kg^{-1})	P NaOH (mg P kg^{-1})	NH_4^+ (mg N kg^{-1})	NO_3^- (mg N kg^{-1})
30 DAS					
CNT	1.7 ± 0.1 c	2.1 ± 0.1 d	47.6 ± 0.4 c	4.2 ± 0.4 b	8.9 ± 0.8 ab
PiL	5.9 ± 0.4 b	7.1 ± 0.1 b	76.2 ± 0.4 b	3.1 ± 0.2 b	6.3 ± 0.5 b
PiH	19.8 ± 1.3 a	30.7 ± 0.2 a	153.5 ± 2.8 a	3.6 ± 0.3 b	1.0 ± 0.3 c
PoL	1.7 ± 0.1 c	2.8 ± 0.1 c	50.7 ± 0.4 c	4.7 ± 0.4 b	9.4 ± 0.5 ab
PoH	1.9 ± 0.1 c	2.8 ± 0.1 c	52.6 ± 1.2 c	6.7 ± 0.7 a	11.5 ± 1.3 a
50 DAS					
CNT	2.7 ± 0.1 c	2.3 ± 0.1 d	49.4 ± 1.4 c	1.9 ± 0.5	3.1 ± 1.1 a
PiL	7.9 ± 0.1 b	7.3 ± 0.1 b	78.5 ± 1.1 b	1.0 ± 0.1	0.2 ± 0.1 b
PiH	27.0 ± 0.5 a	32.8 ± 0.1 a	137.0 ± 2.1 a	1.6 ± 0.1	0.4 ± 0.1 b
PoL	3.0 ± 0.1 c	2.9 ± 0.1 c	49.0 ± 1.7 c	1.1 ± 0.2	0.5 ± 0.1 b
PoH	3.3 ± 0.1 c	2.9 ± 0.1 c	52.7 ± 1.7 c	1.2 ± 0.2	0.5 ± 0.2 b
70 DAS					
CNT	2.0 ± 0.1 c	1.5 ± 0.1 d	47.2 ± 0.7 d	0.9 ± 0.1 ab	0.6 ± 0.3
PiL	7.9 ± 0.1 b	6.1 ± 0.1 b	76.0 ± 0.8 b	1.2 ± 0.2 ab	0.2 ± 0.1
PiH	32.9 ± 1.2 a	27.9 ± 0.2 a	167.7 ± 2.1 a	1.5 ± 0.2 a	0.3 ± 0.1
PoL	2.3 ± 0.1 c	2.2 ± 0.1 c	50.5 ± 0.4 cd	0.9 ± 0.1 ab	0.5 ± 0.2
PoH	3.6 ± 0.1 c	2.3 ± 0.1 c	52.0 ± 0.4 c	0.8 ± 0.1 b	0.2 ± 0.1

R/S ratios of Po-treated soils were not different from those of the control ($p > 0.05$). Application of Pi did not influence root growth but resulted in significantly lower R/S ratios than the control (0.3–0.6; $p < 0.05$). Nodulation was significantly affected by the application of Pi that always led to greater nodule biomass than the control ($p < 0.05$), proportional to the dose of applied P. By day 50, nodule biomass was already 6 and 18 times greater than that in the control for PiL and PiH, respectively (Fig. 1c). In contrast, application of Po did not influence nodulation except for a slightly greater nodule biomass observed for PoH after 70 DAS than the control ($p = 0.052$).

3.3 Plant P Uptake

With the addition of Pi, vetch plants always showed higher total P uptake than the untreated control (Fig. 2a; $p < 0.001$) due to the combined effects of a higher biomass as well as a higher P content. This higher P uptake observed for both above- and belowground biomass was proportional to the amount of applied Pi. The increase in plant P acquisition with increasing P availability however resulted in a PAE that was always lower or equal to the control (Fig. 2b), and a PUE that was always lower than the control (Fig. 2c) throughout the growth period, particularly for PiH. In the case of treatment with Po, only application of high doses resulted in a plant P uptake after 50 DAS that was slightly but significantly higher than that observed for the untreated control ($p < 0.001$; Fig. 2a). This led to a slightly higher PAE than both the control and the Pi-treated plants, which was however only significant at 50 DAS ($p < 0.05$). Nonetheless, this did not correspond to a higher PUE which was often lower than the control, although always higher than that observed for plants receiving Pi that was most evident at 70 DAS, particularly for PoL (Fig. 2).

3.4 Plant N Uptake and BNF Efficiency

Plant N uptake was influenced by the addition of Pi as the supply of plant-available P led to an increase in N uptake that was proportional to the amount of Pi added. This was more appreciable in the shoot N content that was significantly higher than the control already by day 30 ($p < 0.05$), while differences in the root N content were only noted after 70 DAS (Fig. 3; $p < 0.05$). In contrast, application of Po did not affect plant N uptake even when applied at high doses, and shoot and root N contents were generally similar to those observed in the control ($p > 0.05$), except for a higher root N content in plants treated with PoH after 50 DAS (Fig. 3; $p < 0.05$). Similar results were also obtained for the amount of plant N derived from the atmosphere through BNF. Application of Pi had a positive effect on BNF with shoot Ndfa reaching values of around 72–82% by day 70, compared to only 40% in the untreated control and 35–55% in the Po-treated soils (Table 2). Similar trends were observed

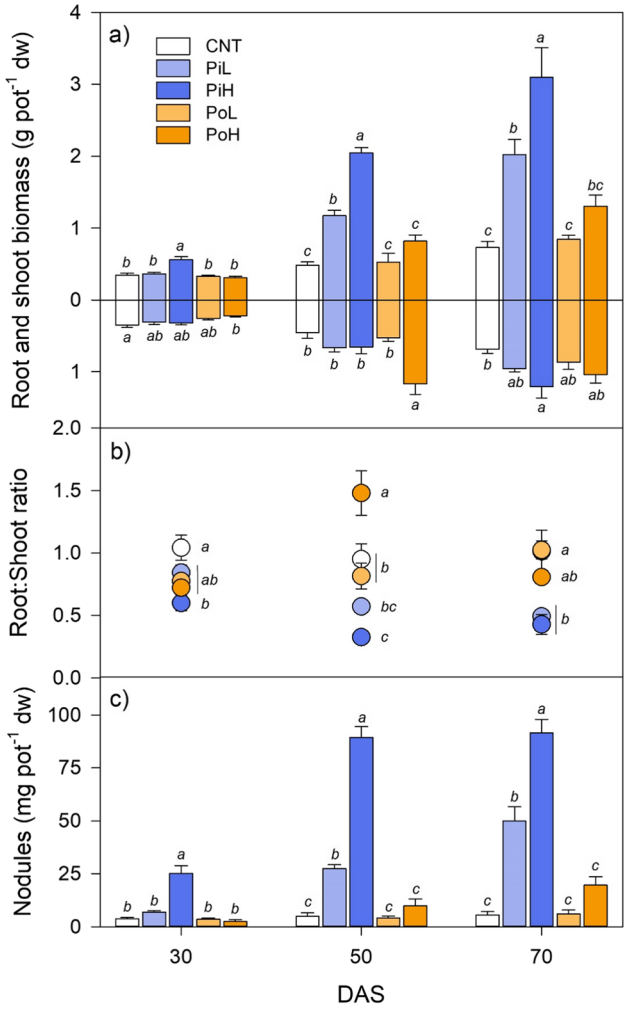

Fig. 1 Root and shoot biomass (**a**), root-to-shoot ratio (**b**), and nodule biomass (**c**), as a function of different forms (Pi, inorganic P; Po, organic P) and amounts (CNT, no P; L, low P; H, high P) of applied P after 30, 50, and 70 days after seeding (DAS). Values represent the mean ($n=5$), while error bars represent the standard error. Different letters indicate significant differences between treatments within each sampling date ($p<0.05$)

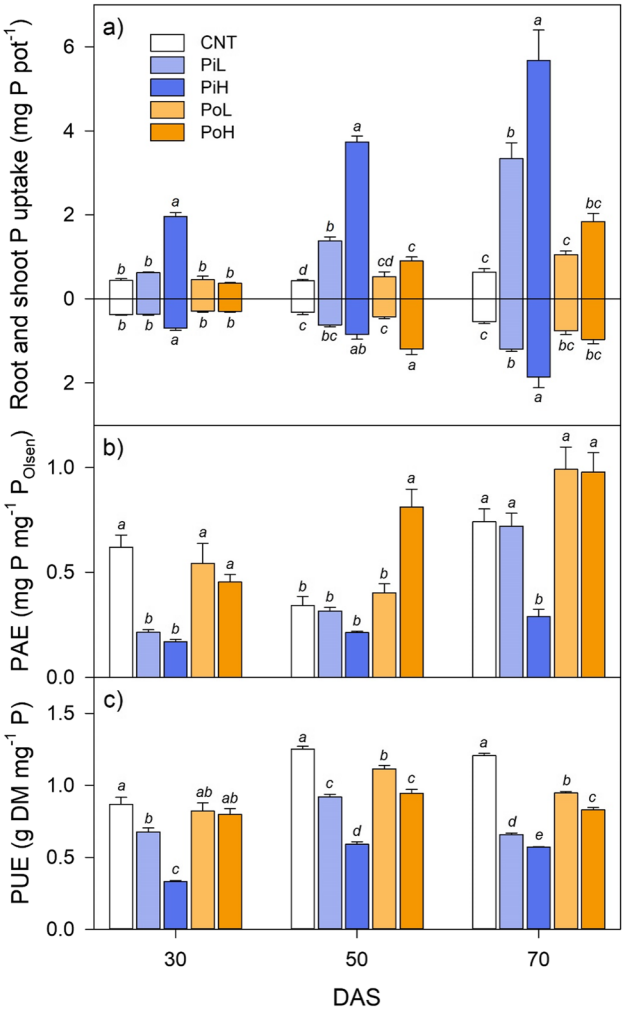

Fig. 2 Root and shoot P acquisition (**a**), P acquisition efficiency (**b**), and P utilization efficiency (**c**) as a function of different forms (Pi, inorganic P; Po, organic P) and amounts (CNT, no P; L, low P; H, high P) of applied P after 30, 50, and 70 days after seeding (DAS). Values represent the mean ($n=5$), while error bars represent the standard error. Different letters indicate significant differences between treatments within each sampling date ($p<0.05$)

for root Ndfa although the differences were less marked as fixed N represented a smaller proportion of total plant N in the roots. Here, a maximum of 67–69% Ndfa was observed in PiH-treated soils, while the proportions of fixed N in the roots of the other treatments were not significantly different from the control (Table 2; $p>0.05$). Root nodules always showed relatively high contents of fixed N (on average 82%) though the difference between treatments was not easy to decipher due to a lack of sufficient sample for analysis leading to non-replicated results. Nonetheless, nodule Ndfa values with the addition of Pi were always somewhat larger than the other treatments.

3.5 Phosphatase and Phytase Activities

Soil enzyme activities related to P cycling did not show large changes as a function of Pi and Po addition when compared to the control (Fig. 4). Enzyme activities were generally rather low with acP and alkP activities ranging between 4.8 and 12.1 and 1.4 and 5.5 nmol g^{-1} h^{-1}, respectively, while inositP activities were always lower than 0.2 nmol g^{-1} h^{-1}. Data revealed a strong evidence that phosphatase activities were affected by P addition only at the earliest time (30 DAS; $p<0.005$), with highest acP and alkP activities observed for the highest dose of Pi. However, by the end of the growth period, there was hardly any

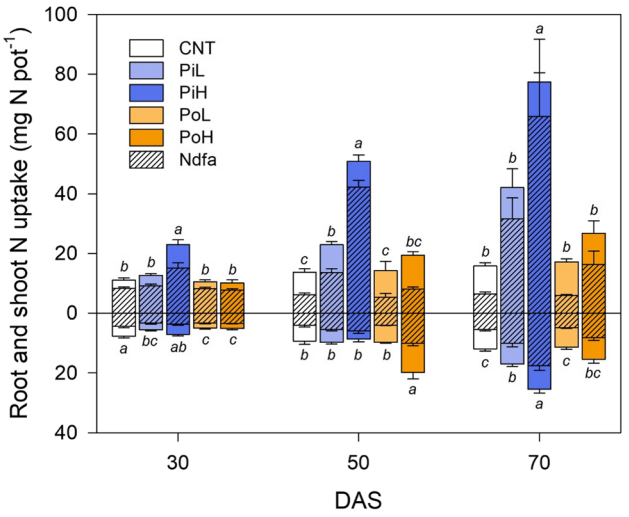

Fig. 3 Total root and shoot N acquisition and proportion of N derived from atmosphere (Ndfa) as a function of different forms (Pi, inorganic P; Po, organic P) and amounts (CNT, no P; L, low P; H, high P) of applied P after 30, 50, and 70 days after seeding (DAS). Values represent the mean ($n=5$), while error bars represent the standard error. Shaded areas represent the proportion of plant N that derives from the atmosphere (Ndfa). Different letters indicate significant differences in both total shoot and root N between treatments within each sampling date ($p<0.05$). The same letters apply for shoot and root Ndfa that showed similar significant differences as total N, except for differences in root Ndfa at 30 DAS that were not significant

Table 2 Proportion of N derived from the atmosphere (Ndfa) in the shoots, roots, and nodules of hairy vetch as a function of different forms (Pi, inorganic P; Po, organic P) and amounts (CNT, no P; L, low P; H, high P) of applied P after 30, 50, and 70 days after seeding (DAS). Values represent the mean ± standard error ($n=5$), while different letters indicate significant differences between treatments within each sampling date ($p<0.05$)

Treatment	Shoot Ndfa (%)	Root Ndfa (%)	Nodule Ndfa (%)
30 DAS			
CNT	76.0 ± 1.7 a	56.8 ± 3.1 ab	86.8†
PiL	72.5 ± 2.1 ab	59.5 ± 2.5 ab	85.7†
PiH	64.7 ± 3.4 b	50.6 ± 3.7 b	83.2 ± 1.6
PoL	78.1 ± 1.0 a	67.4 ± 1.6 a	100.0†
PoH	77.1 ± 2.7 a	67.9 ± 3.5 a	91.8†
50 DAS			
CNT	45.1 ± 3.4 c	43.2 ± 2.7 b	73.1†
PiL	58.7 ± 3.2 b	55.1 ± 2.4 ab	77.7 ± 1.9
PiH	82.9 ± 2.5 a	67.4 ± 7.0 a	90.8 ± 1.0
PoL	38.7 ± 2.2 c	41.1 ± 1.9 b	75.4†
PoH	41.5 ± 2.3 c	51.8 ± 5.5 ab	67.8†
70 DAS			
CNT	40.3 ± 2.9 c	45.2 ± 1.6 bc	72.4†
PiL	72.1 ± 7.0 ab	59.0 ± 5.1 ab	80.1 ± 3.5
PiH	82.4 ± 4.6 a	69.4 ± 5.9 a	86.6 ± 2.4
PoL	34.7 ± 2.4 c	42.4 ± 1.7 c	56.4†
PoH	56.8 ± 7.4 bc	52.7 ± 2.6 bc	73.9†

†Insufficient sample mass for replicated analysis

evidence that enzyme activities related to P cycling were in any way influenced by P additions.

4 Discussion

4.1 Influence of Inorganic P Inputs on Plant Growth and BNF

Plant growth is generally greatly influenced by soil P availability, and in leguminous plants, an increase in P availability can positively affect BNF rates. This can be due to the high P demand of N_2-fixing bacteria as well as to the effect of P availability on the plant photosynthetic capacity and belowground C allocation to roots and nodules (Divito and Sadras 2014, and references within). Similarly, hairy vetch growth was strongly limited by low soil P availabilities, such that increasing P supply through the addition of Pi resulted in a strong positive effect on biomass production, primarily through enhanced shoot growth. This inevitably led to hairy vetch plants investing less resources towards root development (lower R/S ratios), and showing lower P acquisition and utilization efficiencies when compared to plants grown in P-deficient conditions. As reported by Hidaka and Kitayama (2013), the preferential allocation of P to the shoots than the roots under P-sufficient conditions possibly allowed the plants to maintain their productivity and growth, reducing the demand for P.

Increasing N uptake was previously linked with a growth response to P supply in various other leguminous crop species (e.g., *Trifolium repens* L. by Almeida et al. 2000 and Høgh-Jensen et al. 2002, *Medicago* spp. by Püschel et al. 2017). In the presence of readily available P, even hairy vetch enhanced N_2-fixation suggesting an increase in the plant's N demand when P was not limiting, as indicated by the strong positive relationship between Ndfa and shoot P at maturity (i.e., 70 DAS; Fig. 5c). Moreover, the increase in nodule biomass with increasing P availability suggests that whereas nodulation was strongly P limited under deficient conditions, plants allocated more resources to the symbiosis with the N_2-fixing bacteria when P was readily available. This was reflected in a higher BNF efficiency and a greater allocation of fixed N into the aerial parts of the plant, confirming our first hypothesis. In contrast, fixed N in the roots was rather conservative, in line with the lower shoot-to-root C allocation. Nonetheless, even though nodule growth was strongly limited under severe P deficiency (≤ 2 ppm Olsen P), approximately 40% of the total N assimilated by these plants was due to symbiotic N_2 fixation, in line with the findings of Almeida et al. (2000) for white clover. BNF

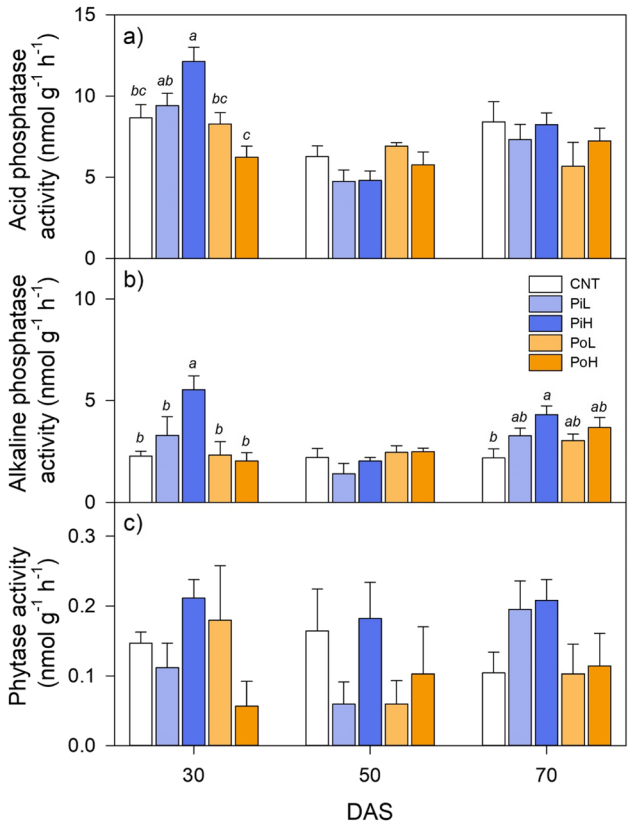

Fig. 4 Acid phosphatase (**a**), alkaline phosphatase (**b**), and phytase (**c**) activity as a function of different forms (Pi, inorganic P; Po, organic P) and amounts (CNT, no P; L, low P; H, high P) of applied P after 30, 50, and 70 days after seeding (DAS). Values represent the mean ($n=5$), while error bars represent the standard error. Different letters indicate significant differences between treatments within each sampling date ($p<0.05$)

showed a stronger relationship with biomass production rather than with Ndfa across all P treatments (Fig. 5a, b), confirming that in P-deficient conditions, BNF was primarily regulated by plant growth rather than a direct effect of P availability on N_2 fixation. Similar effects of P availability on BNF were reported for other vetches like bitter vetch (*Vicia ervilia*) grown in low-fertility soils (Romanyà and Casals 2020), although the effects of P deprivation are also known to depend on the duration of the stress and plant age (Høgh-Jensen et al. 2002).

4.2 Influence of Organic P Inputs on Plant Growth and BNF

Evidence on capability of leguminous plants to access organic P sources and partially offset the dependence on labile inorganic P for satisfying plant P requirements for BNF is still scarce. Our results pointed to an increase in BNF capacity with increasing P uptake as a result of the addition Po (at higher application doses; Fig. 5c), even though only a minimal increase in plant-available P was recorded. Although this effect was much less marked with respect to the addition of Pi, it suggests that hairy vetch can to some extent access organic P sources for its P requirements, and this can partially alleviate the P limitation effect on N_2 fixation. However, only small increases in total P uptake, plant growth, and nodulation were observed after 70 days from the application of higher Po doses. These findings, together with the slightly higher P acquisition efficiency though lower P utilization efficiency compared to the control, suggest that although the plants allocated resources to improve P acquisition from organic forms, this did not lead to a commensurate increase in plant growth over the duration of the experiment. Consequently, neither plant N uptake nor BNF benefitted

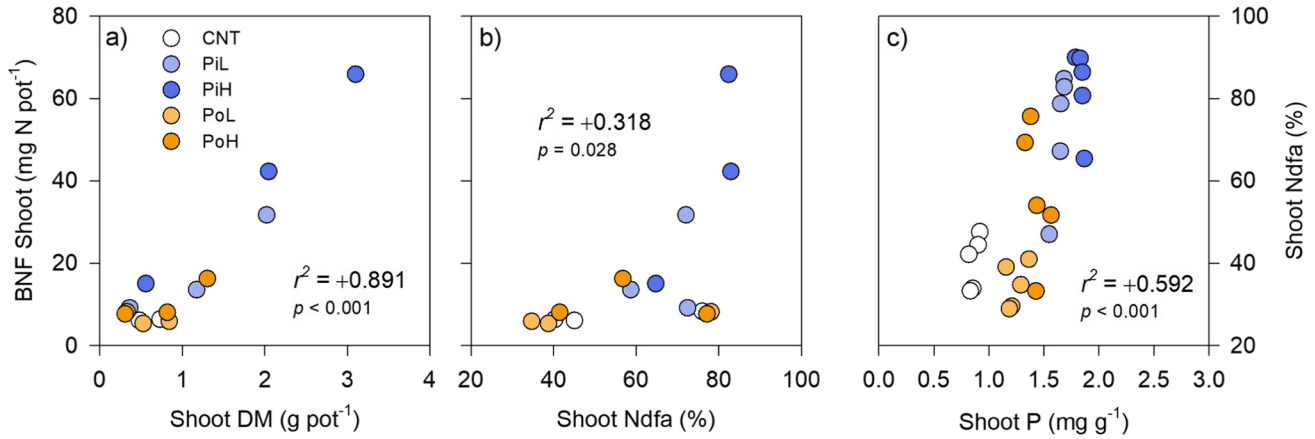

Fig. 5 Relationships between biological N fixation and (**a**) aboveground dry matter, and (**b**) the proportion of shoot N derived from atmosphere (Ndfa) over the whole growth period, and (**c**) relationship between shoot P and Ndfa at maturity, as a function of different forms (Pi, inorganic P; Po, organic P) and amounts (CNT, no P; L, low P; H, high P) of applied P. Values in (a) and (b) represent means for $n=5$ for each sampling time while values in (c) represent single samples at 70 DAS

from the slightly higher P uptake following Po addition, even though the proportion of shoot Ndfa was nonetheless higher in mature plants receiving the highest dose of Po than the control (Fig. 5c; $p = 0.05$).

The acquisition of P from organic resources by hairy vetch over the growth period from seeding to flowering under severely P-limited conditions was thus weak, and BNF was probably limited by the reduced photosynthetic capacity. Although *myo*-inositol phosphate is a potential source of P for plant growth through the up-regulation of root phosphatase (Adams and Pate 1992), it represents a much poorer P source in soils where availability is strongly limited by a preferential interaction with soil minerals (Celi and Barberis 2004; Martin et al. 2004). This could have well been the case in our experiment where even the highest dose of added *myo*-inositol phosphate was well below the maximum sorption capacity of the soil used, and did not result in any substantial increase in plant-available P pools over the duration of the experiment, particularly considering the low P and organic C contents of the soil used in this experiment. The availability of adsorbed Po to microorganisms or enzymes is known to be limited under low surface coverage (Giaveno et al. 2010), while microbial P immobilization in P-deficient (and low organic C) soils may also affect the release of Pi limiting the contribution of mineralization to a plant available P pool (Bünemann 2015). García-López et al. (2021) further showed that the adsorption of phosphate, deriving from the hydrolysis of Po, on soil minerals can also negatively affect plant P uptake even in the presence of elevated hydrolytic activity. Due to these constrains, we can hypothesize that hairy vetch was induced to adopt and protract combined strategies for an efficient activation of mechanisms that scavenge the nutrient from minerals.

The application of Po did not lead to the expected increase in soil enzyme activities related to P acquisition from organic sources, as phosphatase and phytase activities were generally similar to those observed for the control. We speculate that other strategies could have been activated by the legume plants to increase nutrient availability under P-deficient conditions, such as the release low molecular weight organic acids by the roots to increase soil P availability by ligand exchange or dissolution (Egle et al. 2003; O'Sullivan et al. 2021) that were not evaluated in this study. Moreover, Olde Venterink (2011) reported that the plasticity in root phosphomonoesterase activity of leguminous forbs to gradients in P supply was actually less evident under severely P-limited conditions, possibly due to the high costs in terms of N of enzyme synthesis. Application of Pi did however lead to a rapid, albeit temporary, increase in soil phosphatase activity though this was probably due to a microbial response to the higher P availability that could have induced a greater production of extracellular enzymes when compared to P-limited conditions (Malik et al. 2012). Unlike phosphatase activity in N_2-fixing roots (Nasto et al. 2014; Png et al. 2017), the relationship between rhizosphere soil phosphatase activities and available P may depend on the integrated effects of nutrient availability on both plant and microbial response, which may also show different temporal responses (Solangi et al. 2019), and remain rather inconclusive. Andrino et al. (2021) and Santoro et al. (2022) showed that even though the production of low molecular weight organic acids and protons can favor the release of inositol phosphate from mineral surfaces by ligand exchange or dissolution (Egle et al. 2003; O'Sullivan et al. 2021), the necessity to be subsequently hydrolyzed by phosphatases could further delay the incorporation of mobilized P into plant tissues compared to Pi sources. From a practical point of view, the time required for hairy vetch to benefit from Po acquisition strategies is nonetheless constrained by crop termination at flowering stage for green manuring before sowing of the succeeding cash crop.

5 Conclusions

The effects of inorganic and organic P inputs on *Vicia villosa* growth and N_2-fixing capacity are biologically interesting and agronomically relevant, particularly when considering the importance of this leguminous cover crop for improving organic N inputs and reducing mineral N use in agroecosystems. Together our findings confirm that soil N input by hairy vetch cover cropping and green manuring is greatly dependent on the P nutritional status of soils. When grown in P-deficient soils (Olsen $P < 3$ mg kg^{-1}) biological N_2 fixation (BNF) by hairy vetch was primarily limited by plant growth rather than directly due to the high P costs of symbiotic N fixation. On the other hand, under high soil P availability (Olsen $P > 20$ mg kg^{-1}) plant growth, nodule formation, P acquisition, and BNF efficiency were all strongly enhanced (3-, 16-, 6-, and sevenfold, respectively) when compared to P-deficient conditions.

Our findings also suggest that in low fertility cropping systems that rely on organic inputs or those having soils with a high P sorption capacity, organic P inputs alone will not necessarily allow hairy vetch to provide sufficient BNF for improving soil N inputs, and their contribution to P recycling is also limited. In fact, even with the addition of high doses of organic P, hairy vetch was only minimally able to access these organic sources and could only partially alleviate the P limitation effect on plant growth and N_2 fixation, under P-deficient conditions. The strategies that could have been activated by hairy vetch to increase organic P availability under P-deficient conditions however remain elusive as no notable effects of P addition on rhizosphere soil phosphatase activities were observed.

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Data Availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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