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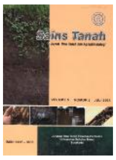

Evaluating soil phosphorus tests and nutrient limitations in Mediterranean pastures

Margarida Arrobas[1,2], Soraia Raimundo[1,2], Almeida Sawimbo[3], Nuno Rodrigues[4], Marjan Jongen[4,5], Ricardo F. M. Teixeira[5], Tiago Domingos[5], Manuel Ângelo Rodrigues[1,2]*

[1] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[2] Laboratório para a Sustentabilidade e Tecnologia em Regiões de Montanha, Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[3] Instituto Superior Politécnico do Cuanza Sul (ISPCS), Rua 12 de Novembro, Sumbe P.O. Box 82, Angola
[4] Terraprima – Serviços Ambientais, Centro de Negócios do Porto Alto, Fracção S, Avenida das Nações Unidas, nº 97, 2135-199 Samora Correia, Portugal
[5] MARETEC – Marine, Environment and Technology Centre, LARSyS, Instituto Superior Técnico, Universi-dade de Lisboa, Av. Rovisco Pais, 1, 1049-001 Lisbon, Portugal

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Corresponding Author
Email address:
** angelor@ipb.pt*

ABSTRACT

Reliable phosphorus (P) fertilization guidelines for Mediterranean pastures remain uncertain due to inconsistent soil testing methods, the complexity of mixed-species systems, and the neglect of other limiting nutrients. This study primarily aimed to identify reliable soil P tests and secondarily to explore potential nutrient limitations by assessing seven acidic Portuguese soils under Mediterranean conditions using subterranean clover (*Trifolium subterraneum*) and ryegrass (*Lolium multiflorum*) as model species. Five P extraction methods (Egner-Riehm, Olsen, Bray II, Mehlich I, and anion exchange resin) were compared alongside a pot experiment with clover, ryegrass, and their mixture. Biomass production, plant nutritional status, and soil–plant relationships were used to assess nutrient availability and plant response. The Egner-Riehm method proved the most reliable, showing linear correlation with the resin method ($R^2 = 0.89$), widely regarded as the best indicator of soil P bioavailability. P availability was therefore not a limiting factor for biomass production. The lowest soil P content (54 mg kg^{-1} P_2O_5, Egner-Riehm) was close to the medium threshold (50–100 mg kg^{-1}), yet plant growth indicated adequacy. In contrast, nitrogen (N) emerged as the main growth constraint, and boron (B) deficiency further restricted clover performance, confirming additional nutritional imbalances. A linear relationship between soil and tissue B supported its association with DMY, and tissue B levels were below sufficiency thresholds. Overall, in acidic Portuguese soils under Mediterranean conditions, soil P was adequate, whereas N and B acted as key constraints to productivity. These findings support more accurate and efficient fertilization strategies for Mediterranean pastures.

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1. INTRODUCTION

Improved pastures are a practical approach to intensifying livestock production systems without relying on the application of external inputs. Essentially, they involve sowing plant species that are potentially more productive than spontaneous vegetation (Schils et al., 2022; Teixeira et al., 2015; van Dobben et al., 2019). Legume species are among the primary vegetation types used in improved pastures, as they have the potential to enhance both the biomass productivity of the agroecosystem and the protein content of the biomass consumed by livestock (McLachlan et al., 2024; Prestes et al., 2017). The introduction of additional N into the system occurs naturally, without the need for synthetic fertilizer application, due to the ability of legumes to establish symbiotic relationships with rhizobia, thereby accessing atmospheric N (Hungria & Nogueira, 2023). In Portugal, Mediterranean dryland pastures also play a crucial role in livestock production systems by providing forage under warming climatic and challenging edaphic conditions, where low soil fertility constrains the persistence of legumes that are essential for pasture productivity and ecosystem functioning

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(Carreira et al., 2022; Carreira et al., 2025; Serrano et al., 2020; Teixeira et al., 2015).

For improved pastures to be successful, it is essential to create conditions that allow the sown species to establish and persist over time, particularly the legume component (McLachlan et al., 2024; Moir et al., 2016; Monjardino et al., 2022). In general, one of the main practices associated with the establishment of legume-rich improved pastures is the application of lime to acidic soils, as legumes tend to have higher calcium (Ca) requirements than grasses (Aguiar et al., 2024; Bryson et al., 2014), and nodulation is highly sensitive to aluminium (Al) toxicity and Ca deficiency (Hungria & Nogueira, 2023; Morton, 2020; Yang et al., 2021). Additionally, during both the establishment and management of legume-rich improved pastures, P is commonly applied in nutrient-poor soils (Cuadro et al., 2022; Moir et al., 2016). This is because biological N fixation requires higher P levels than general plant growth due to the intense respiration of nodules, which rely on energy-rich phosphates (Hungria & Nogueira, 2023).

However, well-defined limits for applying lime and P have not been sufficiently established for many agricultural regions where these farming systems are important. Since pastures consist of a mixture of species, including both natural and cultivated vegetation, it is challenging to use diagnostic tools commonly applied to sole crops, such as plant tissue analysis and interpreting results based on sufficiency ranges (Bryson et al., 2014). Nutritional diagnosis techniques for crops depend on the appropriate definition of the sampling time, which is linked to the phenological stage of the species and the type of tissue sampled, as nutrient concentrations in plants vary depending on the tissue analyzed (Hungria & Nogueira, 2023). In pastures, tissue sampling is inherently complex due to the mixture of species. Different species exhibit highly diverse nutrient concentrations, and pastures lack the minimal homogeneity required to compare results across different world regions. The most rational approach to this issue, and the one most used as the basis for pasture fertilization recommendation systems, is soil analysis (Cuadro et al., 2022; Moir et al., 2016). However, even soil analyses, regardless of the extractants used, may yield less consistent results, as they are not tailored to a specific species but rather to a mixture of plant species that are largely unknown and highly dynamic over time.

Given that lime application tends to have a longer-lasting effect, once pH is corrected, P application is likely the most relevant practice for pasture management (Carreira et al., 2025; Serrano et al., 2020). However, few studies have directly compared the responses of species from different botanical groups (e.g., grasses and legumes) under varying levels of soil P availability, and such studies are notably absent in Portugal. The complexity of this issue is further compounded by the fact that different countries employ different soil P extraction methods (Cuadro et al., 2022; McLachlan et al., 2024), which can lead to inconsistent interpretations of soil fertility. For instance, while the Egner-Riehm method is widely employed in Portugal as well as in Central and Northern European countries, neighbouring Spain, despite having similar soil and climatic conditions, typically relies on the Olsen method (Arrobas et al., 2022; Recena et al., 2017). Moreover, it is often unclear to what extent other ecological or nutritional factors, beyond P availability, may influence the balance between legume and grass components in mixed pastures. Consequently, P fertilization recommendations based on current extraction methods remain relatively uncertain (Cuadro et al., 2022; McLachlan et al., 2024; Moir et al., 2016). Although various extraction methods, including Olsen, Bray II, and Mehlich I, have been used to assess soil P (Cuadro et al., 2022; McLachlan et al., 2024), there remains a significant knowledge gap regarding their reliability and applicability in Mediterranean pasture systems, particularly in the context of complex species interactions between legumes and grasses. Furthermore, the potential limitations imposed by other essential nutrients remain insufficiently investigated. This gap hinders the development of accurate fertilization guidelines and poses a challenge to the sustainable management of soil fertility in these systems.

A broader understanding of P management in agroecosystems is essential due to its agronomic relevance and environmental implications. In many regions of the world, low soil P availability limits plant productivity (Muktamar et al., 2020; Wahba & Zaghloul, 2024). On the other hand, global reserves of phosphate rock, the primary source for phosphate fertilizer production, are being progressively depleted (Hawkesford et al., 2023). At the same time, excessive P application can result in nutrient losses to surrounding water bodies, contributing to environmental issues such as eutrophication (Kleinman et al., 2015; Noor et al., 2023; Toor et al., 2020). To advance understanding P dynamics in rainfed improved pastures, five commonly used soil P extraction methods, Egner-Riehm, Olsen, Bray II, Mehlich I, and Resin, were applied to soils with contrasting P fertilization histories and presumed differences in P availability. A pot experiment was then conducted using a legume, a grass, and a legume–grass mixture to evaluate nutrient bioavailability and to investigate the influence of soil properties on biomass production.

The main hypotheses tested were: (i) the Egner-Riehm method, used in official laboratories in Portugal, accurately assesses soil P availability; (ii) soil P is a key limiting factor in these pastures; and (iii) other factors or soil properties may impact pasture productivity.

2. MATERIAL AND METHODS

2.1. Experimental conditions and soil sampling

The pot experiment was conducted in Bragança, in northeastern Portugal, during the growing season from September 19, 2023, to April 29, 2024. The observed values of mean air temperature and monthly precipitation during this period, along with the climatological normal (1991-2020) values for the location, are presented in Figure 1.

The study used soils from seven locations. Six of these locations are in the agricultural farm of Quinta da França (40.27º, -7.42º), near Covilhã, Portugal. In Quinta da França, soil samples were collected from pastures with distinct P management histories, either due to differences in the timing of their conversion into improved pasture areas (including pastures that have not been improved) or variations in P application rates.

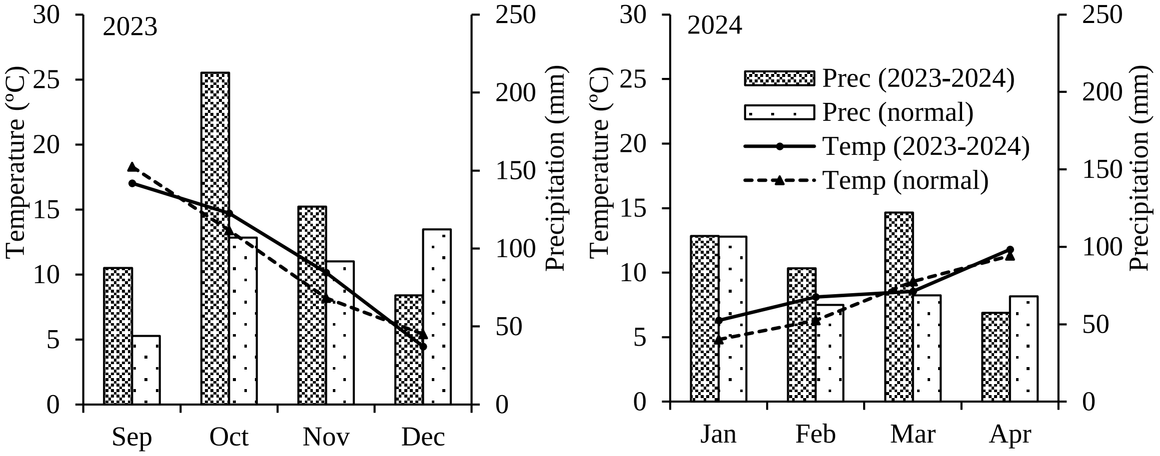

Figure 1. Monthly mean temperature (Temp) and precipitation (Prec) during the experimental period

Table 1. Soil properties (mean ± standard deviation, n = 3) of the soil samples used in this study, collected at a depth of 0–0.15 m.

Soil properties	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6	Soil 7
[1]Sand (g kg^{-1})	855±14.7	794±4.4	767±9.9	806±2.3	822±8.3	845±3.0	709±6.5
[1]Silt (g kg^{-1})	88±21.7	152±18.3	157±7.2	132±4.4	112±3.0	91±4.6	176±9.0
[1]Clay (g kg^{-1})	56±7.0	53±13.9	76±2.7	63±2.1	66±11.3	65±1.6	114±2.5
[2]Texture	Sandy loam	Loamy sand	Loamy sand	Loamy sand	Loamy sand	Loamy sand	Sandy loam
[3]TOC (g kg^{-1})	14.3±1.21	20.0±2.67	18.9±2.55	17.6±1.88	12.6±0.89	19.4±0.83	13.1±2.62
[3]TN (g kg^{-1})	1.1±0.01	1.5±0.10	1.4±0.13	1.4±1.11	1.1±0.04	1.7±0.12	1.1±0.14
[4]pH(H_2O)	5.9±0.08	5.8±0.06	5.7±0.05	5.9±0.07	6.1±0.01	5.9±0.06	6.6±0.06
Exchangeable							
[5]Ca^{++} (cmol$_+$ kg^{-1})	3.7±0.72	5.0±0.13	4.0±0.31	5.2±0.32	5.1±0.30	5.3±0.49	15.1±1.41
[5]Mg^{++} (cmol$_+$ kg^{-1})	0.7±0.14	0.9±0.03	0.6±0.03	0.7±0.07	0.5±0.01	0.7±0.07	5.7±0.78
[5]K$^+$ (cmol$_+$ kg^{-1})	0.3±0.03	0.4±0.25	0.2±0.04	0.3±0.02	0.2±0.01	0.2±0.01	0.4±0.19
[5]Na$^+$ (cmol$_+$ kg^{-1})	0.2±0.08	0.2±0.01	0.1±0.01	0.2±0.02	0.2±0.03	0.1±0.02	0.2±0.10
[6]Acidity (cmol$_+$ kg^{-1})	0.2±0.06	0.1±0.00	0.2±0.00	0.2±0.00	0.1±0.00	0.1±0.00	0.1±0.00
[7]CEC (cmol$_+$ kg^{-1})	5.0±0.99	6.6±0.30	5.8±1.31	6.6±0.39	6.1±0.30	6.6±0.59	21.5±2.20
Extractable							
[8]K (mg kg^{-1}, K_2O)	145±5.6	119±9.0	109±3.2	141±9.9	108±14.6	122±5.1	170±46.1
[8]P (mg kg^{-1}, P_2O_5)	116±4.2	54±0.7	70±7.7	115±12.0	118±25.6	186±10.6	114±27.7
[9]B (mg kg^{-1})	0.7±0.19	0.7±0.02	0.6±0.03	0.6±0.25	0.4±0.01	0.7±0.17	0.8±0.15

Notes: [1]Robinson pipette method; [2]USDA (United States Department of Agriculture); [3]Total Organic Carbon and Total Nitrogen (Dumas dry combustion); [4]Potentiometry; [5]Ammonium acetate; [6]Potassium chloride; [7]Cation exchange capacity; [8]Egner-Riehm; [9]Hot-water, azomethine-H.

The seventh location (41.797º, -6.761) corresponds to an agricultural plot that had not been cultivated for the previous two years. It was included in the experiment to introduce additional variability and serve, to some extent, as a control. Soil samples were collected from the 0–0.15 m layer, as these acidic Portuguese soils are typically shallow and rarely exceed 15 cm of effective depth, making this layer representative of the main rooting and nutrient-uptake zone. Samples were then oven-dried at 40 °C and sieved through a 2 mm mesh. Some of the initial properties of the sieved samples are presented in Table 1.

2.2. Experimental design and trial management

The pot experiment was arranged in a randomized block design with seven soils (representing the seven origins), three types of vegetation (grass, legume, and mixture), and three replicates (Fig. 2). The definition of vegetation types considers a mixture of a grass species with a legume to simulate the interaction that occurs in a pasture, as well as the cultivation of each species as a sole crop to better assess the effect of soil on both the grass and the legume. Two of the most relevant species commonly used in rainfed improved pastures in Mediterranean regions were selected: annual ryegrass (*Lolium multiflorum* Lam., cv. Falladino) and subterranean clover (*Trifolium subterraneum*, ssp. *subterraneum*, cv. Denmark).

Pots with a diameter and depth of 0.14 m and a dry soil mass of 3 kg were used. The amount of seed applied per pot was calculated based on the pot's surface area, considering the typical recommended seeding rates for these two species (50 kg ha^{-1} for ryegrass and 25 kg ha^{-1} for subterranean clover). Therefore, for pots with ryegrass, 80 mg of seed was used; for pots with clover, 40 mg of seed was used; and for pots with the mixture, 40 mg of ryegrass seed and 20 mg of clover seed were used, respectively.

Figure 2. Appearance of the vegetation (grass, legume, and mixture) during the growing season.

After filling the pots with soil, they were moistened, and the seeds were placed at a depth of 1 cm. During the two weeks following the start of germination, spontaneous vegetation seedlings that could be distinguished from the sown species were removed to ensure greater biomass homogeneity at harvest. Sowing occurred on September 19, 2023, and vegetation harvesting occurred on April 29, 2024. The pots were kept in an open space but were enclosed with wooden fences to ensure that only the top portion of the pots received sunlight. Although the conditions under which the pots were kept were relatively homogeneous regarding solar exposure, they were rotated weekly to change their positions. During this period, the water availability in the pots was monitored, and irrigation was provided whenever long dry spells without precipitation were observed.

2.3. Soil analysis

The sieved and dried soil samples were analyzed for pH (H_2O and KCl) (soil: solution ratio, 1:2.5), exchangeable bases (ammonium acetate, pH 7.0), and exchangeable acidity (potassium chloride). Soil B was extracted using hot water and determined by the azomethine-H method. Extractable potassium (K) was determined using the Egner-Riehm method described below in the P extraction methods. These analytical procedures are provided in detail in van Reeuwijk (Van Reeuwijk, 2002). The Dumas dry combustion method was used to determine total organic carbon (C) (FAO, 2019) and total N (FAO, 2021b).

Five P extraction methods were used: Resin-P, Egner-Riehm, Olsen, Bray II, and Mehlich I. Briefly, the method using an anionic exchange resin (Resin-P) involves extracting P from 1 g of soil using a 1 × 2 cm anionic exchange resin sheet (product 55164 2S BDH, England) in the presence of water overnight with stirring. The resin is then eluted with 20 mL of 0.5M HCl, and P is determined in the extract. The Egner-Riehm method involves extracting P with a solution of ammonium lactate and acetic acid, buffered at pH 3.5 (soil:solution ratio 1:20). The Olsen method uses a 0.5M $NaHCO_3$ extractant solution at pH 8.5 (soil:solution ratio 5:100). The Bray II method uses a solution of 0.03M NH_4F + 0.025M HCl (soil:solution ratio 1:7). The Mehlich I method uses an extractant solution of 0.05M HCl + 0.0125M H_2SO_4 (soil:solution ratio 1:10). More details on the P extraction methods, including P quantification, can be found in FAO (2021a).

2.4. Plant sampling and elemental chemical analysis

On April 29, 2024, 212 days after sowing, the aboveground biomass was cut as close to the soil surface as possible. The samples were briefly washed in water to remove any soil particles that might have adhered to the vegetation. The samples were then oven-dried at 70 °C and weighed. Afterward, the samples were ground using a 1 mm mesh. The dried and ground plant tissues were analyzed for their elemental composition. N concentration in plant tissues was determined using the Kjeldahl method, while B and P were determined by colorimetry. K was measured by flame emission spectrometry, and Ca, magnesium (Mg), manganese (Mn), iron (Fe), copper (Cu), and zinc (Zn) were determined by atomic absorption spectrophotometry after digesting the samples with nitric acid in a microwave oven. For a detailed description of these methodologies, the reader is referred to Temminghoff and Houba (2004).

2.5. Data analysis

The data analysis was conducted using the statistical software SPSS Statistics (version 25, IBM SPSS, Armonk, NY, USA). Data were tested for normality and homogeneity of variances using the Shapiro–Wilk and Bartlett's tests, respectively. Data were analyzed using a two-way ANOVA, with soils from different sites and vegetation type as factors. When the means of the treatments showed significant differences ($p < 0.05$), they were separated using the Tukey HSD test ($\alpha = 0.05$). In cases of significant interaction, the main effect of the soil factor was analyzed separately (one-way ANOVA) to better understand its influence on vegetation type, and the same multiple mean separation test was used. The data was also examined for the presence of linear or other relationships between measured variables. Depending on the data, simple linear regression models and asymptotic exponential models were used.

3. RESULTS

3.1. Relationship between phosphorus extraction methods

The results from the soil P availability assessment methods (Egner-Riehm, Olsen, Mehlich I, Bray II, and Resin) showed significant linear relationships between all possible combinations. However, the Egner-Riehm method resulted in significant linear relationships with high determination coefficients for all other methods (Fig. 3). The relationship with the highest determination coefficient occurred between the results of the Egner-Riehm method and the Resin method ($R^2 = 0.89$).

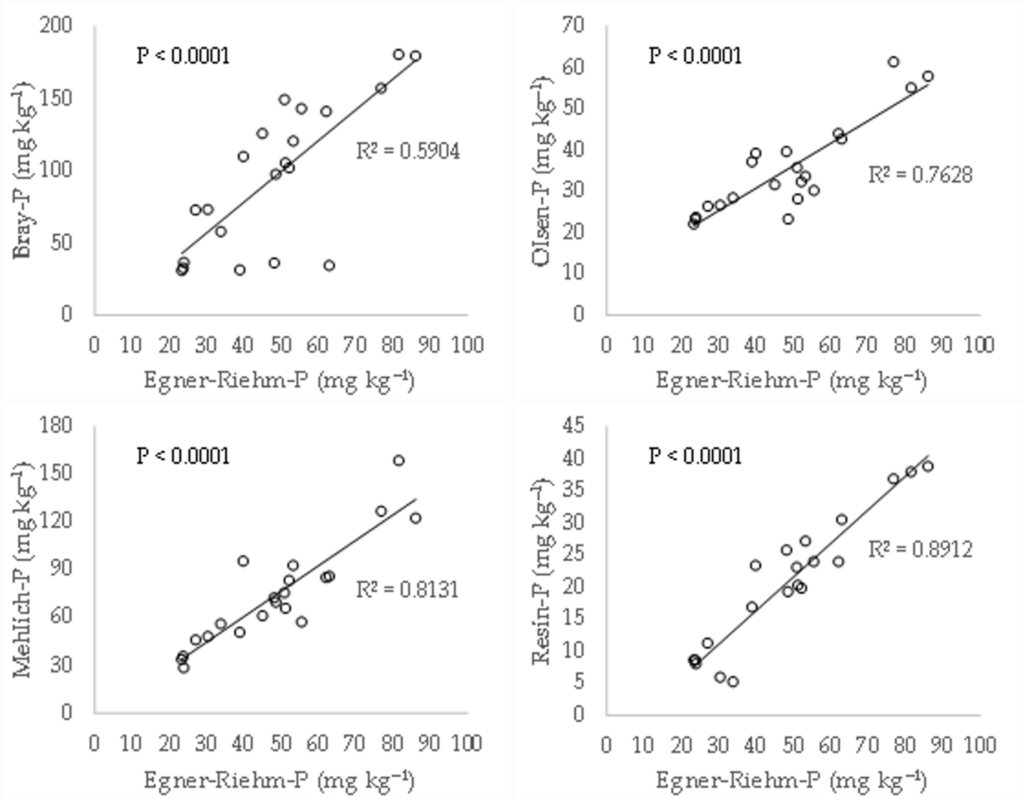

Figure 3. Relationship between the soil's phosphorus (P) levels obtained by the Egner-Riehm method and the other P extraction methods used in this study.

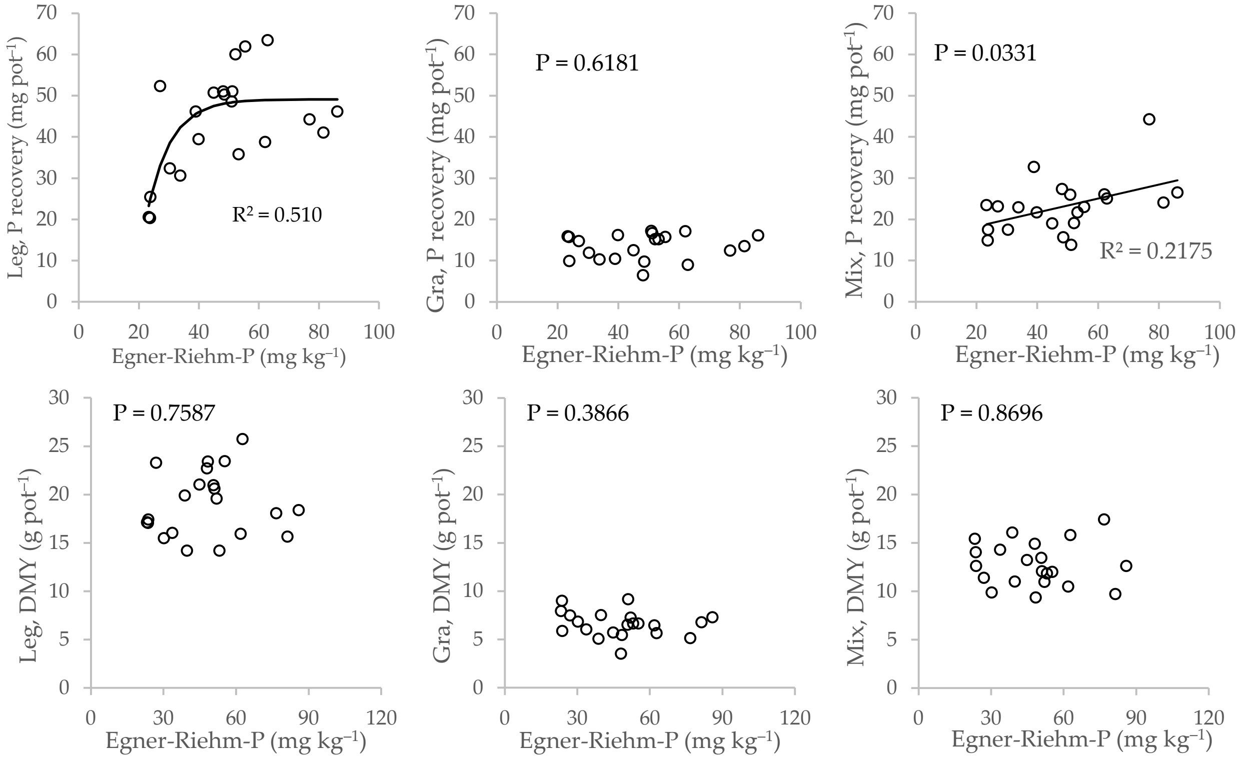

Figure 4. Relationship between soil phosphorus (P) levels (Egner-Riehm method) and P concentration in plant tissues, total P recovered in aboveground biomass, and dry matter yield (DMY) of the legume, grass, and mixture.

3.2. Relationship between soil P, tissue P concentration and total P, and dry matter yield

An asymptotic exponential relationship was observed between all the laboratory methods for assessing soil P availability and the P concentration in legume tissues. However, the Egner-Riehm method (Fig. 3) best fits the model, with a high coefficient of determination ($R^2 = 0.77$). The relationship between soil P levels, as determined by laboratory methods, and P concentration in grass tissues did not conform to any theoretical model. Only the Egner-Riehm method showed a significant linear relationship ($P = 0.0496$), but with a low coefficient of determination ($R^2 = 0.19$).

The relationship between extractable P, as determined by different extraction methods, and the total P content in the aboveground biomass of the legume generally followed an asymptotic exponential model. In contrast, no significant relationship was observed for the grass species (Fig. 4). The coefficients of determination for the asymptotic exponential model were generally lower for P recovery than for tissue P concentration, with the highest values obtained using the Egner-Riehm method.

No significant relationship was established between soil P, as extracted by any of the methods, and the DMY of the legume, grass, or mixture (Fig. 3).

3.3. Effect of soil and crop on dry matter yield

A significant interaction ($P = 0.0006$) was observed in DMY between the soil factor and vegetation type (Fig. 5). While the significant effect of soil, and particularly of vegetation type, on DMY, was evident, with legume DMY (19.1 g pot^{-1}) being 2.9 times higher than that of the grass (6.6 g pot^{-1}), the occurrence of a significant interaction warranted the separate evaluation of soil effects on the legume and the grass. This analysis clarified that the influence of soil on DMY was primarily driven by its impact on the legume, as the variance analysis conducted separately did not identify significant differences between soils in grass DMY. Soil 5 resulted in the lowest DMY, with significant differences from soils 1, 4, and 7.

3.4. Effect of soil and crop on nutrient concentration in plant tissues

Tissue P concentration was significantly affected by soil origin and vegetation type, and their interaction (Fig. 6). Although soil 2 exhibited markedly lower average values than the other soils, and the legume showed higher average values than the grass, a separate analysis of variance was conducted for each species. The analysis of the soil effect for each cultivated species confirms the findings of the joint analysis, highlighting significant differences in P concentration between the grass and the legume. A noteworthy aspect of this figure is that the soil that resulted in the lowest average P concentration in the legume tissue, soil 2, does not coincide with the soil that led to the lowest DMY, which was soil 5 (Fig. 4).

The N concentration in plant tissues did not vary significantly with the soil but showed substantial differences between vegetation types, with the legume exhibiting a higher N concentration (28.1 g kg^{-1}) than the mixture (20.8 g kg^{-1}), and the mixture having a higher concentration than the grass (10.5 g kg^{-1}), although a significant interaction was observed (Fig. 7). When analyzing the effect of soil separately for each vegetation type, no significant differences were observed in N concentration in the legume. Still, a significant soil effect was found for N concentration in the grass tissue.

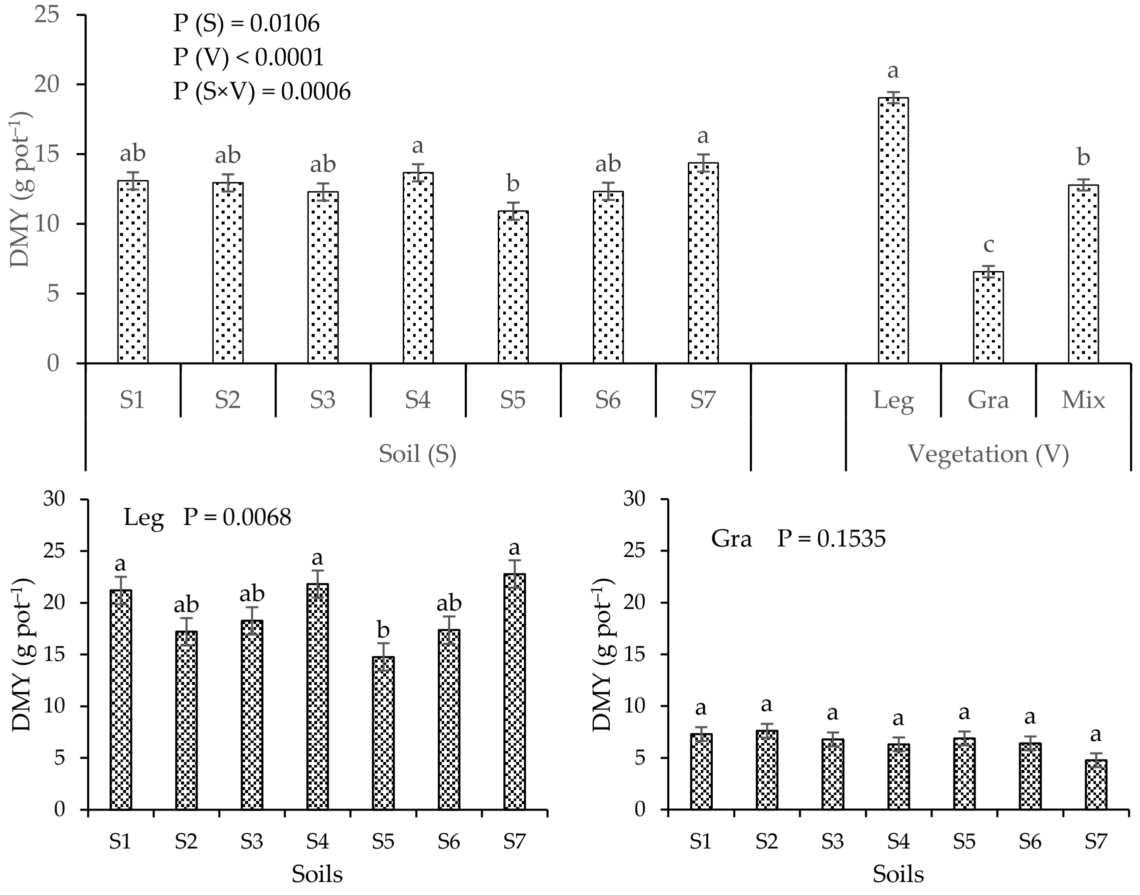

Figure 5. Effect of the soil × vegetation type factorial (top) on dry matter yield (DMY) and effect of soil factor on DMY of the legume (Leg) and grass (Gra) when analyzed separately (bottom). Error bars are the standard errors.

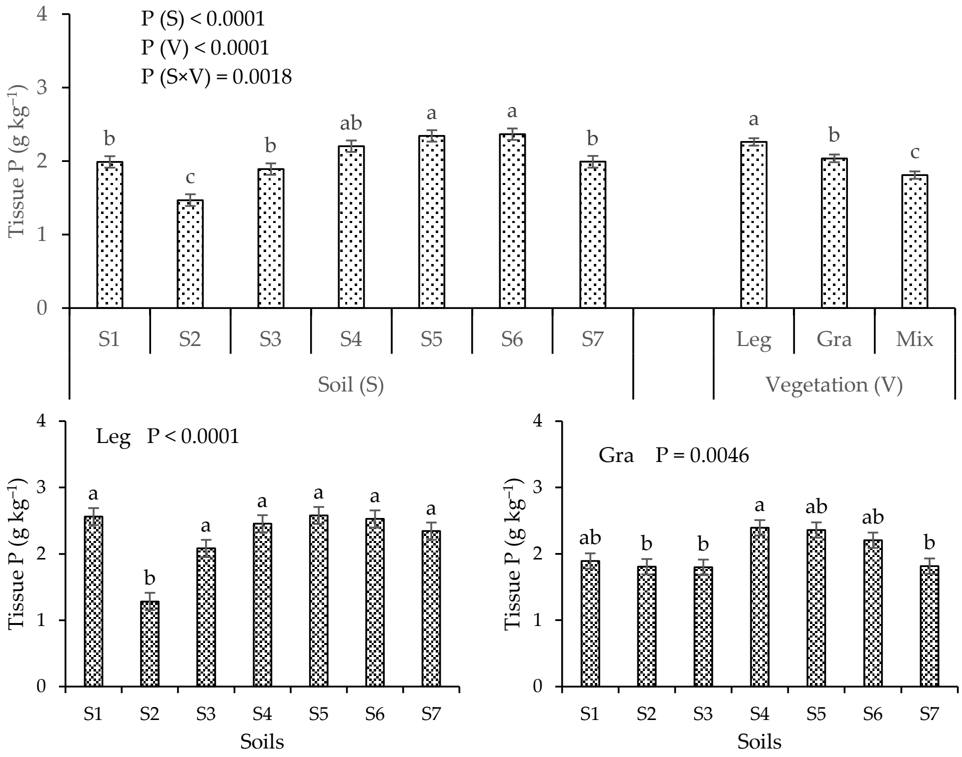

Figure 6. Effect of the soil × vegetation type factorial (top) on tissue phosphorus (P) concentration and effect of soil factor on tissue P concentration of the legume (Leg) and grass (Gra) when analyzed separately (bottom). Error bars are the standard errors.

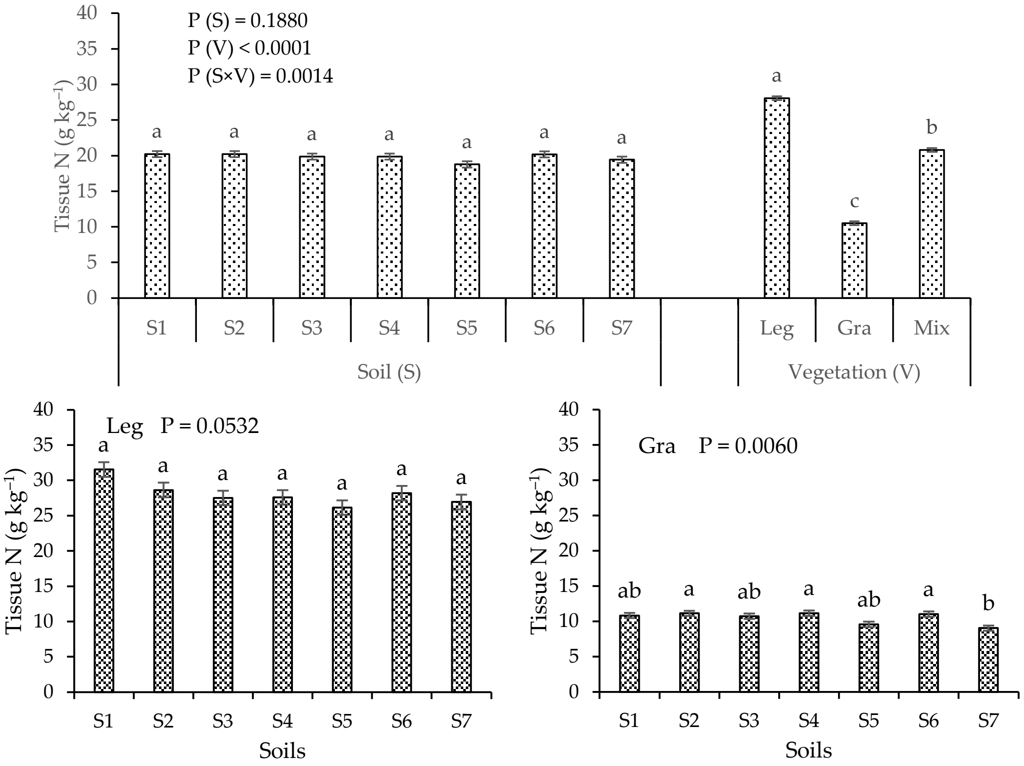

Figure 7. Effect of the soil × vegetation type factorial (top) on tissue nitrogen (N) concentration and effect of soil factor on tissue N concentration of the legume (Leg) and grass (Gra) when analyzed separately (bottom). Error bars are the standard errors.

B concentration in the tissues varied significantly with the soil and the cultivated species, although a significant interaction was also observed, indicating that the response to soil depended on the species and vice versa (Fig. 8). The differences between vegetation types were so pronounced that no further analysis was required, with mean values of 13.6, 11.5, and 4.5 mg kg^{-1} for the legume, mixture, and grass, respectively. The soil effect was analyzed separately, and the differences remained consistent with the values for soil 5, which resulted in the lowest DMY (Fig. 4), being the lowest. The grass also showed significantly lower mean values in soil 5 compared to soil 7.

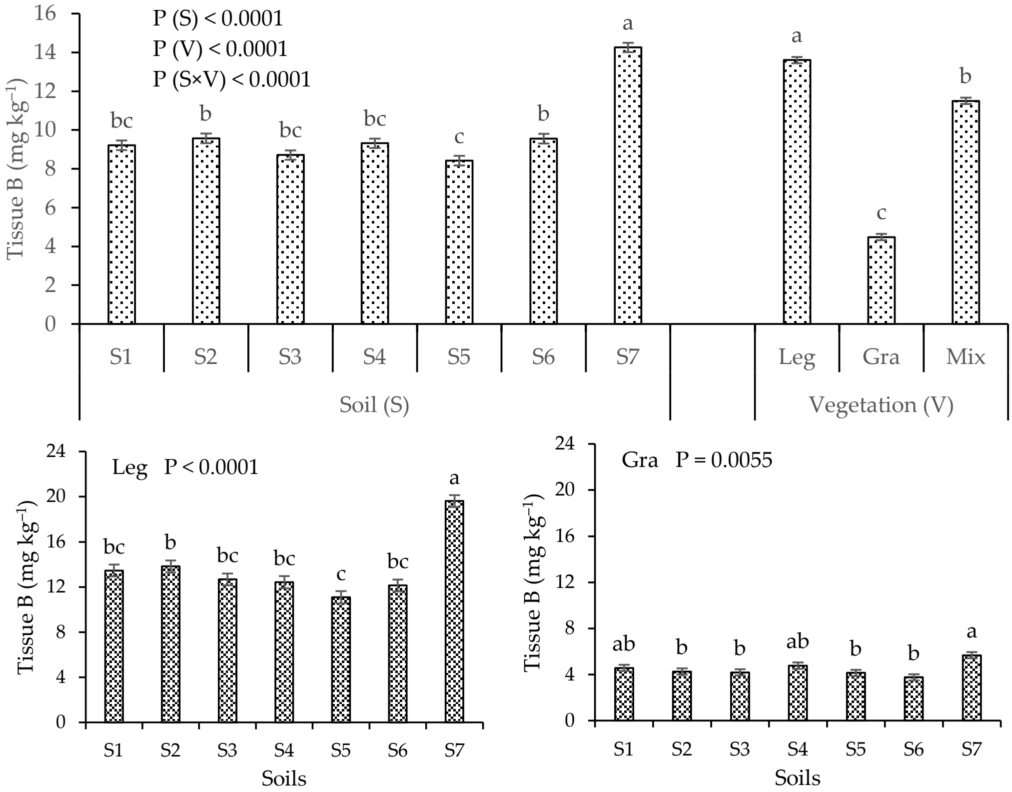

Figure 8. Effect of the soil × vegetation type factorial (top) on tissue boron (B) concentration and effect of soil factor on tissue B concentration of the legume (Leg) and grass (Gra) when analyzed separately (bottom). Error bars are the standard errors.

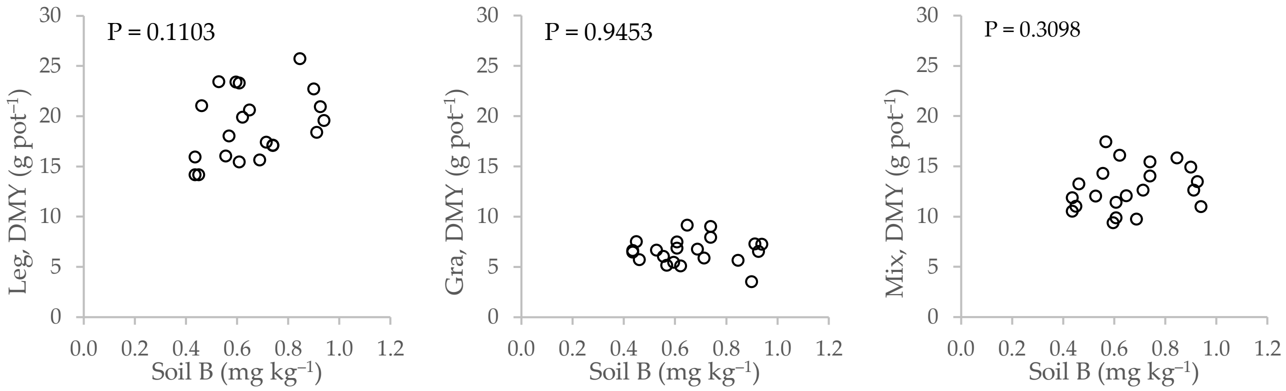

Figure 9. Relationship between soil boron (B) and the dry matter yield (DMY) of legume (Leg), grass (Gra), and mixture (Mix). P is the probability of the linear relationship.

In this study, the concentration of other nutrients in the tissues was determined, namely K, Ca, Mg, Fe, Mn, Cu, and Zn. However, the information proved to be of limited relevance in helping to explain the hypotheses posed for this study. Nonetheless, the concentration of some nutrients was significantly different between the grass and the legume, with Ca levels, for instance, being particularly higher in the legume species.

3.5. Relationship between individual soil properties and dry matter yield

The soil significantly influenced the DMY of the legume (Fig. 5). However, it was not possible to establish any significant relationship between an individual soil property and the DMY for any of the cultivated species or their mixture. Of all the relationships tested, the one that most closely approached a significant linear relationship was the B content in the soil and the DMY of the legume, with P = 0.1103 (Fig. 9).

3.6. Relationship between soil properties and nutrient concentration in plant tissues

Figure 4 illustrates the relationship between soil P content and P concentration in legume tissues, revealing an asymptotic pattern. In contrast, no significant relationship was found between soil organic C and total N with N concentration in plant tissues. The B content in the soil was linearly related to the B concentration in the legume tissues ($P = 0.0287$), although with a relatively low coefficient of determination ($R^2 = 0.2276$) (Fig. 10). On the other hand, soil B did not show a significant relationship with B concentration in the tissues of either the grass or the mixture.

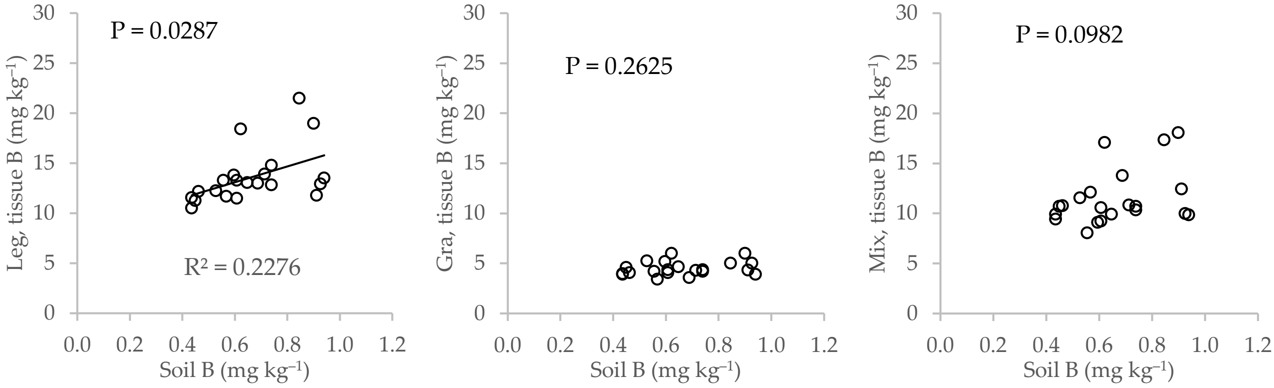

Figure 10. Relationship between soil boron (B) and the concentration of B in the tissues of the legume (Leg), grass (Gra), and mixture (Mix). P is the probability of the linear relationship

Table 2. Nitrogen (N) recovery in aboveground biomass and N derived from the atmosphere (Ndfa)

	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6	Soil 7
N recovery	(mg pot^{-1})	(mg pot^{-1})	(mg pot^{-1})	(mg pot^{-1})	(mg pot^{-1})	(mg pot^{-1})	(mg pot^{-1})
Legume	667.9±56.0	492.7±33.5	502.2±121.8	602.0±40.3	386.0±32.7	493.7±111.8	613.9±84.2
Mixture	197.0±23.7	292.0±24.9	252.1±42.3	268.5±1.9	228.9±12.8	279.3±68.9	347.9±26.5
Grass	79.8±26.1	84.4±14.8	72.5±3.4	70.6±10.0	65.6±4.8	70.2±9.7	43.0±10.6
Ndfa	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Legume	88.1±4.6	82.9±4.0	85.6±2.6	88.3±1.6	83.0±2.1	85.8±4.4	93.0±2.1
Mixture	59.5±8.7	71.1±5.1	71.2±5.2	73.7±3.9	71.3±2.4	74.9±8.8	87.6±2.4

3.7. Nitrogen derived from the atmosphere

The legume exhibited exceptionally high N levels in its tissues, approximately twice the amount of N found in the mixture and eight times the amount in the grass (Table 2). The N in the legume is primarily derived from the atmosphere, with values ranging between 83 and 93%. Soil 5 resulted in the lowest N content in the legume biomass (386 mg pot^{-1}) and a lower percentage of N derived from the atmosphere (83%). In the mixture, a smaller proportion of the N in the biomass originated from the atmosphere, with values ranging from 60 to 88%.

4. DISCUSSION

This study aimed to evaluate the performance of different P extraction methods in predicting P availability in soils intended for pasture establishment, and to investigate how this availability relates to nutrient uptake and plant productivity, as well as the potential influence of other soil properties on pasture performance. The main findings revealed that all P extraction methods showed significant linear relationships with one another, with the Egner-Riehm method exhibiting the strongest correlations, especially with the ion-exchange resin method (R^2 = 0.89) (Fig. 3), which is often considered the most accurate proxy for bioavailable P in soils (Bouray et al., 2022; Zaryab et al., 2024). However, despite its reliability, the resin method is rarely used in routine analysis due to its time-consuming procedure. In contrast, the Egner-Riehm method is routinely applied in Portugal, as well as in several Central and Northern European countries, because it allows the simultaneous extraction of P and K and performs well in acidic soils (Arrobas et al., 2015; Arrobas et al., 2022). The strong performance of the Egner-Riehm method in this study supports its continued use in acidic pasture soils, even though the Olsen method remains more common internationally (Higgins et al., 2021; Vistoso et al., 2021).

When examining the relationship between soil P and plant uptake, all extraction methods revealed an asymptotic exponential relationship with P concentration in legume tissues (Fig. 4). P concentrations in the plants increased rapidly at low soil P levels and plateaued around 2.5 g kg^{-1}. Although this value is slightly below the critical range reported for subterranean clover (2.5–3 g kg^{-1}), it is important to note that such references are based on leaf analysis only (Bryson et al., 2014), whereas the present study considered whole shoots. Leaves generally exhibit higher P concentrations than stems due to their lower structural component content (Bell, 2023), which could explain this discrepancy. Nonetheless, the stabilization of P concentration at relatively high levels suggests that the soils provided adequate P supply for optimal legume nutrition.

Despite the agronomic importance of P in legume systems, no significant relationship was observed between soil P levels and DMY of legumes. Similarly, total P content in plant tissues followed the same asymptotic trend as P concentration, reinforcing the notion that biomass production was not P-limited under the studied conditions. This suggests that even soils with lower extractable P levels supplied sufficient P for plant growth, potentially because all tested soils exceeded the critical threshold for P response. This interpretation is supported by the findings of Cuadro et al. (2022), who reported that above this threshold, further increases in P availability do not lead to higher biomass production. Furthermore, Soil 5, which exhibited the lowest DMY, did not show the lowest P concentrations in plant tissues, confirming that another factor was responsible for the observed yield reduction.

A strong candidate for this limiting factor is B, which has been widely recognized as a critical micronutrient for legumes, especially in regions with naturally low B availability, such as Portugal (Arrobas et al., 2024; Portela et al., 2015). Soil 5 presented both the lowest B concentrations in the soil (Table 1) and in legume tissues (Fig. 8), with an average B content of 11.1 mg kg^{-1} in plant tissue, well below the sufficiency range of 25 to 50 mg kg^{-1} established for this species (Bryson et al., 2014). Although B concentrations in ryegrass were even lower, this was expected due to the inherently lower B requirements in grasses compared to dicots. The higher demand in dicots is linked to their greater proportion of cis-diol compounds in cell walls, which require B for structural integrity (Cakmak et al., 2023). Therefore, the limited productivity observed in Soil 5 is best explained by B deficiency, which likely restricted plant physiological processes as well as biological N fixation.

Beyond its role in cell wall structure, B is essential for the physiological processes involved in biological N fixation. Soil 5 not only showed lower biomass and total N content in legumes but also a lower percentage of N derived from the atmosphere, suggesting that B deficiency may have impaired nodule formation and/or functioning. Previous research has demonstrated that B deficiency in nodulated legumes induces physiological stress that compromises the efficiency of N fixation (Hamilton et al., 2015). These results underscore the importance of B in supporting legume productivity not only directly, by contributing to plant structure, but also indirectly, by sustaining the symbiotic relationship with N-fixing bacteria.

The N dynamics in grasses contrasted with those observed in legumes. While the soil factor did not influence N concentrations in legume tissues, it significantly affected N concentrations in grasses. Among soil properties, organic C exhibited a significant positive linear relationship with N concentration in grasses, even though it did not significantly affect DMY. This suggests that organic matter mineralized sufficient N to increase tissue concentrations, but not to enhance biomass accumulation. The pot setup, which involved soil sieving and increased aeration, likely stimulated microbial activity, thereby enhancing N mineralization in soils with higher organic matter content (Weil & Brady, 2017). This explains the positive association between organic C and N uptake in grasses, which are more dependent on mineral N sources than legumes.

DMY differed substantially among the plant types evaluated. Legumes produced significantly more biomass than the legume–grass mixture, which in turn outperformed the grass grown alone. This pattern is primarily attributed to the legumes' access to atmospheric N, which eliminates a major ecological constraint to growth and leads to higher productivity (Aguiar et al., 2024; Rodrigues et al., 2015). Additionally, legumes typically have higher N concentrations in their tissues (Bryson et al., 2014), which enhances the nutritional quality of pasture for livestock (Castro-Montoya & Dickhoefer, 2020; Solomon, 2022). Other nutrients, including P and B, followed similar trends and were found in higher concentrations in legume tissues than in grasses. This also held true for Ca and, to a lesser extent, Mg. These differences are well-documented between dicots and monocots and are primarily driven by physiological factors. For instance, the lower Ca concentration in grass tissues is due to their lower demand for this element, since monocots have reduced pectate content and cell wall cation exchange capacity (Hawkesford et al., 2023). Consequently, legume-rich pastures provide more nutrient-dense forage, particularly in essential elements such as N, P, Ca, and B, thereby contributing to improved animal nutrition.

Although P is essential for legume productivity, particularly because of its role in supplying energy to root nodules and supporting biological N fixation (Hungria & Nogueira, 2023), the results of this study indicate that additional P fertilization would not have increased productivity in the tested soils. This has important implications, as P fertilization is a widespread practice aimed at boosting legume performance in pastures (Cuadro et al., 2022; McLachlan et al., 2024). However, the continued debate regarding the most appropriate indicators for determining P responsiveness (Cuadro et al., 2022; Olson-Rutz & Jones, 2018) highlights the complexity of developing universally reliable fertilization strategies. Inappropriate application of P not only wastes resources but also poses environmental risks, such as water eutrophication (Kleinman et al., 2015; Lewis et al., 2013; Toor et al., 2020). Furthermore, phosphate rock, the primary source of P fertilizers, is a finite resource expected to become increasingly scarce within this century (Hawkesford et al., 2023), making efficient use of P is not only an agronomic priority but a global sustainability issue.

Despite these insights, the study has limitations. It was conducted under controlled greenhouse conditions using potted soils, which may not fully replicate field dynamics. The sieving and handling of soil samples likely increased aeration, promoting microbial activity and nutrient mineralization beyond what would typically occur in situ. Lastly, nutrient concentrations were measured in whole plant tissues rather than specific organs (e.g., leaves), which limits direct comparisons with literature thresholds.

5. CONCLUSION

The Egner-Riehm method appears to be a reliable routine method for assessing P availability in acidic soils, as its results were linearly correlated with those of other extraction methods, particularly with the resin method, which is widely regarded as the most suitable for evaluating soil P bioavailability. In the soil samples analyzed in this study, P availability was not a limiting factor for biomass production in either the grass or the legume. The soil with the lowest P levels, as determined by the Egner-Riehm method, had an average value of 54 mg kg^{-1} P_2O_5, which is close to the lower threshold of the medium range (50–100 mg kg^{-1} P_2O_5). Thus, based on this value, soil testing laboratories would typically recommend a relatively high application of phosphate fertilizers. Instead of P, N was the primary limiting factor for plant productivity. In legumes, N fixation eliminated the soil N limitation, which led to an average 2.9 times increase in biomass production compared to grasses. Importantly, soil B was identified as a potential limiting factor. A significant linear relationship was established between soil B content and B concentration in legume tissues. Further, B was the soil property closest to showing a significant linear relationship with DMY (P = 0.1103). B concentrations in plant tissues were well below the sufficiency threshold, indicating a nutrient deficiency.

Declaration of Competing Interest

The authors declare that no competing financial or personal interests may appear to influence the work reported in this paper.

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