International Journal of Agronomy

WILEY

RESEARCH ARTICLE OPEN ACCESS

Assessing the Role of Phosphorus, Starter Nitrogen and Boron in Subterranean Clover and Annual Ryegrass Grown in Acidic Soils Under Mediterranean Conditions in Portugal

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Received: 13 June 2025 | **Revised:** 26 February 2026 | **Accepted:** 27 February 2026

Academic Editor: Maria Serrano

Keywords: biological nitrogen fixation | dry matter yield | *Lolium multiflorum* | sown pastures | *Trifolium subterraneum*

ABSTRACT

In Mediterranean pasture systems, maintaining legume persistence and productivity is often constrained by phosphorus (P) deficiency in acidic soils. Although small doses of starter nitrogen (N) are commonly used in pulse crops to support early growth before N fixation begins, this practice has not been widely tested in legume–grass mixtures. In addition, boron (B), a micronutrient particularly important for dicot species, may also influence legume performance under these conditions. Thus, this study investigated the effects of P application on dry matter yield (DMY) and nutrient uptake in subterranean clover (*Trifolium subterraneum* L., cv. Denmark), annual ryegrass (*Lolium multiflorum* Lam., cv. Falladino) and their mixture, using a pot experiment with acidic soils from two distinct locations in Portugal. The study also evaluated the impact of a small N starter dose and B application. Although Soil 2 had higher acidity (pH 5.0) than Soil 1 (pH 5.4), it supported greater biomass production, likely due to its higher organic carbon (C) content (50.8 g kg^{-1}), which may have buffered soil acidity. P application significantly increased P uptake, DMY and N accumulation in legumes, suggesting a critical role in nodulation and/or nodule function. The N starter had no significant effect, likely because sufficient N was released from soil organic matter prior to the onset of biological N fixation. While the B application increased tissue B concentrations, it did not affect DMY, possibly because B was adequately available from organic matter. These findings highlight the importance of P in legume management in pasture systems and suggest that, in soils with sufficient organic matter, the application of starter N and B may not be necessary.

1 | Introduction

Grasslands occupy a crucial position in land use, accounting for approximately 69% of the global arable land [1]. These ecosystems are vital for livestock production, thus supporting the livelihoods of millions, particularly in developing nations [2]. Beyond their primary role in providing forage for farm animals, grasslands deliver indispensable ecosystem services, including

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soil erosion control, biodiversity preservation and C sequestration [1, 3, 4]. Notably, grassland soils can store up to 50% more C than forest soils, making them a critical component of climate change mitigation strategies [5].

Three primary categories of grasslands can be distinguished based on the extent of human intervention: natural, seminatural and improved [3]. Improved pastures generally involve intensive management practices, such as the introduction of more productive species compared to native vegetation, often coupled with fertilization strategies [6, 7]. A key element of improved grasslands is the sowing of legumes, which can access atmospheric N through a symbiotic association with rhizobial bacteria, thereby enhancing productivity and reducing the need for synthetic fertilizers [7–10].

The primary challenge in managing grasslands containing annual legumes lies in ensuring the persistence of the sown legume species. Beyond implementing appropriate grazing strategies, meticulous soil fertility management is essential to support the legume component. One of the most critical agronomic measures is correcting soil pH, as this mitigates the adverse effects of excessive aluminium on legume nodulation while enhancing calcium (Ca) availability, an essential element for both legumes and the nodulation process [11, 12]. Furthermore, the application of lime typically improves the bioavailability of key nutrients, particularly P [11, 13], collectively contributing to enhanced pasture productivity [14, 15]. However, since liming primarily mitigates soil acidity over extended periods, particularly in Mediterranean climates characterized by low rainfall and limited base leaching, P availability remains a persistent management challenge that must be addressed annually in legume-rich pastures [16–18]. Legumes typically exhibit higher P concentrations in their tissues compared to grasses [19, 20]. Under P-deficient conditions, nodule mass is significantly reduced, and biological N fixation is adversely affected [21]. This is because nodule function requires higher P levels than those needed for general plant growth, due to the high respiratory demands of nodules, which depend on energy-rich phosphate compounds to sustain their activity [12].

Legumes typically do not receive N fertilization, as they can access N from the atmosphere for their own growth. Furthermore, they can transfer N to other crops grown in intercropping systems or those that follow them in rotations [20, 22]. Legumes obtain atmospheric N through a symbiotic relationship with microorganisms of the Rhizobiaceae family, commonly known as rhizobia [12]. The symbiotic process begins with the recognition between the plant and the bacteria, initiated by the plant's secretion of secondary metabolites called flavonoids. These flavonoids are recognized by the bacteria, which respond by releasing lipochitooligosaccharides known as nodulation factors. However, from this initial interaction to the point when the bacteria transform into bacteroids, after synthesizing nitrogenase and leghaemoglobin, several weeks may pass [12, 23]. During this period, the entire process of nodulation occurs at the plant's expense. The plant must have all the necessary growth factors, including soil nutrients, to maintain high photosynthetic activity and produce photosynthates for both growth and nodule formation [13]. Therefore, when soil N levels are expected to be low, it is common to apply a small amount of N fertilizer to ensure N availability during the early growth stages before the plant can access atmospheric N. This application is often referred to as a starter, and it has been shown not to inhibit nodulation and to increase legume productivity [24–26]. In pastures, where there is a high mixture of species from various botanical groups, using N as a starter fertilizer is not common. However, pastures in Mediterranean regions are often dominated by annual species that complete their cycle in spring and summer, producing seeds that are expected to self-reseed with the first autumn rains [7, 27]. In these cases, the success of nodulation is a yearly concern, and it is important to determine whether N limitation is a constraint to the establishment of annual legumes.

B is an essential micronutrient for plant development. It plays a critical role in biosynthesis and in maintaining the structural integrity of cell walls, with a portion of B in plants complexed with cis-diol esters associated with pectins. The higher B requirement in dicots is attributed to their greater proportion of cis-diol-containing compounds in cell walls compared to monocots [28, 29]. Legumes, being dicots, tend to accumulate higher levels of B in their tissues [19, 20]. Experimental evidence also indicates that B is essential for nodule growth and N fixation, as B deficiency in nodulated legumes induces physiological conditions within the plant and/or its rhizobia that impair N fixation [8, 30]. B deficiency has been identified as a significant nutritional disorder in the inland regions of Portugal, affecting various agricultural and forest crops. Studies have shown that B deficiency is highly prevalent in soils derived from granite and schist rocks, which are typically acidic and low in organic matter [31–33]. However, to our knowledge, no studies have addressed the nutritional B status of Mediterranean pastures, in contrast to the attention given to B in other crops [28, 34]. Since legumes have much higher B requirements than grasses [8, 19, 29], increasing knowledge about the role of B in legume-based pastures is important to steer farm management.

In the annual management of improved legume-rich pastures, P application is considered the most critical factor because it plays an essential role in nodulation and biological N fixation, which gives legumes a competitive advantage. While applying a small dose of N is a common practice in pulse crops to support their growth before biological N fixation begins, its use has not been tested in pastures dominated by annual species. Additionally, although B is regarded as equally important as macronutrients in annual fertilization programs in the inland regions of Portugal, its role in improved legume-rich pastures remains largely unexplored. Thus, this study tested the hypothesis that the application of P, starter N and/or B would enhance legume performance relative to grasses in a pot experiment using subterranean clover, annual ryegrass and a mixture of both species.

In the annual management of improved legume-rich pastures, P application is considered the most critical factor due to its essential role in nodulation and biological N fixation, which gives legumes a competitive advantage over grasses. While applying a small dose of N is a common practice in pulse crops to support early growth before biological N fixation begins, its use has not been thoroughly evaluated in pastures dominated by annual species. In addition, although B is considered an important nutrient in annual fertilization programs in inland regions of Portugal, its role in improving legume-rich pastures remains largely unexplored. Therefore, the objective of this study was to evaluate the effects of P, starter N and B on the performance of

subterranean clover and annual ryegrass, grown separately and in mixture, under acidic soils from Mediterranean Portugal.

2 | Materials and Methods

2.1 | Local Climate and Properties of the Soils

The experiment was conducted in pots between 19 September 2023 and 29 April 2024, at the agricultural experimentation unit of the Polytechnic Institute of Bragança (IPB). The region is characterized by a Mediterranean climate with Atlantic influence, classified as Csb according to the Köppen–Geiger system, marked by rainy winters and hot, dry summers [35]. The average annual temperature is 12.9°C, and the total annual precipitation amounts to 790.0 mm [35]. The meteorological records observed during the experimental period, along with the region's climatological normals, are presented in Figure 1.

In this study, two soils were used: one collected from a pasture in central Portugal (Soil 1) and another from a mountain pasture in northern Portugal (Soil 2). Both soils were sampled from the 0- to 0.15-m layer and sieved through a 2-mm mesh. Soil 1 is classified as a Dystric Leptosol, while Soil 2 is classified as a Umbric Dystric Leptosol [36].

Soil 1 has a sandy loam texture (123.8, 123.7 and 752.5 g kg^{-1} of clay, silt and sand, respectively), is acidic (pH_{H2O} 5.4) and has a medium organic C content (17.7 g kg^{-1}, Walkley–Black method). It presents medium and high extractable P (76.5 mg kg^{-1} P_2O_5) and potassium (K) (130.5 mg kg^{-1} K_2O) levels, respectively (Egner–Riehm method), and a medium level of extractable B (0.72 mg kg^{-1}, azomethine-H method). Exchangeable Ca and magnesium (Mg), as well as the cation exchange capacity (CEC), are very low at 1.87, 0.61 and 3.76 $cmol^+$ kg^{-1}, respectively.

Soil 2 also has a sandy loam texture (148.4, 107.0 and 744.7 g kg^{-1} of clay, silt and sand, respectively), is strongly acidic (pH_{H2O} 5.0) and has a very high organic C content (50.8 g kg^{-1}). This soil exhibits very high levels of extractable P (244.1 mg kg^{-1} P_2O_5) and K (376.0 mg kg^{-1} K_2O) and a high level of extractable B (1.05 mg kg^{-1}). Exchangeable Ca and Mg and the CEC are low at 4.40, 1.15 and 7.54 $cmol^+$ kg^{-1}, respectively. The analytical methods and classification criteria used follow standard procedures adopted by Portuguese soil analysis laboratories [37].

2.2 | Experimental Design and Management of the Trial

The experiment was arranged in a completely randomized factorial design (CRD) with three factors: soil type, vegetation type and fertilization. The soil factor comprised two levels: Soil 1 and Soil 2, consisting of soils from different origins. The vegetation type factor included three levels: an annual legume, subterranean clover (*Trifolium subterraneum* ssp. *subterraneum*, cv. Denmark), annual ryegrass (*Lolium multiflorum* Lam., cv. Falladino) and a mixture of both species. The fertilization factor included five treatments: unfertilized control, starter N, P, B and a combination of P and N. The nutrients N, P and B were applied as a basal fertilization. N was supplied at a reduced rate of 20 kg N ha^{-1}, equivalent to 0.044 g N pot^{-1} (considering the pot surface area of 0.022 m^2), using ammonium nitrate (27% N) at 0.16 g pot^{-1}. P was applied at a rate equivalent to 80 kg P_2O_5 ha^{-1} (37.4 kg P ha^{-1}), corresponding to 0.076 g P (0.174 g P_2O_5) pot^{-1}, supplied as 1 g of single superphosphate pot^{-1} (18% P_2O_5). Boron (B) was applied at a rate of 1.5 kg ha^{-1}, corresponding to 0.02 g pot^{-1} of Neobor (14.9% B as disodium tetraborate pentahydrate). Each treatment was replicated three times.

The trials were conducted in pots with a diameter of 0.14 m, each with a capacity to hold 3 kg of air-dried and sieved soil (2-mm mesh). The seed quantities were calculated based on the surface area of the pot opening, considering recommended seeding rates for sole crops of 25 and 50 kg ha^{-1} for subterranean clover and ryegrass, respectively. Accordingly, the amounts of seed applied per pot were 40 mg for subterranean clover and 80 mg for ryegrass. In the mixed treatment, each species was sown at half of its respective sole crop rate.

Following sowing, which took place on September 19, 2023, the pots were monitored during the initial weeks for the emergence of weeds. These were removed once they could be unequivocally distinguished from the sown species, to maintain a more homogeneous vegetation cover for analysis. The pots were surrounded with wooden boards to prevent lateral radiation and overheating. Their positions were rotated weekly to ensure uniform exposure to incident radiation. During extended periods without rainfall, the pots were irrigated to maintain optimal growth conditions for the vegetation. The volume of irrigation water applied was adjusted based on regular monitoring of the pots' moisture content, thereby ensuring that water did not limit plant growth. Water loss varied with ambient temperature and plant vegetative development, which differed among treatments, as did the amount of water applied.

2.3 | Sample Harvesting and Laboratory Analyses

On April 29, 2024, at full bloom of the legume, the aboveground biomass was harvested at the soil surface and briefly rinsed with clean water to remove any adhering soil particles. The samples were then oven-dried at 70°C until constant weight. Subsequently, the plant material was ground to pass through a 1-mm mesh and analysed for its elemental composition.

N concentration in the plant tissues was determined using the Kjeldahl method. P and B were measured by colorimetry, while K was determined by flame emission spectrometry. The remaining cations [Ca, Mg, copper (Cu), iron (Fe), zinc (Zn) and manganese (Mn)] were quantified by atomic absorption spectrophotometry following nitric acid digestion of the samples, according to the procedure described by Temminghoff and Houba [38].

Initial soil samples were analysed for pH (soil-to-solution ratio of 1:2.5), CEC using ammonium acetate at pH 7.0, organic C via wet digestion following the Walkley–Black method and extractable P and K using the Egner–Riehm method (ammonium lactate extraction), as well as extractable B determined by the azomethine-H method. Soil particle size distribution (clay, silt and sand fractions) was determined using the Robinson pipette method. These analytical procedures are described in detail by van Reeuwijk [39].

2.4 | Data Analysis

Statistical analysis was performed using SPSS Statistics software (Version 25, IBM SPSS, Armonk, NY, USA). The data

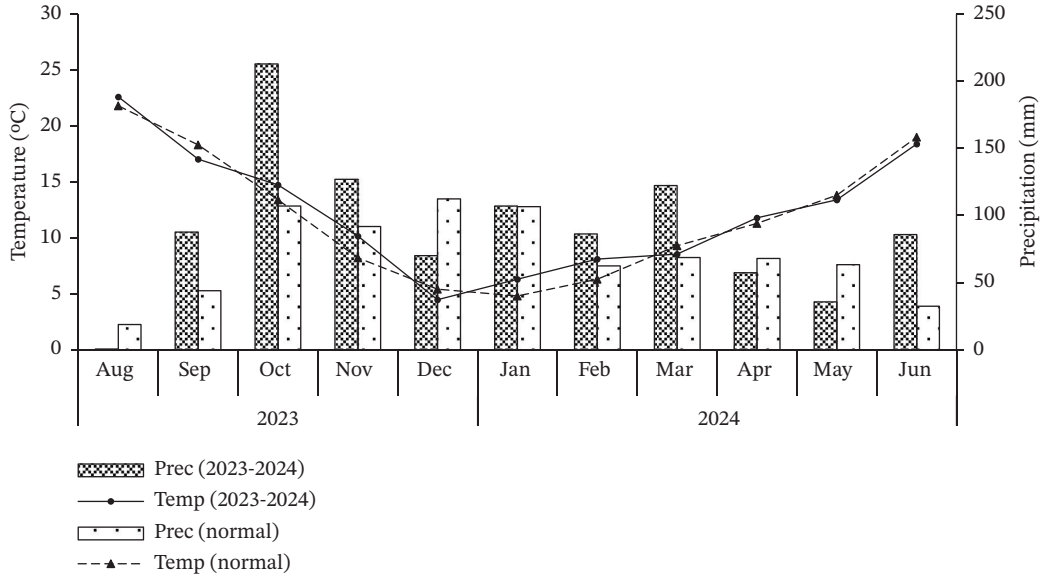


FIGURE 1 | Monthly mean air temperature (Temp) and precipitation (Prec) during the study period and the climatological normal (1991–2020) of the region.

were initially tested for normality and homogeneity of variances using the Shapiro–Wilk test and Levene's test, respectively. Data were analysed using three-way analysis of variance (ANOVA), with factors soil type, vegetation type and fertilization treatments. Given that the pot experiment allows for complete randomization, in cases where significant interactions occurred, factors were removed from the analysis, and a two-way ANOVA was applied. The means of treatments with significant differences ($\alpha < 0.05$) were separated using the Tukey HSD test ($\alpha = 0.05$).

The percentage of N derived from the atmosphere (%Ndfa) was estimated using the difference method, which compares the N content in legume or mixed plant tissues with that of nonfixing reference grasses [40].

3 | Results

3.1 | Dry Matter Yield (DMY)

There was a significant interaction between all pairwise combinations of the three factors under study (Figure 2), although no significant three-way interaction was observed. Nonetheless, analysis indicates a clear soil effect on DMY, with Soil 2 yielding significantly higher values. Significant differences were also found for the vegetation types, with the legume yielding significantly greater DMY than the mixture and the pure grass cultivation. Mean dry matter values were 16.0, 15.0 and 3.8 g pot^{-1} for legume, mixture and grass, respectively. Fertilizer treatments also had a significant effect. Treatments that received P, with or without additional N, showed significantly higher

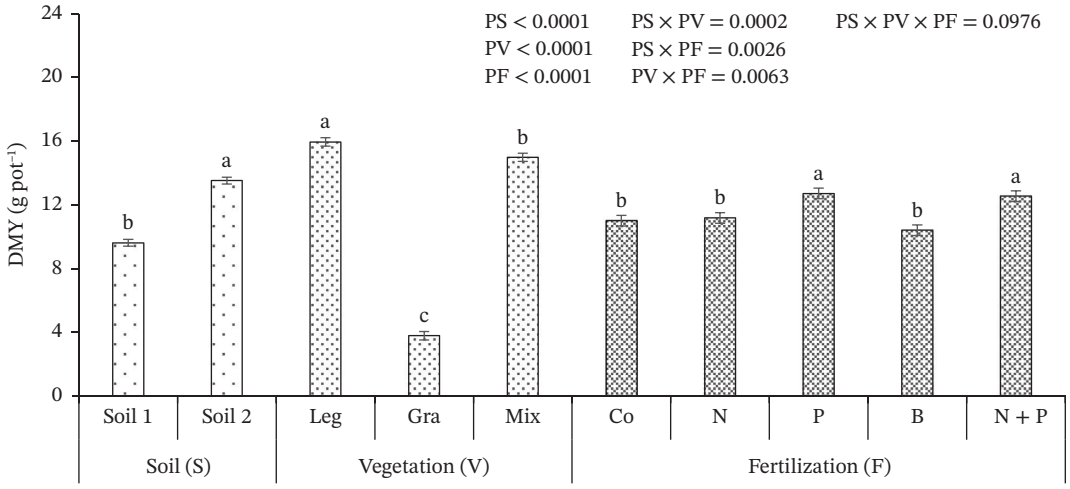


FIGURE 2 | Dry matter yield (DMY) in response to soil (Soil 1, Soil 2), vegetation type (Leg, legume; Gra, grass; and Mix, mixture) and fertilization (Co, control; N, nitrogen; P, phosphorus; B, boron; and N + P). Probability (P) associated with three-way ANOVA of the factors soil (S), vegetation (V), fertilization (F) and their interaction. Within each factor, means with the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$). Error bars represent the standard error.

DMY than all other treatments. In contrast, DMY in pots that received N or B before sowing was not significantly different from the control.

To further elucidate the effects of the treatments, given the significant interaction observed between factors, the data were analysed separately by cultivated species (Figure 3). Results indicate no significant interaction between soil types and fertilization treatments. However, for each species, significant differences between soils and fertilization treatments persisted, both for the legume and the grass. In the case of the legume, the positive effect of P stands out, although significantly higher DMY was only observed for P, when applied in conjunction with starter N. B, on the other hand, did not improve DMY in either the legume or the grass.

3.2 | Nitrogen Concentration and Recovery in Plant Tissues

Significant interactions were observed between all pairs of factors for both tissue N concentration and recovered N, although no significant three-way interaction was observed (Figure 4). N concentration in plant tissues was higher in Soil 2 compared to Soil 1. Significant differences were also found between vegetation types, with the legume showing the highest tissue N concentration, followed by the mixture, and lastly the grass. Regarding fertilization treatments, tissue N concentration was not affected by the small amount of N applied before sowing. However, it was influenced by P application, especially when combined with N.

Recovered N reflects the multiplicative effect of N concentration and DMY. Thus, in terms of total N recovered in the aboveground biomass, the effect of soil became considerably more evident, with Soil 2 resulting in substantially higher values than Soil 1. Differences between vegetation types were also more pronounced, with legumes, mixtures and grasses yielding 380.3, 327.3 and 75.2 g pot^{-1}, respectively. Among the fertilization treatments, it became clear that P application to the soil was the main driver of increased N recovery in the aboveground biomass. N application, by contrast, did not affect the amount of N contained in the aboveground biomass at the time of harvest.

The %Ndfa ranged from 70.7% to 87.2% across all cultivation conditions (Table 1). The effect of fertilization treatments was

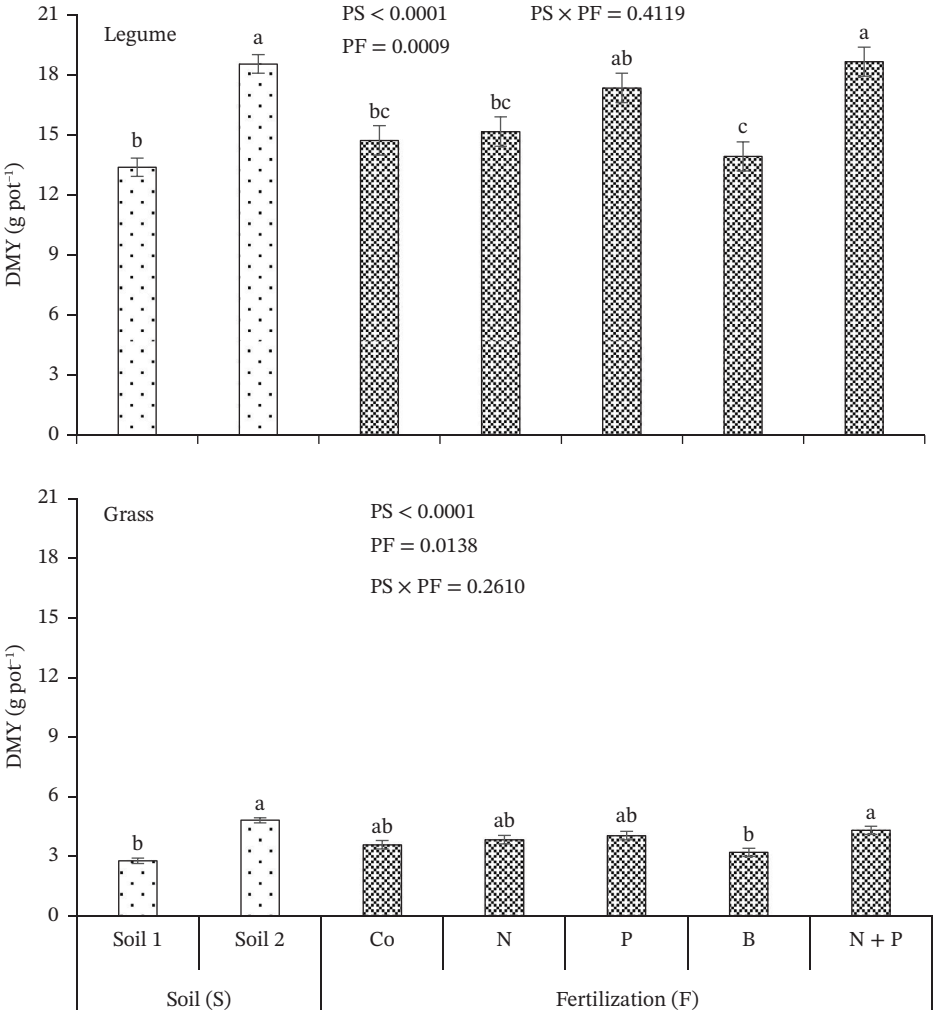


FIGURE 3 | Dry matter yield (DMY) in response to soil (Soil 1, Soil 2), vegetation type (Leg, legume; Gra, grass; and Mix, mixture) and fertilization (Co, control; N, nitrogen; P, phosphorus; B, boron; and N + P). Probability (P) associated with two-way ANOVA of the factors soil (S), fertilization (F) and their interaction. Within each factor, means with the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$). Error bars represent the standard error.

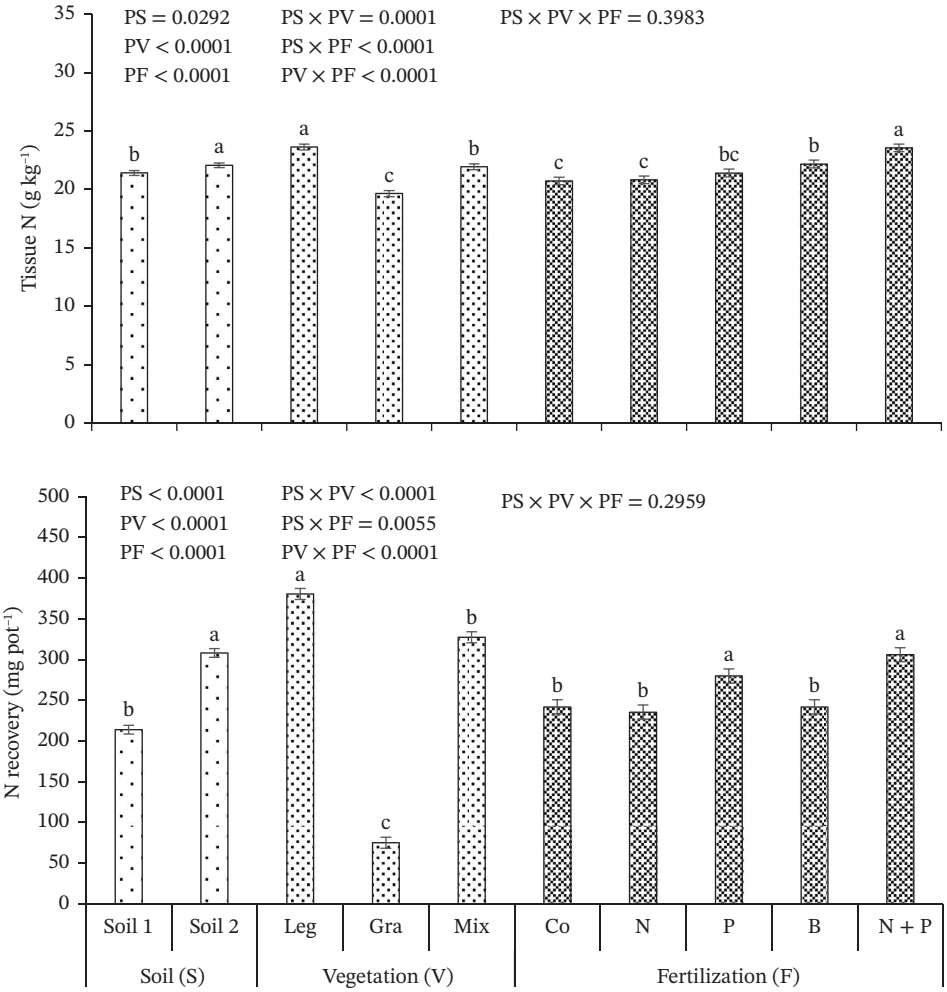


FIGURE 4 | Nitrogen (N) concentration and recovery in plant tissues in response to soil (Soil 1, Soil 2), vegetation type (Leg, legume; Gra, Grass; and Mix, mixture) and fertilization (Co, control; N, nitrogen; P, phosphorus; B, boron; and N + P). Probability (P) associated with three-way ANOVA of the factors soil (S), vegetation (V), fertilization (F) and their interaction. Within each factor, means with the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$). Error bars represent the standard error.

inconsistent. The most consistent trend was the effect of soil. Plants grown in Soil 1 obtained a higher proportion of their N from the atmosphere compared to those grown in Soil 2. On average, 81.4% of the N in Soil 1 was derived from the atmosphere, whereas in Soil 2, the value was only 76.0%.

3.3 | Phosphorus Concentration and Recovery in Plant Tissues

Soil type significantly influenced P concentration in plant tissues, with higher values observed in Soil 2 (Figure 5). Given that Soil 2 also produced more biomass, the difference in total P recovered in the aboveground biomass was even more pronounced, with higher values again recorded in Soil 2. P concentration in plant tissues was significantly higher in the grass than in the legume and the mixture. However, due to the substantially greater biomass production of the legume, the total P recovered in the aboveground biomass was higher in the legume than in the grass. P application significantly increased tissue P concentration as compared to the control and starter N. Since these treatments were also associated with greater DMY, the amount of P recovered in the aboveground biomass was markedly higher in those receiving P.

3.4 | Boron Concentration and Recovery in Plant Tissues

Soil type did not significantly influence B concentration in plant tissues. However, since Soil 2 supported higher biomass production, it resulted in a significantly greater amount of B recovered in the aboveground biomass (Figure 6). The legume exhibited significantly higher B concentrations in its tissues than the grass. Considering the legume's much greater DMY, the total amount of B recovered (0.279 mg pot^{-1}) was more than 10 times higher than that recovered in the grass (0.027 mg pot^{-1}). B application resulted in a significantly higher tissue B concentration and total B recovery, as compared to all the other fertilizer treatments, even though B-treated plants were associated with relatively low DMY.

3.5 | Tissue Concentration and Recovery of Nutrients Not Included as Treatments in the Experiment

Soil type significantly influenced K concentration in plant tissues (Figure 7). K levels were higher in Soil 2, and given that this soil also supported greater DMY, the amount of K in the aboveground biomass was particularly high in Soil 2. The grass exhibited

TABLE 1 | Percentage of nitrogen derived from the atmosphere (average ± standard deviation) in response to fertilization (Co, control; N, nitrogen; P, phosphorus; B, boron; and N + P).

		Treatment				
		Co	**N**	**P**	**B**	**N + P**
Legume	Soil 1	83.3 ± 2.3	76.2 ± 8.5	79.3 ± 5.6	86.4 ± 2.3	83.4 ± 5.2
	Soil 2	79.5 ± 0.5	74.3 ± 7.4	78.2 ± 1.2	80.4 ± 4.1	80.3 ± 1.0
Mixture	Soil 1	82.7 ± 3.5	76.9 ± 6.6	80.5 ± 2.5	87.2 ± 2.1	77.9 ± 3.6
	Soil 2	76.5 ± 1.4	72.2 ± 6.5	75.5 ± 5.5	72.5 ± 2.8	70.7 ± 3.2

significantly higher tissue K concentrations than the legume. However, because the legume produced more biomass, the total amount of K in the aboveground biomass was significantly higher in the legume, overriding the effect of concentration alone. Fertilization treatments, on the other hand, did not significantly affect either tissue K concentration or the total amount of K recovered in the aboveground biomass.

Soil type also significantly influenced both Ca concentration in plant tissues and the total Ca recovered, with higher values observed in Soil 2 compared to Soil 1. Generally, soil and vegetation types led to significant differences in several other nutrients analysed. These differences are attributed to both the availability of nutrients in the soil and the typical variation in nutrient concentrations between grass and legume tissues. In contrast, fertilization treatments did not result in significant differences among treatments. The corresponding results were not presented, as they were not considered relevant to addressing the hypotheses posed in this study.

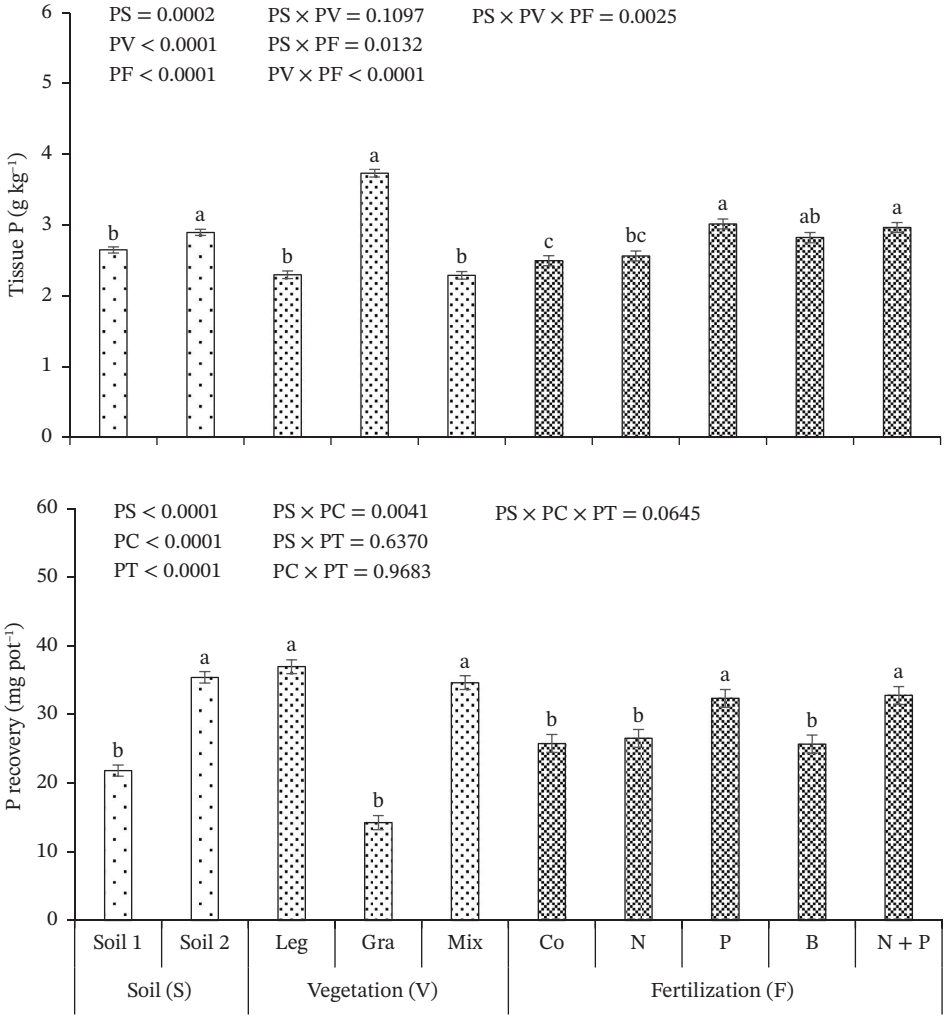


FIGURE 5 | Phosphorus (P) concentration and recovery in plant tissues in response to soil (Soil 1, Soil 2), vegetation type (Leg, legume; Gra, Grass; and Mix, mixture) and fertilization (Co, control; N, nitrogen; P, phosphorus; B, boron; and N + P). Probability (P) associated with three-way ANOVA of the factors soil (S), vegetation (V), fertilization (F) and their interaction. Within each factor, means with the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$). Error bars represent the standard error.

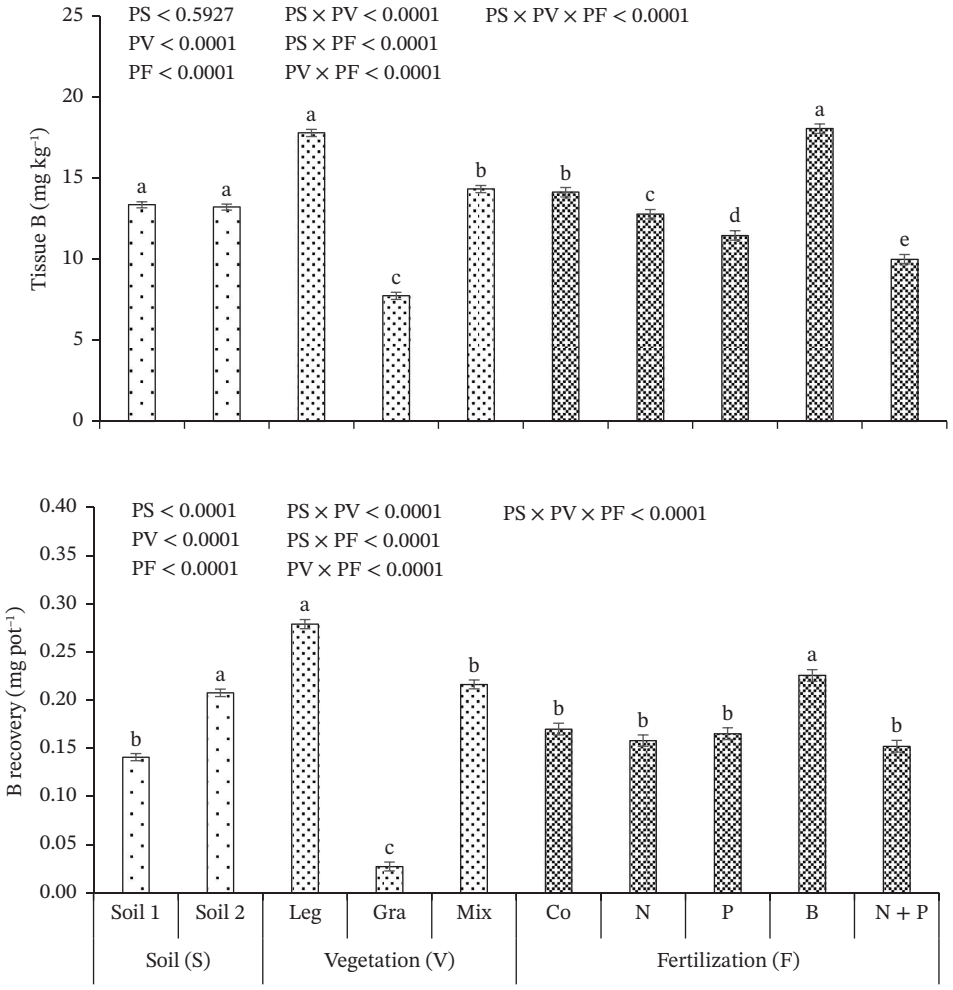


FIGURE 6 | Boron (B) concentration and recovery in plant tissues in response to soil (soil 1, soil 2), vegetation type (Leg, legume; Gra, Grass; and Mix, mixture) and fertilization (Co, control; N, nitrogen; P, phosphorus; B, boron; and N + P). Probability (P) associated with three-way ANOVA of the factors soil (S), vegetation (V), fertilization (F) and their interaction. Within each factor, means with the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$). Error bars represent the standard error.

4 | Discussion

4.1 | Effect of Soil on Plant Nutrition and DMY

Soil type influenced DMY, with Soil 2 resulting in the highest yields for both the grass and the legume species. Among the principal soil properties that could potentially have affected DMY, the acidity, organic matter content, and P and K levels are noteworthy. Soil 2 exhibited higher values for organic matter, P and K, although Soil 2 was also more acidic. Generally, Soil 2 enhanced the concentration of several nutrients in plant tissues, notably P, K and Ca. Additionally, it increased N content in plant tissues while decreasing the %Ndfa in the legume. The observed increases in soil-derived N and tissue Ca suggest that soil acidity was not a significant limiting factor for productivity. Highly acidic soils typically exhibit low Ca availability, which can lead to nutrient deficiencies [41, 42]. Furthermore, strong soil acidity often reduces N availability to plants by impairing biological activity and inhibiting organic matter mineralization and nitrification [11, 15, 43]. However, such limitations were not observed in this study. It is likely that the high organic matter content of Soil 2 exerted a substantial buffering effect, reducing the solubility of potentially toxic elements such as aluminium and/or manganese [41], thus preventing acidity from becoming a limiting factor.

Moreover, the mineralization of organic matter likely contributed to the increased availability of N. In this study, soil was collected and sieved for use in the pot experiment, procedures that disrupted some soil aggregates and enhanced aeration, conditions known to stimulate organic matter mineralization and N release to plants [41, 44].

4.2 | Effect of Phosphorus Application

The grass species exhibited a higher P concentration in its tissues as compared to the legume; however, the legume achieved greater total P recovery due to its higher DMY. This outcome is attributed to a dilution effect of P within the legume tissues. The dilution/concentration effect is well documented in cases where, for a given nutrient availability, variations in biomass accumulation occur due to factors other than nutrient supply [45, 46].

P application increased P concentrations in both the grass and legume tissues, a direct consequence of enhanced soil P availability. Plants can absorb nutrients in varying amounts depending on their availability in soil, sometimes even exceeding

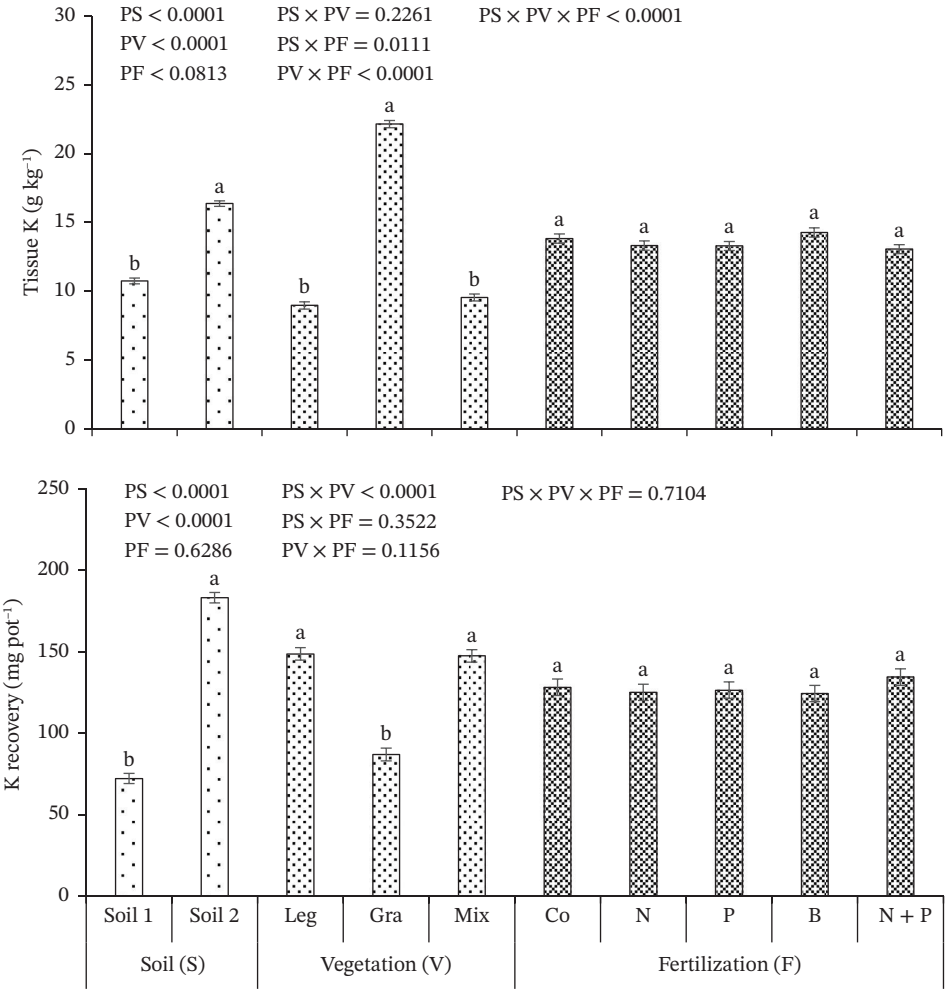


FIGURE 7 | Potassium (K) concentration and recovery in plant tissues in response to soil (Soil 1, Soil 2), vegetation type (Leg, legume; Gra, Grass; and Mix, mixture) and fertilization (Co, control; N, nitrogen; P, phosphorus; B, boron; and N + P). Probability (P) associated with three-way ANOVA of the factors soil (S), vegetation (V), fertilization (F) and their interaction. Within each factor, means with the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$). Error bars represent the standard error.

their immediate metabolic needs, a phenomenon referred to as luxury consumption [19, 41]. Furthermore, P application also stimulated DMY. Increases in biomass production following P application are frequently reported [14, 47, 48], particularly when P is a limiting factor and its concentration in plant tissues falls below the sufficiency range [19, 49].

In this study, P application increased both N concentration in plant tissues and, more notably, total N recovery. Comparable enhancements in N fixation and biomass yield following P fertilization have also been reported in other legume studies [10, 14, 21]. This finding clearly indicates that the primary effect of P on DMY was not merely through its role in plant metabolism, but predominantly through its stimulation of N fixation. This hypothesis is further supported by the fact that the positive effect of P application on DMY was more pronounced in the legume than in the grass. In biodiverse pastures, P is commonly applied to promote the growth and persistence of the legume component [16, 48, 50]. Nodulation and nodule growth require greater P inputs than general plant metabolism due to their high energy demands, as energy-rich phosphates are essential for the intense respiration associated with N fixation in root nodules [12].

4.3 | Effect of Nitrogen Application

The application of starter N did not increase N concentrations in plant tissues or enhance N recovery. This outcome is somewhat unusual, as nitrogen application typically leads to increased crop productivity [51, 52]. The amount of N applied was very low relative to the quantity fixed from the atmosphere and thus did not significantly impact DMY. Starter N applications have generally been recommended for situations where soil N levels are extremely low, potentially compromising early legume growth [24–26].

From the initial recognition between the microorganism and the host plant to the formation of functional bacteroids capable of N fixation, a period of three to 4 weeks may elapse. During this phase, the plant does not yet have access to atmospheric N, and the relationship is characterized by parasitism of the plant by the rhizobium [12]. A limitation in the plant's photosynthetic capacity during this period can impair both its growth and the nodulation process. Consequently, it has been demonstrated that the application of small amounts of N during the early growth stages can stimulate legume development without inhibiting nodulation and subsequent nodule activity [24–26].

Even in the grass species, no positive effect of N application was observed when compared to the control. The small quantity of N applied, combined with the timing of application, immediately prior to the winter season characterized by high rainfall, likely contributed to this outcome, since such conditions favour high nitrate leaching and denitrification losses [53, 54]. Therefore, the combination of a low N dose applied during a period of poor efficiency explains the absence of significant differences in N recovery between the starter N treatment and the control.

Another factor contributing to the ineffectiveness of starter N application in both the legume and the grass was the organic matter content of the soils. Moreover, the soils used in this study were collected from the 0–0.15 m depth and subsequently sieved, a process that disrupts soil aggregates and promotes strong aeration. As previously mentioned, these conditions favour organic matter mineralization [41]. Thus, under these conditions, the legume likely had sufficient N during its early stages to ensure proper nodulation, while the effect on the grass was overshadowed by the low efficiency of the N applied as fertilizer.

4.4 | Effect of Boron Application

The legume exhibited higher B concentrations in its tissues than the grass, a pattern typically observed for dicot species [19, 30]. A significant portion of B in plants is complexed with cis-diol esters associated with pectins in the cell walls. The greater B requirement of dicots is attributed to their higher proportion of cis-diol-containing compounds in cell walls compared to monocots [29, 55].

B application to the soil increased B concentrations in plant tissues but did not enhance DMY. Typically, B fertilization increases crop productivity [31–33]; however, this was not observed in the present study. This finding indicates that B was not a limiting factor, as a positive biomass response to nutrient application is generally expected only when the nutrient is deficient [8, 31, 32]. Initial Soil B levels were not low. Furthermore, the organic matter in these soils may have served as a B reservoir. B can bind to functional groups of organic matter, such as hydroxyl and carboxyl groups, through reversible interactions, allowing it to be gradually released into the soil solution as needed by plants [29, 56]. Additionally, the experimental conditions, specifically, soil collection and sieving, likely promoted mineralization of organic matter [41], further contributing to B availability. As a result, while B application increased tissue B concentrations, it did not translate into increased DMY, as B levels were already sufficient even in treatments that did not receive additional B.

4.5 | Effect of Treatments on Nutrients Not Applied in the Experimental Protocol

Tissue nutrient concentrations were primarily influenced by the availability of each nutrient in the soil. For instance, Soil 2, which was richer in K, resulted in higher K concentrations in plant tissues. The two plant species exhibited different nutrient concentrations according to their specific anatomical and physiological traits. Grasses, for example, have a lower root CEC and therefore tend to absorb monovalent ions, such as K, more efficiently [41]. K uptake in plants is highly selective and tightly coupled to metabolic activity. In plants, K predominantly exists as a free ion and exhibits high mobility at all cellular and tissue levels, including long-distance transport via both the xylem and the phloem [49].

In contrast, dicots generally exhibit a higher root CEC, which favours the absorption of divalent cations, such as Ca [41]. A significant proportion of total Ca in plant tissues is typically located in the cell walls, where it binds to $R\text{-}COO^-$ groups of polygalacturonic acids (pectins). Monocots generally have lower Ca requirements compared to dicots because their cell walls contain lower concentrations of pectate and exhibit reduced CEC [49]. Nevertheless, the total amount of both K and Ca recovered was higher in the legume species, largely due to their greater DMY, an outcome attributed to the dilution effect.

As a general pattern, nutrient concentrations in plant tissues often varied according to their availability in the growth medium, whereas nutrient recovery depended on DMY, which in turn was influenced by N availability. Factors that facilitated nodulation and enhanced access to atmospheric N, particularly P availability, were those that most strongly promoted plant growth in this study.

5 | Conclusions

This study evaluated how soil properties and the application of phosphorus (P), starter nitrogen (N) and boron (B) affect plant nutrition and DMY of a legume (subterranean clover) and a grass (annual ryegrass) grown under mixed pasture conditions. Soil type had a decisive influence on plant performance, with the soil richer in organic matter and available P and K (Soil 2) producing the highest DMY for both species. Despite its greater acidity, Soil 2 did not impose nutritional limitations, likely due to the buffering effect of its high organic matter content, which enhanced N availability and prevented calcium deficiency. Among the applied nutrients, P was the only factor that significantly increased biomass production, especially in the legume. This effect was largely indirect, resulting from the stimulation of biological N fixation rather than from P's direct metabolic roles. The increases in tissue N concentration and total N recovery confirm that P availability is a key driver of N fixation efficiency and legume competitiveness in mixed pastures. Starter N application did not affect DMY or tissue N concentration, likely due to the combination of a low N dose, high soil organic matter mineralization and potential N losses under wet winter conditions. Similarly, B application increased tissue B concentrations but did not enhance growth, indicating that B was not limiting under the experimental conditions.

Overall, the results demonstrate that, in soils with moderate to high organic matter content, P availability is the primary nutritional factor governing legume performance, whereas starter N and B fertilization provide no agronomic benefit. These findings underscore the importance of prioritizing P management to optimize N fixation and the productivity of grass–legume mixtures in pasture systems.

Author Contributions

Almeida Sawimbo: investigation, data curation and draft preparation. Peltier Aguiar: investigation and draft preparation. Margarida Arrobas: conceptualization, methodology, funding acquisition and writing–review and editing. Carlos Aguiar: conceptualization, resources and

writing–review and editing. Nuno Rodrigues: methodology and writing–review and editing. Marjan Jongen: methodology and writing–review and editing. Ricardo F. M. Teixeira: methodology and writing–review and editing. Tiago Domingos: conceptualization, project administration, funding acquisition and writing–review and editing. Manuel Ângelo Rodrigues: conceptualization, project administration, data curation and writing–review and editing.

Acknowledgements

This work was supported by national funds through FCT/MCTES (PIDDAC), through the projects UIDB/50009/2025, UIDP/50009/2025, LA/P/0083/2020, UIDB/00690/2020 (DOI: 10.54499/UIDB/00690/2020) and LA/P/0007/2020 (DOI: 10.54499/LA/P/0007/2020). It was also funded by the project AVALON (COMPETE2030-FEDER-02288900).

Funding

No funding was received for this manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The original contributions presented in the study are included in the article; further enquiries can be directed to the corresponding author.

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