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Combining Zinc Biofortification and Native *Trichoderma* Inoculation Strategies for Subterranean Clover

Carlos García-Latorre [1,*], Rocío Velázquez [1], Alejandro Hernández [2], Paula Tejero [2] and Maria J. Poblaciones [1,*]

[1] Department of Agronomy and Forest Environment Engineering, University of Extremadura, Avenida Adolfo Suárez s/n, 06007 Badajoz, Spain; rvotero@unex.es
[2] Department of Animal Production and Food Science, School of Agricultural Engineering, Universidad de Extremadura, Avenida Adolfo Suárez s/n, 06007 Badajoz, Spain; ahernandez@unex.es (A.H.); paulatc@unex.es (P.T.)
* Correspondence: cgarcialn@unex.es (C.G.-L.); majops@unex.es (M.J.P.)

Abstract: Using beneficial microorganisms along with sustainable strategies such as agronomic biofortification offers eco-friendly alternatives to combat climate change in ecosystems like dehesas. This study analyzes the combined effects of four wild *Trichoderma* spp. isolated from Extremadura, Spain (*T. koningiopsis*, two *T. gamsii*, and *T. koningii*, with negative and positive controls) and four Zn biofortification treatments (no Zn application; soil application of 5 mg of $ZnSO_4 \cdot 7H_2O$ per kg of soil, labeled soil Zn; two foliar applications of 5 mL 0.5% $ZnSO_4 \cdot 7H_2O$, labeled foliar Zn; and soil + foliar combination, labeled SF) on *Trifolium subterraneum* performance. The combination of *T. koningiopsis* and *T. gamsii* with foliar Zn improved plant growth by up to 34.4%. Zinc accumulation was about 30% higher when *T. gamsii* and *T. koningii* were applied with SF, and their inoculation resulted in a 2.5-fold increase in ash. *Trichoderma* spp. affected nodulation differently; both *T. gamsii* inhibited nodulation by 24%, whereas neither *T. koningiopsis* nor *T. koningii* showed differences from the controls. These results highlight the potential of combining beneficial microorganisms with biofortification strategies to address nutrient deficiencies and improve agricultural sustainability. However, the complex interactions between both factors underscore the importance of strain selection and call for further research to optimize application strategies and elucidate underlying mechanisms.

Keywords: bioinoculation; pasture management; zinc sulfate; nutrient uptake

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1. Introduction

Pastures are integral parts of the ecological and agricultural landscapes of the Southwestern Iberian Peninsula, covering approximately 2.07 million hectares in the Spanish region of Extremadura, which constitutes about 70% of the region's total land area [1]. This extensive land area comprises a variety of ecosystems, most notably dehesas, grasslands, and areas characterized by dense tree cover [2]. These ecosystems provide a variety of ecosystem services that are critical to both agricultural sustainability and environmental well-being. Well-managed pastures play a key role in fire prevention and control through proper grazing planning that includes the establishment of firebreaks [3]. Moreover, these pastures provide other valuable resources to nearby agricultural areas, including the commercialization of by-products such as crop residues and straw. Beyond their economic contributions, these pastures also serve as habitat and food sources for a wide range of faunal species, including storks, vultures, lynx, eagles, lizards, and cranes, among others, contributing to the rich biodiversity of the region [4]. Agroforestry systems, including dehesas, are vital for carbon sequestration and biodiversity preservation [5–9]. Appropriate agroforestry management practices, including sustainable grassland–livestock systems and legume incorporation, are essential for increasing productivity and diversity, especially under increased climate variability [10]. These practices could play an important role in

climate change mitigation [9]. However, pastures in the southwest of the Iberian Peninsula have been frequently perceived as being of low quality due to poor soil conditions and irregular rainfall patterns [11,12]. As a result, these pastures have been used directly as livestock feed regardless of their growth stage. This traditional approach has resulted in minimal management and capital investment, mainly for livestock feeding purposes [13], leading to widespread pasture degradation, reduced soil fertility, and economic losses [14,15].

Among active management strategies to improve pasture conditions in the southwest of the Iberian Peninsula, the promotion of *Trifolium subterraneum*, commonly known as subterranean clover, plays a key role. Its adaptability to the Mediterranean climate and the high quality of its forage makes it essential for supporting livestock [16]. Furthermore, its ability to self-regenerate and adapt to varying climatic conditions, as demonstrated in studies on climatic adaptation and salt tolerance mechanisms, further underlines its significance in sustainable pasture management [17]. Another promising approach to improve pasture sustainability is represented by the adoption of biofortification strategies. Biofortification aims to increase the nutritional value of crops, particularly with micronutrients that are essential to the ecosystem, such as zinc (Zn), in soils that are naturally deficient or have low bioavailability [18]. This addresses Zn deficiency in animal and human diets by increasing its concentration and bioavailability in food crops [19,20]. Agronomic biofortification programs have demonstrated the potential to improve pasture sustainability by increasing the nutritional quality of forage crops [21–23]. This improvement not only benefits the livestock consuming the fortified feed but also contributes to enhancing overall soil health and ecosystem sustainability [24,25]. It represents a promising way to improve pasture sustainability by enhancing the nutritional quality of forage crops like *Trifolium subterraneum*, thereby promoting more resilient and nutrient-rich pasture ecosystems. Finally, the use of native *Trichoderma* isolates has been highlighted to enhance nutrient uptake and crop yield, promoting sustainable agricultural practices [26].

The combination of Zn biofortification and the use of native *Trichoderma* isolates represents a holistic approach to addressing nutrient deficiencies and improving crop productivity. The simultaneous biofortification of crops with micronutrients through microbial interventions has already demonstrated additive and synergistic effects on plant quality [27]. For example, the application of Zn biofortification, along with seed co-inoculations of plant-growth-promoting bacteria, has been found to enhance growth, yield, biofortification, and Zn use efficiencies in legumes such as common bean [28]. However, there is still limited information available on the efficacy of combining both techniques, especially concerning subterranean clover. Recent studies have demonstrated the positive effect of selenium biofortification in natural pastures containing *T. subterraneum*, among other species [29,30], as well as the impact of fungal endophytes on the performance of this legume [31]. However, to our knowledge, no studies have incorporated both strategies for *T. subterraneum*. Therefore, this research holds significant importance due to the scarcity of data in this area. Additionally, this integrated approach is consistent with the goal of sustainable agriculture as it promotes soil health, reduces the dependence on external inputs, and improves the nutritional quality of crops. Integrating agronomic biofortification to increase the nutrient content of *T. subterraneum* with the application of native *Trichoderma* isolates to stimulate plant growth and manage soil-borne pathogens could enhance pasture sustainability. Thus, the use of native *Trichoderma* isolates as natural biofortification agents underscores the potential for environmentally friendly and cost-effective agricultural strategies [32]. This approach would not only improve the nutritional quality of forage crops but also increase plant resilience and productivity through biocontrol mechanisms.

Therefore, based on the premise that the improvement of pasturelands can favor their sustainability and resilience to climate change, this study aimed to evaluate the effects of soil and foliar Zn biofortification, coupled with the simultaneous use of different *Trichoderma* isolates obtained from soils of Extremadura, on plant growth and nutrient concentration in *Trifolium subterraneum*, a legume that is well adapted to dehesa ecosystems.

2. Materials and Methods

2.1. Site Details

The experiment was conducted in a naturally lit greenhouse at the School of Agricultural Engineering, University of Extremadura, situated in Badajoz, Spain (38°89′ N, 6°97′ W; elevation: 186 m above sea level). Throughout the duration of the experiment (from 9 October 2023 to 26 January 2024), the greenhouse maintained average temperatures of 18 ± 6 °C during the day and 12 ± 4 °C during the night.

2.2. Soil Characterization

The soil used in this experiment was collected from the area of Elvas in the Alentejo region of Portugal. This soil was first air-dried under laboratory conditions and then sieved through a 2 mm mesh sieve. Following this, four aliquots underwent oven-drying at 50 °C until they reached a constant weight, and they were then analyzed to determine their physicochemical characteristics using standardized procedures. The soil was characterized by a sandy loam texture with constituent proportions of 14.9% clay, 57.1% sand, and 28.0% silt. The soil pH was 6.5 ± 0.1 (average ± standard error). Organic matter content was 1.2 ± 0.1 g/kg [33], carbonate content was <1%, available phosphorus was 19.5 ± 3.4 mg/kg, and potassium content was 0.8 ± 0.1 meq/kg [34]. The extractable Zn in the soil, 0.34 ± 0.04 mg/kg, was extracted with DTPA (diethylenetriamine penta acetic acid) according to the method of Lindsay and Norvell [35] and determined by inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Fisher Scientific iCAPQ, Bremen, Germany). Internal references and blanks were included in each batch of samples, and all the results were reported on a dry weight basis.

2.3. Fungal and Plant Material

In this study, four different fungal isolates of the genus *Trichoderma* were selected to evaluate their potential to enhance subterranean clover growth and nutrient uptake. These isolates were specifically obtained from soils at different locations in the Valle del Jerte region of Extremadura, Spain, where cherry cultivation is prevalent. Selection criteria included factors such as isolation frequency and observed bioactivity in previous assays in relation to biocontrol and plant growth promotion in vitro. Table 1 shows the identification details of these isolates. In addition, a commercially available product (Bactiva®, Bactiva GmbH, Straelen, Germany), referred to as Tc, was used as a positive control. This product contained a combination of three different *Trichoderma* species (*T. harzianum*, *T. eesei* and *T. viride*) that have already been shown to be effective, allowing us to assess the efficacy of our wild isolates. Sterilized distilled water (designated as T0) was used as the negative control in the experiment.

Table 1. *Trichoderma* isolate identification and experimental controls in this study.

Code	Identification	GenBank Accession Number
T05	*Trichoderma koningiopsis*	MT520626
T09	*Trichoderma gamsii*	MT557537
T14	*Trichoderma koningii*	KT715712
T18	*Trichoderma gamsii*	MT557537
T0	Negative control: Sterilized distilled water	
Tc	Positive control: *Trichoderma* consortium (Bactiva®): *T. harzianum*, *T. eesei*, *T. viride*	

Prior to plant inoculation, fungal spores were obtained from *Trichoderma* isolates. Spore samples from previous plates of each fungal isolate were seeded on autoclaved potato dextrose agar (PDA; 39 g/L of distilled water) and incubated in a germination chamber at 23 °C for seven days in the dark, followed by exposure to direct sunlight and room temperature for another seven days to induce sporulation. The same procedure was used for the commercial product (Bactiva®), internally designated as Tc. Once sufficient growth

and spore presence were confirmed on the plates, spore collection was performed by adding 3–5 mL of sterile distilled water to the plates. The spore concentration was determined by using a hemocytometer (Neubauer chamber). From these initial concentrations, each concentration was diluted to obtain the final application concentration of 10^6 spores per mL for the different *Trichoderma* spp. isolates used in the experiments.

Trifolium subterraneum var. Antas seeds were surface-sterilized by successive immersion in a solution of 70% ethanol and a solution of 2% sodium hypochlorite, and then washed three times with sterile distilled water, according to the method described by Zabalgogeazcoa et al. [36].

2.4. Experimental Design and Treatments

The experiment followed a completely randomized block design with four Zn treatments combined with six fungal treatments, and four replicates per combination, resulting in a total of 96 pots. Each pot was seeded with 3 seeds of *T. subterraneum* pretreated with one of the fungal treatments. The Zn biofortification treatments included the following: no Zn application (no Zn); soil application of 5 mg of $ZnSO_4 \cdot 7H_2O$ per kg of soil (soil Zn); foliar application of 5 mL 0.5% $ZnSO_4 \cdot 7H_2O$ per pot performed once, three weeks after sowing, and again four weeks later (foliar Zn); and the combination of both soil and foliar $ZnSO_4 \cdot 7H_2O$ applications (SF Zn). Foliar treatments were applied by continuous spraying to wet the plants evenly. Soil moisture content was maintained at an adequate level by daily irrigation with 50 mL of deionized water. No pests or disease incidents were observed during this study.

2.5. Microorganism Re-Isolation

In greenhouse trials, the efficacy of fungal treatments on *Trifolium subterreum* was assessed at two critical time points. The first assessment occurred just before sowing and focused on the viability of the fungal inoculum applied to the seed, establishing a baseline for the study and confirming the initial success of seed inoculation. At harvest, two assessment methods were used. First, fungi were re-isolated from soil samples to study their persistence and adaptation within the soil ecosystem. Second, plant tissues were examined to determine the presence and development of fungi within the plants and their potential behavior as endophytes. In all cases, seed inoculation with each isolate proved effective, resulting in fungal growth and development both in the soil and within the plants. These results are significant because they provide a solid basis for establishing a confident link between the effects observed in subsequent results and the action of the selected fungal strains.

2.6. Measurements

Plant Growth Parameters

Plants were harvested three weeks after the second foliar application of Zn, approximately 10 weeks after sowing, and carefully washed with deionized water. Prior to harvest, the number of plants per pot was recorded, and indirect chlorophyll concentration was measured using a SPAD meter (Minolta SPAD 502). During plant collection, many *Rhizobium* sp. nodules were observed in most of the roots. An evaluation based on the number and activity of nodules was conducted, with samples categorized on a scale of 1 to 4: 1 represents samples without nodules; 2 represents samples with few green or white nodules, indicating low N-fixing activity; 3 represents samples with numerous pink nodules, indicating good fixation activity; and 4 represents samples with roots practically covered with nodules, indicating high fixation capacity. After harvesting, the herbage and root parts were separated, and their fresh weights were recorded. Both parts were then air-dried for three days and then oven-dried at 50 °C until a constant weight was reached to determine their dry weights. Following complete drying of the samples, determinations of neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), and total ash were conducted. NDF, ADF, and ADL analyses were performed using a fiber analyzer

(ANKOM 8-98, ANKOM Technology, Macedon, NY, USA), while total ash analysis was carried out by ignition in a muffle furnace at 600 °C. In all cases, the determinations were made by following the official procedures [37]. Dry matter digestibility was then calculated from the ADF percentage as follows [38]:

$$Dry\ matter\ digestibility,\ DMD\ (\%) = 88.9 - (0.779 * ADF)$$

2.7. Nutrient Concentration

The concentrations of iron (Fe), magnesium (Mg), phosphorus (P), selenium (Se), and Zn were measured in the herbage dry matter of the plants, as well as in samples obtained from the potted soils for each treatment. For this, 20 mg of dried soil or herbage dry matter from each sample was digested using a mixture of nitric acid (2 mL, 70%) and hydrogen peroxide (2 mL, 50%) in a closed-vessel microwave system (Anton Paar Gmbh, Graz, Austria). Each digestion run included two blanks and two certified reference materials (CRM: tomato leaf SRM 1573a NIST, Gaithersburg, MD, USA). The digested samples were analyzed using ICP-MS (Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany).

2.8. Statistical Analysis

Two-way analysis of variance (ANOVA) was used to evaluate the effect of *Trichoderma* spp. isolates, biofortification treatment, and the interaction of both factors on the yield and nutritional parameters of *Trifolium subterraneum* plants. Fisher's protected least significant difference (LSD) test at a significance level of $p < 0.05$ was used for mean comparisons when significant differences were detected. Normality was assessed using the Shapiro–Wilk test, while homoscedasticity was assessed using the Levene test. The relationship between each parameter was evaluated by Pearson correlation at a significance level of $p < 0.05$. All statistical analyses were performed with the STATISTIX 8.1 statistical package (Analytical Software, version 8.1, Tallahassee, FL, USA, 2005). The correlogram (shown as Figure S1) was performed with the R 4.3.3 statistical package (R Core Team, Vienna, Austria, 2024) [39].

3. Results

3.1. Influence on Growth and Quality Parameters of Trifolium subterraneum

The results of the two-way ANOVA are summarized in Table 2. The inoculation with the different *Trichoderma* spp. isolates revealed significant effects on all studied parameters except for the herbage dry matter (HDM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and dry matter digestibility (DMD). Regarding the effect of the biofortification treatment on the yield and quality parameters, Zn application significantly affected the root dry matter (RDM) and the rhizobia nodulation index, as well as the ADF, acid detergent lignin (ADL), and DMD, while its effects on the HDM, the SPAD value, as well as the NDF and ash percentages were not statistically significant.

When examining the effect of *Trichoderma* isolates combined with biofortification treatments on the HDM (Figure 1a), it can be noticed that the plants inoculated with T09 (*Trichoderma gamsii*), T14 (*Trichoderma koningii*), and T18 (*Trichoderma gamsii*) showed increased plant yield in the absence of Zn applications (no Zn) compared to the negative control without Zn (T0 and no Zn), although no significant differences were observed compared to the T0 group treated with SF Zn. In addition, the combinations of T05 (*T. koningiopsis*) with a double Zn application (SF) and T18 (*T. gamsii*) with a foliar Zn application significantly increased the HDM compared to the T0 control with no Zn (23.4% and 34.4%, respectively), reaching similar levels to those obtained with the sole Zn application by soil and foliar methods (T0 and SF).

Regarding the effect on the RDM, the improvements were more limited. In particular, the only *Trichoderma* spp. isolate that significantly improved this parameter without Zn application was T14 (*T. koningii*) compared to both controls (10.64 mg). In addition, the combinations of T14 with a foliar Zn application, T09 with a double Zn application (SF), and

T18 inoculation with either foliar or soil Zn application (but not SF) significantly increased the RDM in the treated *T. subterraneum* plants, although this effect was significantly lower than that of the T14 (*T. koningii*) application with no Zn (Figure 1b).

For the indirect measurement of the chlorophyll content through the SPAD values recorded immediately before harvest (Figure 1c), it is noteworthy that the inoculation with any *Trichoderma* spp. isolate significantly increased this parameter in the absence of a Zn application compared to T0 with no Zn (with increases ranging from 77.6% for T09 to 129% for T14). Similarly, all three biofortification treatments significantly increased this parameter in the plants that were not inoculated with any fungal isolate compared to the T0 control with no Zn (T0 and no Zn). Furthermore, different combinations of fungal isolates and biofortification treatments significantly increased the SPAD values. In particular, the application of T18 (*Trichoderma gamsii*) with foliar Zn significantly increased all three parameters shown in Figure 1. It is also worth noting that, when studying the correlation matrix (shown in Figure S1 in the Supplementary Materials), significant positive correlations of 0.2467 (p-value < 0.00001) and 0.4679 (p = 0.0367) were found between the SPAD value and HDM and RDM, respectively. Additionally, a positive relationship was found between both the HDM and RDM with a value of 0.3448 (p-value = 0.003). These results are indicative of a directly proportional relationship between the chlorophyll content (as measured by the SPAD value) and both the herbage dry matter (HDM) and root dry matter (RDM) production.

Table 2. A summary of the two-way ANOVAs assessing the effect of the application of the *Trichoderma* spp. isolates, the biofortification treatment, and their interaction on the growth values and fiber and nutrient contents of *Trifolium subterraneum* plants. The degrees of freedom (DFs) and the F-values, including the level of significance (* $p \leq 0{,}05$; ** $p \leq 0{,}01$; *** $p \leq 0{,}001$), when appropriate, are shown for each factor.

	***Trichoderma* (T)**	**Biofortification (B)**	**T ∗ B**
DF	5	3	15
Herbage dry matter (HDM) (mg)	0.87	1.78	4.59 ***
Root dry matter (RDM) (mg)	7.68 **	6.17 *	3.63 **
Rhizobia nodulation index	28.04 ***	7.55 *	1.48
SPAD measurement	15.00 **	0.53	8.78 **
Neutral detergent fiber (NDF) (%)	1.76	2.52	3.23 ***
Acid detergent fiber (ADF) (%)	0.92	5.44 *	3.81 ***
Acid detergent lignin (ADL) (%)	9.92 ***	14.22 ***	15.90 ***
Ashes (%)	9.71 ***	3.53	6.77 ***
Digestibility (DMD) (%)	0.92	5.44 *	3.81 *
Fe content (mg kg^{-1})	13.48 ***	4.09 *	2.11 *
Mg content (mg kg^{-1})	5.45 **	2.49	3.23 **
P content (mg kg^{-1})	22.33 ***	4.29	4.28 ***
Se content (µg kg^{-1})	11.91 ***	10.34 **	0.74
Zn content (mg kg^{-1})	9.55 ***	67.05 ***	2.20 *

Figure 2 shows the effects that the *Trichoderma* spp. inoculation and the biofortification treatment had on the rhizobia nodulation index. Inoculation with *Trichoderma* spp. isolates T09 and T18 (both identified as *Trichoderma gamsii*) led to a significant reduction in nodulation in subterranean clover plants compared to the negative control (Figure 2a). Regarding Zn application, the SF treatment significantly increased root nodulation in the treated *T. subterraneum* plants compared to the control and Soil treatments, although no significant difference was observed when compared to the foliar application (Figure 2b).

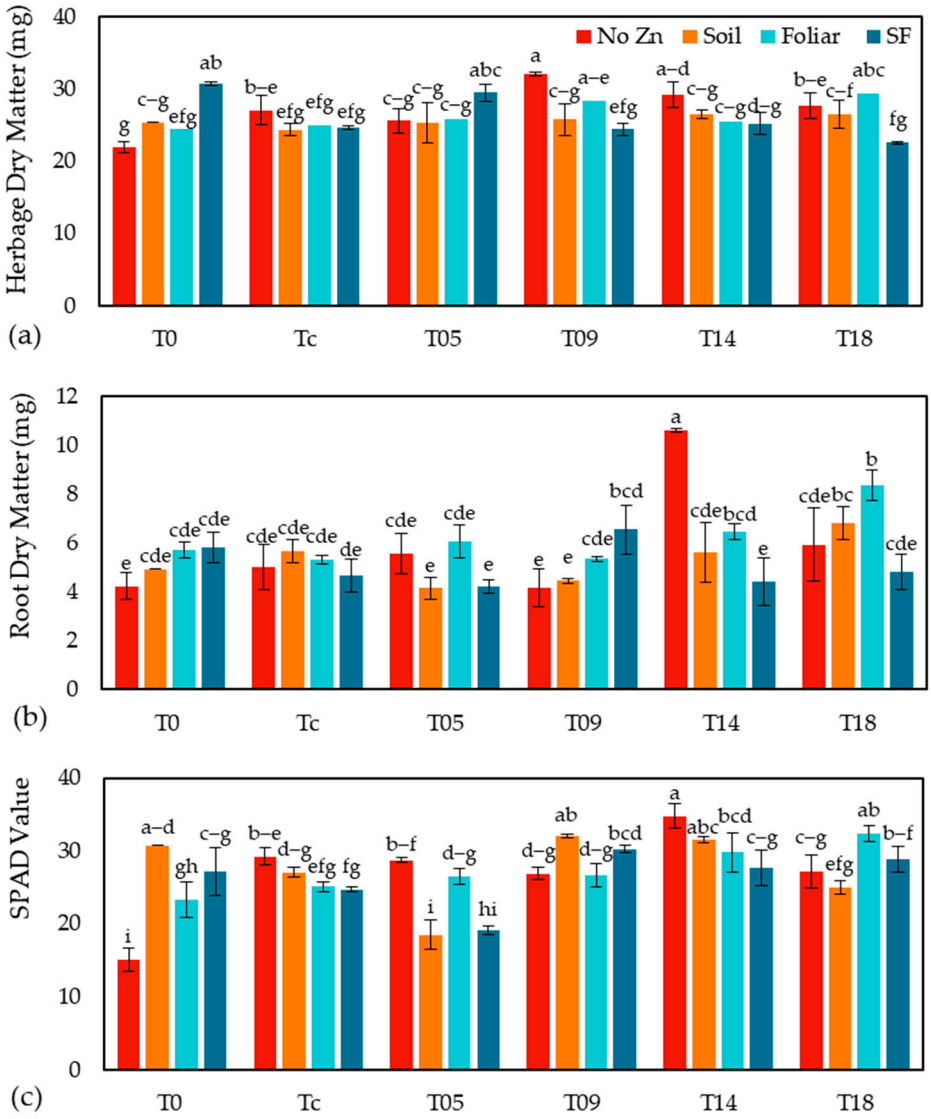

Figure 1. The effect of the interaction between the application of the *Trichoderma* spp. isolates and the biofortification treatment on (**a**) the herbage dry matter, HDM; (**b**) root dry matter, RDM; and (**c**) the SPAD value measured before the harvest of *Trifolium subterraneum* plants. The results are shown as the mean ± standard error of the mean (n = 3). Different letters indicate significant differences according to the Fisher's protected lsd (least significant difference) test at $\alpha = 0.05$.

Regarding the effect of the combined application of the *Trichoderma* isolates and biofortification treatments on the fiber content and digestibility, the results are presented in Table S1 in the Supplementary Materials. When reviewing the results, the acid detergent lignin (ADL) and ash percentages are of particular interest, since only the sole application of isolate T14 (*T. koningii*) resulted in a reduction in the ADF content and, consequently, a 27% improvement in subterranean clover digestibility compared to T0 with no Zn. In terms of the ADL content, different combinations of *Trichoderma* spp. inoculations and biofortification treatments significantly reduced this parameter compared to T0 with no Zn. The combinations included T05 and soil Zn, T09 and foliar treatments (both foliar and SF), T14 alone or combined with SF Zn, and all of the treatments incorporating isolate T18 (*T. gamsii*) except T18 with SF Zn. In any case, the lowest value was obtained with the sole application of Zn via soil. Regarding the ash content, a significant positive effect was more generalized, with most combinations of isolate inoculation and biofortification treatment significantly increasing the total content of minerals. Inoculation with isolate T14 (*T. koningii*) increased the ash content in all combinations with or without Zn compared

to T0 with no Zn. Again, the sole application of foliar treatments of Zn (both foliar and SF) significantly increased this parameter. Regarding the correlation between these parameters, there was a strong positive correlation between the NDF and ADF contents (0.8182, p-value < 0.0001) and a strong negative correlation between those parameters and the dry matter digestibility (−0.8182, p-value < 0.0001).

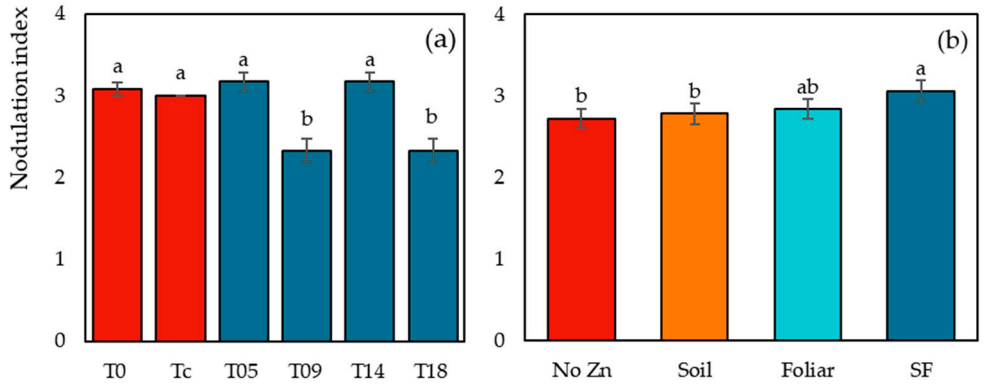

Figure 2. *Rhizobia* nodulation index ± standard error in *Trifolium subterraneum* plants as affected by the application of (**a**) the *Trichoderma* spp. isolates (control treatments in red and wild isolates in blue) and (**b**) the biofortification treatment. Different letters indicate significant differences according to the Fisher's protected lsd (least significant difference) test at $\alpha = 0.05$.

3.2. Influence on Plant Nutrient Accumulation in Trifolium subterraneum Plants

In terms of the nutrient content in *T. subterraneum* plants, Table 2 shows the significant effect of fungal inoculation on the accumulation of all determined nutrients. Additionally, the different Zn treatments significantly affected the contents of Fe, Se, and Zn, with no significant impact on the Mg and P contents. Moreover, the interaction between the application of the *Trichoderma* spp. isolates and the biofortification treatments had a significant effect on all nutrients studied, except for the Se content in *T. subterraneum* plants.

As shown in Figure 3, the effect of the interaction between the *Trichoderma* spp. isolate inoculation and biofortification treatments on the plant nutrient concentrations varied depending on the studied nutrient. Thus, compared to the negative control with no Zn (T0 and no Zn), the treatments generally increased Fe and Zn accumulation but decreased the Mg and P levels in the treated plants. All treatments involving T05 (*T. koningiopsis*) inoculation significantly increased the Fe content. In addition, the application of T05 when combined with Zn also significantly increased the levels of Zn on the *T. subterraneum* plants when compared to T0 with no Zn. Similar results were observed for the other isolates, where the combined applications of T14 (*T. koningii*) and Zn in any form significantly increased the concentrations of both Fe and Zn compared to the T0 control with no Zn. In this sense, regarding the application of T09 and T18 (both identified as *T. gamsii*), their combination with the Zn treatments significantly increased the uptake of Fe and Zn when compared to T0 with no Zn. However, exceptions were observed for the combinations of T09 with soil (which did not increase the Zn content) and T18 with SF (which did not significantly increase the Fe content).

The most effective strategy for Fe uptake involved the combination of soil Zn with T05 and T14, resulting in increases of 1.79 times and 1.36 times, respectively, compared to T0 and no Zn. Conversely, the combined treatment of soil and foliar Zn (SF) showed promising trends for Zn accumulation in plants, particularly when applied together with the inoculation of T09 (*T. gamsii*) or T14 (*T. koningii*). This combined approach significantly increased the Zn levels in the treated plants, surpassing those of the negative control with the same Zn treatment (T0 and SF), with remarkable increases of around 30% in both cases.

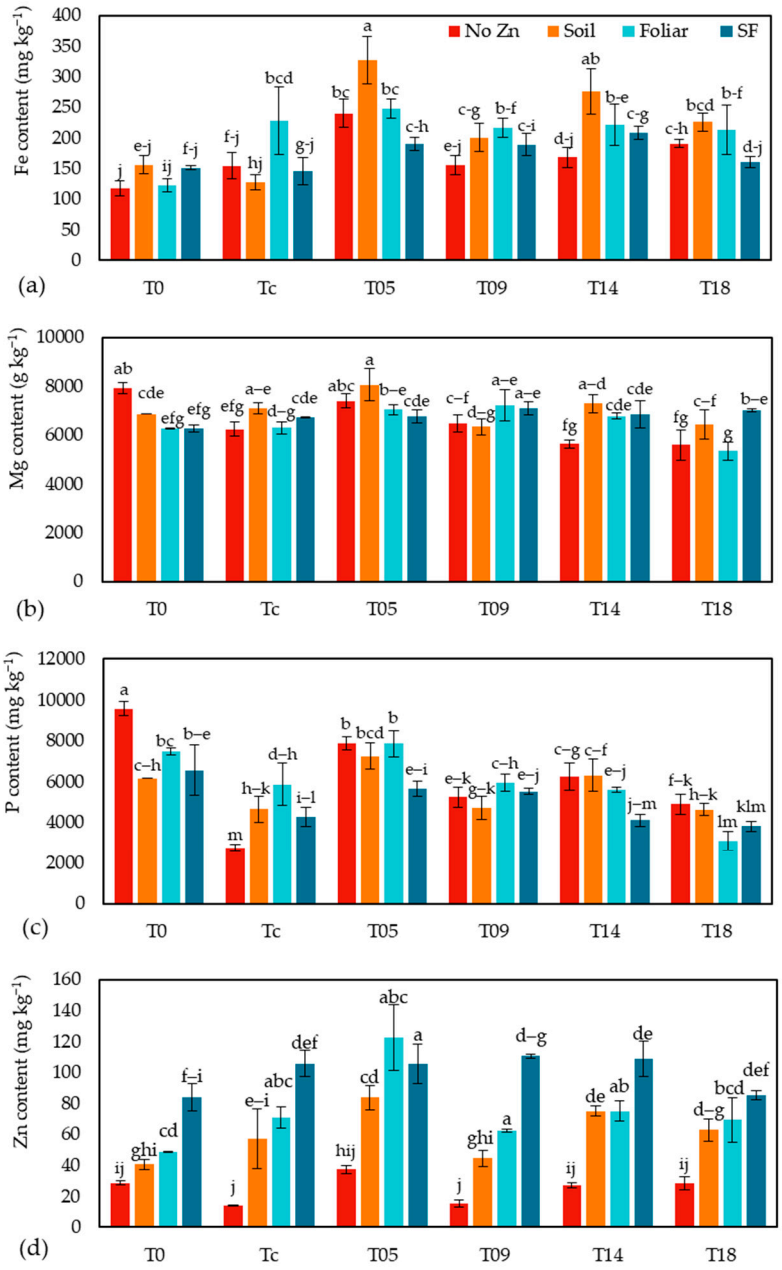

Figure 3. The effects of the interaction between the application of the *Trichoderma* spp. isolates and the biofortification treatment on the nutrient content, specifically (**a**) Fe, (**b**) Mg, (**c**) P, and (**d**) Zn, of the *Trifolium subterraneum* plants. The results are shown as the mean ± standard error of the mean (n = 3). Different letters indicate significant differences according to the Fisher's protected lsd (least significant difference) test at $\alpha = 0.05$.

Conversely, all of the combinations significantly reduced the P uptake in the treated plants, while the effect on the Mg content was less uniform, with different combinations of *Trichoderma* spp. isolates and Zn treatments, especially those involving the inoculation with T05 (*T. koningiopsis*), did not show any differences when compared to T0 with no Zn. A positive correlation between (0.4815, $p < 0.0001$) the Mg and P contents was found. However, the contents of both nutrients were negatively correlated with the SPAD value (−0.4225, *p*-value = 0.0002 and −0.4239, *p*-value = 0.0002, respectively). Additionally, the Mg content was negatively correlated with the HDM and RDM values (−0.3457, $p = 0.0029$ and −0.4454, *p*-value < 0.0001).

Figure 4 shows the effect that the main factors, the *Trichoderma* spp. inoculation and the biofortification treatment, had on the Se concentration in subterranean clover plants. Regarding the inoculation with the *Trichoderma* spp. isolates, only the plants treated with T18 (*T. gamsii*) showed a significant increase in Se concentration compared to T0, although without a significant difference compared to Tc. Regarding the biofortification treatments, while the soil application did not show a significant effect on the Se content, the foliar treatments (foliar and SF) significantly increased this parameter compared to no Zn and the soil Zn application.

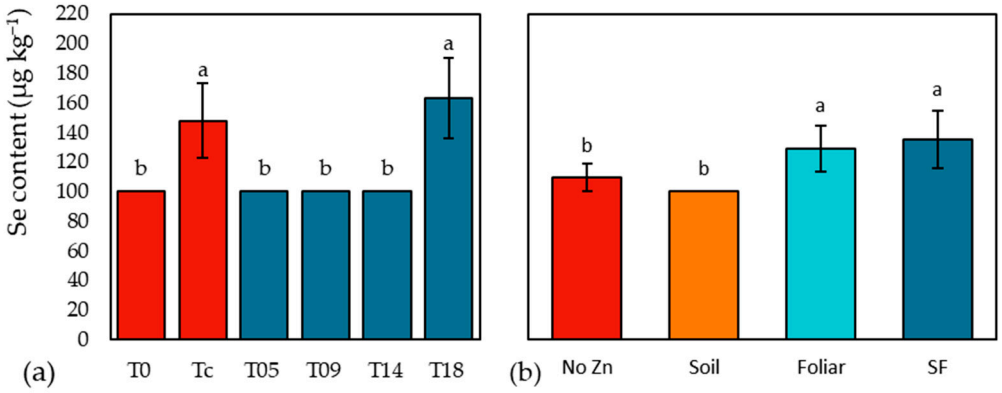

Figure 4. The Se content (µg kg^{-1}) ± standard error in the *Trifolium subterraneum* plants as affected by the application of (**a**) *Trichoderma* spp. isolates (control treatments in red and wild isolates in blue) and (**b**) the biofortification treatment. Different letters indicate significant differences according to the Fisher's protected lsd (least significant difference) test at $\alpha = 0.05$.

The results presented in Table 3 provide insights into the effects of *Trichoderma* spp. inoculation and the biofortification treatment on the soil bioavailable Zn concentration in the treated pots after the experiment. In general, in the absence of a Zn application, *Trichoderma* spp. inoculation decreased the available soil Zn content compared to the negative control (T0 and no Zn), with reductions ranging from 7.6% to 49.3% for T05 (*T. koningiopsis*) and the Tc, respectively. A similar trend was observed for the foliar application of Zn, where all of the isolates, except for T18 (*T. gamsii*), reduced the soil Zn content by percentages ranging from 15.7% to 46.8%. A mixed effect was observed for the soil application, where the inoculations with T05 (*T. koningiopsis*) and with T18 (*T. gamsii*) decreased the Zn bioavailability by almost 31% and 5%, respectively. The SF application only showed a slight decrease of 2% in the Zn concentration when the plants were inoculated with isolate T05 (*T. koningiopsis*), while the rest of the inoculations had a positive effect on Zn accumulation, with increases ranging from 2.3% to 33.7%.

Table 3. Soil DTPA–Zn content matrix following trials: interaction of *Trichoderma* spp. isolate application and Zn biofortification. Values are presented as mg kg^{-1} and relative variation compared to corresponding negative control (T0) for each biofortification treatment.

Biofortification	**No Zn**		**Soil (S)**		**Foliar (F)**		**SF**	
Trichoderma	**DTPA-Zn (mg kg^{-1})**	**Var. (%)**	**DTPA-Zn (mg kg^{-1})**	**Var. (%)**	**DTPA-Zn (mg kg^{-1})**	**Var. (%)**	**DTPA-Zn (mg kg^{-1})**	**Var. (%)**
T05	168.5	−7.6	430.4	−30.9	169.8	−21.6	583.0	−2.0
T09	105.9	−41.9	717.2	15.1	115.3	−46.8	741.9	24.8
T14	140.8	−22.8	723.3	16.1	178.1	−17.8	707.9	19.1
T18	127.3	−30.2	593.6	−4.7	229.8	6.1	608.2	2.3
Tc	92.4	−49.3	816.3	31.1	182.6	−15.7	794.8	33.7
T0	182.3	-	622.9	-	216.6	-	594.6	-

4. Discussion

In response to climate change and evolving agricultural policies, future farming systems, especially in ecosystems that are already vulnerable, such as dehesas [40], must prioritize both crop productivity and sustainability. To achieve this goal, harnessing the potential of beneficial microorganisms to complement other sustainable strategies like agronomic biofortification is gaining traction as a more environmentally friendly alternative [41,42].

This article should be taken as a first approximation that provides a comprehensive analysis of the combined effects of four wild *Trichoderma* spp. isolates, sourced from cherry tree fields in Extremadura (West Spain), and Zn biofortification on the growth, nutrient uptake, and overall status of subterranean clover (*Trifolium subterraneum*). This analysis highlights the intricate interactions between *Trichoderma* spp., Zn biofortification, and plant growth responses, emphasizing the importance of specific combinations for optimal outcomes. Further research, including field trials, should be carried out to verify the promising results obtained.

The positive results recorded, which may be attributed to the well-known Zn-solubilizing activities observed in many strains of this fungal genus in soil [43,44], support the initial hypothesis that the inoculation of these microbial isolates could enhance the bioavailability of applied Zn while simultaneously promoting plant growth. Additionally, the favorable results associated with using well-adapted microorganisms obtained from the same ecosystem where they would be inoculated, in conjunction with an essential legume for dehesas like *T. subterraneum*, show that this may be a promising alternative for enhancing the sustainability of this ecosystem by improving the quality of its pastures. In this regard, it is worth noting that inoculating dehesa soils with native *Trichoderma* spp. isolates through the application of myco-primed *T. subterraneum* seeds might facilitate the colonization of soils by these microorganisms and their long-term survival [45,46]. Therefore, one of the main goals of future studies should be to evaluate the survival rates of the chosen strains under field conditions. This approach could potentially lead to the naturalized presence of these beneficial *Trichoderma* spp. isolates in dehesas, thereby reducing the cost of biofortification treatments, especially after optimizing the dosage and method of Zn applications.

The consistency of three of the four isolates (T14, *T. koningii*; T09 and T18, both *T. gamsii*) in promoting the HDM and SPAD values in the absence of a Zn application aligns with previous studies on *Trichoderma's* multifaceted roles, which include promoting plant growth [47,48]. Similarly, this study highlights the potential of biofortification to not only improve the uptake of essential micronutrients but also to enhance plant growth, as supported by previous research [49,50]. The observed positive effect with the sole application of Zn (T0) may explain the increases in the SPAD values, as Zn is crucial for photosynthesis, with its deficiency leading to a 50% decrease in photosynthetic activity [51]. Furthermore, the role of Zn in increasing stomatal conductivity, facilitating sufficient water and CO_2 intake for sustained photosynthesis and nutrient absorption from the soil [52], might explain the observed increase in the HDM in the case of the SF application in the negative control (T0 and SF).

The combination of *Trichoderma* spp. with Zn biofortification had a beneficial effect on nutrient uptake by plants depending on the combination of isolates and biofortification methods, which agrees with findings from previous studies [53,54]. In particular, the results of the interaction between both factors revealed varying effects on the plant nutrient concentrations depending on the mineral studied. The treatments generally increased Fe and Zn accumulation but decreased the Mg and P levels in the treated plants compared to the negative control without Zn (T0 + no Zn). The general decrease in P uptake may be explained by the inhibitory effect of Zn on the root uptake and shoot accumulation of P, leading to a decrease in P uptake with increased Zn fertilization [55]. Meanwhile, studies have shown that increased Zn application can indeed lead to a decrease in Mg uptake by plants [56]. Additionally, the competition between Zn and Mg for uptake by plant roots can further contribute to the reduction in Mg uptake following Zn application [57].

This varying effect on nutrient uptake could have been affected by the combined effects of *Trichoderma* spp. with other beneficial microbes, such as the soil microbial consortium, as evidenced by studies such as that of Moradtalab et al. [58]. Considering the natural nodulation with Rhizobia recorded in the soil, the lack of a significant effect of the interaction between *Trichoderma* inoculation and Zn biofortification on this parameter could be due to the independent mechanisms through which *Trichoderma* spp. and Zn biofortification influence plant nodulation, as well as to the complex interactions among plants, microbes, and soil in biofortification processes [43]. As for the independent effect of both factors on nodulation, the inhibitory effect in the plants inoculated with T09 and T18 (both identified as *T. gamsii*) may be a result of the colonization dynamics in the rhizosphere, considering that nodulation success may be influenced by the timing and sequence in rhizosphere interactions between different microorganisms [59]. These results are consistent with a related study by Khan et al. [60], where some *Trichoderma* strains enhanced plant growth and nutrient uptake by promoting root development and nutrient solubilization, whereas others inhibited plant growth and nodulation. This underscores the potential influence of combining different beneficial microbes to enhance plant growth. On the other hand, the biofortification treatments did not show significant differences in nodulation compared to the plants of the negative control without added Zn (T0 and no Zn), except for the combination of soil and foliar Zn application, which notably increased root nodulation. This different impact from the biofortification treatments on nodulation could be due to the higher application rate in the SF treatment, as it has been identified in previous studies as one of the interacting factors between biofortification and nodulation [61].

In this sense, the combination of T05 and T14 (together with the positive control, Tc) with the soil Zn application, and the combination of T05 and T18 with the foliar application of Zn led to similar levels of Mg to those of the negative control without Zn (T0 and no Zn) being maintained. In the first case, these fungi did not affect the rhizobial nodulation index, and therefore, they could have modulated the soil nutrient dynamics together with microbial communities [62] and buffered the negative effect of the increased Zn concentration in the soil colloid on the Mg content. Conversely, in the cases of T05 and T18 (both *T. gamsii*), which significantly reduced rhizobial nodulation, the same buffering effect only occurred with foliar applications since the beneficial effect with the soil microbial consortium seemed to have been less important. This underscores the potential additive benefits of combining different beneficial microbes to enhance plant growth.

Additionally, the use of different wild strains of *Trichoderma*, which may have different abilities to promote plant growth and nutrient uptake, as well as the complexities of Zn uptake pathways in plants [63,64], may have contributed to the observed variability in the results. The same variable results, depending on the specific combination of fungal strain and biofortification treatment, were found by Marra et al. in their study on the biofortification of lentils [53]. At the same time, this result emphasizes the importance of the correct formulation and timing of the application of these biological products containing living microorganisms, as well as the importance of optimizing the application of inorganic Zn fertilizers, which can be costly and whose efficacy may vary depending on the plant being fertilized, rendering it insufficient as a standalone strategy for improving zinc deficiency and enhancing crop yield [65]. Thus, based on the preliminary results of our study, future experiments should consider the optimization of both the microbial inoculation and the Zn application conditions of the most promising combinations. In this regard, the results obtained by Anwar et al. [42] show that the application of *Trichoderma harzianum* together with copper as a biofortification strategy, using a lower dosage of the mineral, produced a higher accumulation of said nutrient in the treated wheat plants.

Notably, the combination of *T. koningiopsis* (T05) inoculation with Zn significantly increased the Fe levels, with similar results being observed for other isolates such as T14 (*T. koningii*). The most effective strategy for Fe uptake was the combination of soil Zn with T05 and T14, resulting in substantial increases compared to the control. In addition, the SF treatment, particularly in combination with the inoculation of T09 (*T. gamsii*) or T14

(*T. koningii*), significantly increased the Zn levels in the plants, surpassing those of the negative control that underwent the same Zn treatment. The combined treatment of Zn biofortification with *Trichoderma* spp. may have enhanced the nutrient uptake efficiency of subterranean clover. *Trichoderma* spp. can solubilize nutrients in the soil, making them more accessible to plants [64]. By inoculating subterranean clover with different *Trichoderma* spp., the plants may have experienced improved root colonization and nutrient mobilization, leading to increased Zn and Fe uptake. Furthermore, the interactions between *Trichoderma* spp. and plant roots can trigger systemic responses in plants, including the activation of defense mechanisms and stress tolerance pathways [66]. These responses can optimize nutrient uptake processes and mitigate the negative effects of Zn application on plant growth. The combined treatment of Zn biofortification and *Trichoderma* spp. inoculation may have induced a more robust plant defense and nutrient acquisition system, resulting in the observed increase in Zn and Fe uptake in subterranean clover.

Regarding Se uptake, the different effects of the biofortification treatments may be explained by prior studies that have shown that foliar Zn application can notably impact the Se content in plants [21,67], whereas the positive effect of isolate T18 (*T. gamsii*) may be related to a potential ability to mobilize this nutrient more effectively than the other isolates.

The observed reduction in the soil bioavailable Zn content when *Trichoderma* spp. was inoculated without a Zn application, as well as with a foliar application, could be attributed to the ability of *Trichoderma* to alter soil conditions [68]. It has been reported that *Trichoderma* spp. promotes the solubilization of complex compounds by stimulating the secretion of organic acids from plant roots, leading to a significant decrease in the soil pH [69]. This change in soil pH may have influenced the availability and mobility of the Zn that is naturally present in the soil, as none of these plants received additional Zn from the soil, potentially resulting in decreased Zn levels in the soil.

On the other hand, when Zn was applied to the soil (soil and SF) in combination with *Trichoderma* inoculation, a general positive effect on bioavailable Zn accumulation was observed, especially for T09 (*T. gamsii*) and T14 (*T. koningii*). This could be due to the beneficial effects of soil Zn applications and *Trichoderma* inoculation on plant nutrient uptake, which may have facilitated the uptake and translocation of Zn within the plant, leading to increased Zn accumulation despite the initial reduction in the soil Zn content. Overall, the contrasting effects of *Trichoderma* inoculation and Zn application on the soil Zn content highlight the complex interactions between microbial activity, nutrient availability, and plant responses. Further research into the specific mechanisms underlying these interactions, such as changes in the soil pH, nutrient solubilization, and plant–microbe interactions, could provide valuable insights into optimizing agricultural practices for improved nutrient uptake and biofortification.

5. Conclusions

In conclusion, this study highlights several key findings that contribute to our understanding of the beneficial effects of *Trichoderma* spp. inoculation and Zn biofortification on plant growth and nutrient uptake. Firstly, the combined application of *Trichoderma* spp. and Zn biofortification led to increased Fe and Zn accumulation in plants, demonstrating the potential of these strategies to enhance nutrient uptake efficiency. However, different *Trichoderma* strains exhibited variable effects on plant growth and nodulation, emphasizing the importance of strain selection in agricultural practices. While some strains (T05, *T. koningiopsis* and T14, *T. koningii*) promoted plant growth and nutrient uptake, others (T09 and T18, both identified as *T. gamsii*) inhibited nodulation.

Moreover, this study revealed the complex interactions between *Trichoderma* spp., Zn biofortification, and soil dynamics, leading to variable outcomes depending on factors such as soil conditions and application methods. These results suggest that optimization opportunities exist to maximize the benefits of microbial inoculants and biofortification in sustainable agriculture. Additionally, integrating locally adapted microorganisms like

Trichoderma spp. with Zn biofortification presents a promising approach to enhance crop productivity and sustainability, particularly in vulnerable ecosystems like dehesas. To this end, their long-term survival may be a key factor that needs to be taken into account in future studies.

In summary, these findings underscore the potential of combining beneficial microorganisms with biofortification strategies to address nutrient deficiencies and improve agricultural sustainability. However, further research is needed to elucidate the underlying mechanisms and optimize application strategies to maximize these benefits.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/su16093730/s1, Table S1. Effect of the interaction between the application of the *Trichoderma* spp. Isolates and the biofortification treatment on fiber content, measured through Neutral Detergent Fiber (NDF) Acid Detergent Fiber, Acid Detergent Lignin (ADL) and the content of total ashes. Additionally, the percentage of digestibility of the treated plants is shown; Figure S1. Correlation Matrix showing the significant Pearson correlations ($p \leq 0.05$) between parameters investigated in the study. The robustness of the correlations is depicted using a gradation of colors, with darker colors indicating stronger correlations.

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