






Article

A Spray Foliar Containing *Methylobacterium symbioticum* Did Not Increase Nitrogen Concentration in Leaves or Olive Yield Across Three Rainfed Olive Orchards

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Abstract: Biological nitrogen (N) fixation has been advocated in agricultural fields due to being considered a more sustainable way to introduce N into agrosystems than industrial N fertilizers. In this study, a foliar spray inoculant containing the microorganism *Methylobacterium symbioticum* was applied. This microorganism is known for fixing N in the phyllosphere, regardless of the cultivated species. This study was conducted in three rainfed olive orchards over three years. In two orchards managed according to European Union (EU) integrated production rules, the experiment was organized as a factorial design with inoculant (applied at two levels, yes and no) and N fertilization (applied to the soil at three levels, 0, 40, and 80 kg ha⁻¹ of N). The third trial, managed according to EU organic farming rules, was organized in a completely randomized design with three treatments: with (yes) and without (no) inoculant and with a treatment involving a seaweed extract, also for foliar application. The microbiological inoculant did not consistently influence olive yield or N concentration in leaves across the three trials. Conversely, N application to the soil significantly influenced N concentration in leaves and olive yield. In one of the trials, in the third year of the study, soil N application (80 kg ha⁻¹) resulted in an olive yield of ~eight times higher than the unfertilized control treatment. The seaweed extract also did not lead to significant differences in leaf mineral composition or olive yield compared with the other treatments. These findings from the on-farm research highlight the importance of accurately determining the conditions under which commercial products can deliver effective results. It is crucial to acknowledge that these products involve expenses not only in their acquisition but also in their application.

Keywords: plant biostimulant; *Olea europaea*; nitrogen fertilization; organic farming; integrated production; seaweed extract; biological nitrogen fixation



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1. Introduction

Some prokaryotic microorganisms from the Bacteria and Archaea domains can fix atmospheric dinitrogen, as they have the nitrogenase enzyme complex [1]. Plants can indirectly access fixed N after being released into the environment or by establishing symbiotic

associations with these microorganisms [2]. The atmosphere is an inexhaustible source of elemental N, representing approximately 78% by volume of all gases [3]. Thus, unlike the raw materials for the manufacture of phosphorus (P) and potassium (K) fertilizers, there is no risk of the resource running out, although the manufacturing of N fertilizers by the Haber–Bosch process continues to have a very high energy consumption [1]. The energy demands of manufacturing N fertilizers, along with reduced crop N use efficiency [4] and the environmental impacts of N losses from fields [4,5], underscore the importance of promoting biological N fixation for sustainable agriculture [3–7].

In recent decades, significant research effort has been dedicated to nodulated legumes. Due to the protection provided by the host plant to the microorganisms, they exhibit a high N fixation capacity [1], potentially eliminating the need for N fertilization [2]. Other N-fixing systems have also been strongly promoted in agricultural fields, such as the symbiotic relationship between the aquatic fern of the genus *Azolla* and the cyanobacterium *Anabaena azollae* in rice paddies [8,9]. Increasing importance has also been attributed to the N-fixing capability of non-nodule-producing and endophytic N-fixing bacteria in tropical grasses such as sugarcane (*Saccharum officinarum* L.) [8,10]. N-fixing microorganisms that live freely in the soil or on plant surfaces are also gaining increasing attention, although they typically exhibit lower fixation capacity [11,12].

Recently, it has been suggested that a microorganism capable of establishing itself in the leaf surface or phyllosphere may exhibit a high N fixation capacity, regardless of the host plant [13,14]. The microorganism was isolated from spores of *Glomus iranicum* var. *tenui-hypharum* and classified as *Methylobacterium symbioticum* sp. nov. (strain SB0023/3T) [15]. Various other microorganisms of the genus *Methylobacterium* are found in diverse habitats, including soil, water, and plants [16,17], and some of them are also recognized for their N-fixing capabilities [18]. The possibility of applying a microorganism to the phyllosphere through foliar spray, which can establish itself regardless of the cultivated species and prevailing environmental conditions and fix N in quantities significant to the agroecosystem, would be revolutionary for agricultural systems worldwide. However, studies on *M. symbioticum* applied to lettuce (*Lactuca sativa* L.) [19], potted olive trees (*Olea europaea* L.) [20], and maize (*Zea mays* L.) [21] have shown that, while N fixation can be detected, the amounts that are fixed are comparable with those of free-living N fixers. These findings hardly justify using the commercial product as an external input by farmers. Nevertheless, the relevance of the topic warrants further studies to better assess the actual N-fixing capabilities of this microorganism.

Current agriculture urgently requires such products to reduce the reliance on inputs with significant environmental impacts, such as N fertilizers. Olive cultivation in the Mediterranean basin is also facing considerable challenges due to climate change, driving the need to adjust cultivation practices to enhance sustainability [22,23]. Alongside the use of legumes as cover crops to boost N levels in the agrosystem, various commercial products have been promoted, particularly those categorized as plant biostimulants, including seaweed extracts [24–27]. Although these products contain only trace amounts of nutrients, they can support plants in managing environmental stresses and enhancing nutrient use efficiency [26–28].

In this study, a commercial inoculant containing *M. symbioticum* was applied in three mature olive groves using the growers' standard spray equipment, simulating real-world application conditions through on-farm research. Two of the olive groves are managed according to European integrated production standards (Regulation (EU) 2021/2115), and one is managed according to European organic farming rules (Regulation (EU) 2018/848). The first two trials were arranged in a factorial design, with two levels of inoculant application (yes and no) and three levels of mineral N application (0, 40, and 80 kg ha⁻¹). In

the organic olive grove, the experiment was arranged in a completely randomized design with three treatments: application of the inoculant, application of an algae-based foliar spray authorized for organic farming, and an unfertilized control. By conducting these three trials over three years and simulating various nutritional conditions for the trees, the main hypothesis is that the microbial inoculant and seaweed extract have a measurable and significant effect on the trees' mineral nutrition and/or olive yield.

2. Materials and Methods

2.1. Characterization of the Experimental Plots

The field experiments were conducted in the municipality of Mirandela in North-eastern Portugal. The region features a Mediterranean climate, with an average annual air temperature of 14.3 °C and annual precipitation of 508.6 mm. Monthly records of air temperature and precipitation and climatological normal values (1981–2010) are shown in Figure 1.

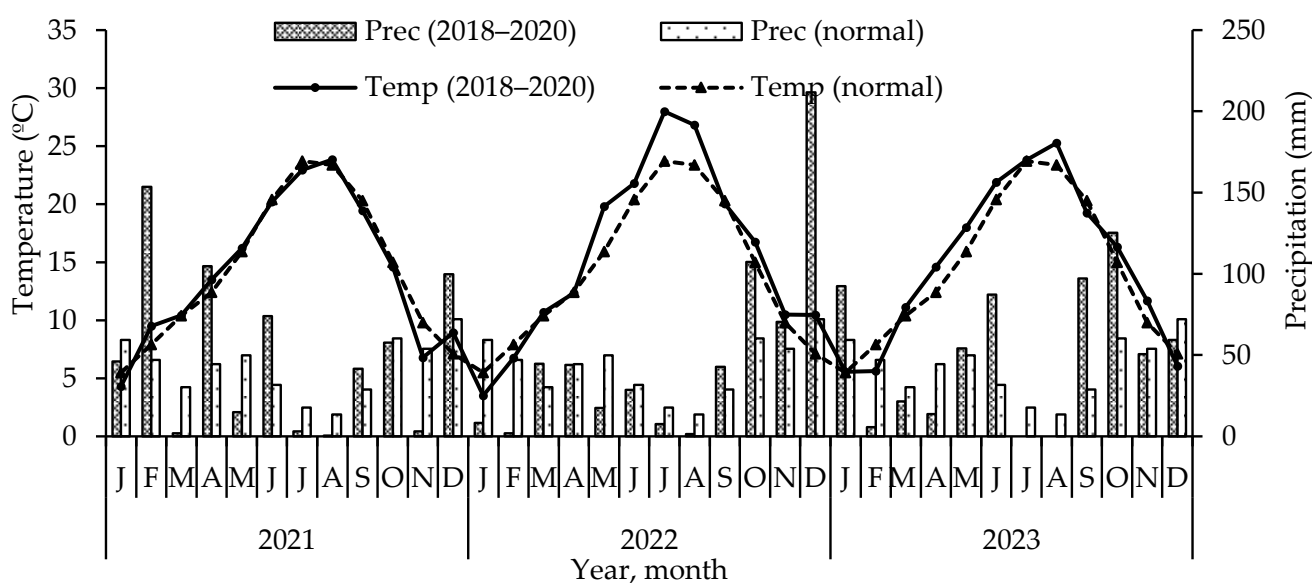


Figure 1. Climatological normal values (1981–2010) for Mirandela and monthly records of average air temperature and precipitation during the experimental period.

The trials were conducted in three olive groves of the cv. Cobrançosa, the most widely grown in the region. The trees were planted in a 7 m × 7 m spacing, totaling 204 trees per hectare. One of the trials, henceforth referred to as Valongo, is located at Valongo farm (41.513880, −7.187417), in the parish of Carvalhais. This grove consists of mature trees aged 40 years. Before the trial installation, the soil was managed with conventional tillage, and the trees received annual fertilization with approximately 40 kg ha^{−1} of N, P₂O₅, and K₂O, applied as a 10:10:10 compound fertilizer. Another trial was established in a grove with 25-year-old trees in the village of Marmelos (41.439850, −7.217300). This trial, named Marmelos Prodi hereafter, has been managed by clearing natural vegetation using an interrow shredder in the spring. Before the trial installation, the olive grove was managed with low-intensity practices and rarely received mineral fertilizers to the soil, although foliar sprays were sometimes applied. The third trial was in a 25-year-old olive grove (41.439714, −7.215640) in the same locality, henceforth referred to as Marmelos Bio. The soil of this grove was managed by clearing natural vegetation once a year at the end of spring. It has been handled according to the organic farming rules of the European Union. No fertilizers have been applied to the soil, but occasionally, foliar fertilizers authorized for organic farming were used.

The soils of the olive groves are all Leptosols [29] derived from schist. Before the trials began, three composite soil samples were collected from each olive grove. The results of the analyses conducted on these samples are presented in Table 1.

Table 1. Soil properties (average \pm standard deviation, $n = 3$) determined from composite soil samples (10 cores per composite sample) taken at a 0–0.20 m depth at the beginning of the field experiments.

Soil Properties	Valongo	Marmelos Prodi	Marmelos Bio
¹ Organic carbon (g kg ⁻¹)	6.92 \pm 1.14	5.94 \pm 0.14	6.16 \pm 0.99
² Total nitrogen (g kg ⁻¹)	0.30 \pm 0.04	0.29 \pm 0.04	0.31 \pm 0.02
³ pH (H ₂ O)	5.61 \pm 0.17	5.45 \pm 0.15	5.48 \pm 0.25
⁴ Extract. phosphorus (mg kg ⁻¹ , P ₂ O ₅)	56.05 \pm 8.86	57.33 \pm 3.04	63.33 \pm 6.81
⁴ Extract. potassium (mg kg ⁻¹ , K ₂ O)	115.33 \pm 29.09	147.67 \pm 24.50	138.67 \pm 21.08
⁵ Exchang. calcium (cmol _c kg ⁻¹)	3.49 \pm 0.69	2.49 \pm 0.56	2.53 \pm 0.40
⁵ Exchang. magnesium (cmol _c kg ⁻¹)	0.60 \pm 0.18	0.43 \pm 0.04	0.42 \pm 0.07
⁵ Exchang. potassium (cmol _c kg ⁻¹)	0.26 \pm 0.07	0.29 \pm 0.03	0.31 \pm 0.02
⁵ Exchang. sodium (cmol _c kg ⁻¹)	0.35 \pm 0.09	0.26 \pm 0.02	0.24 \pm 0.02
⁶ Exchang. acidity (cmol _c kg ⁻¹)	0.33 \pm 0.06	0.30 \pm 0.00	0.27 \pm 0.06
⁷ CEC (cmol _c kg ⁻¹)	5.03 \pm 0.88	3.77 \pm 0.57	3.76 \pm 0.40
⁸ Sand	78.37 \pm 1.92	74.87 \pm 2.97	73.90 \pm 3.17
⁸ Silt	15.37 \pm 1.01	15.07 \pm 2.14	15.67 \pm 2.00
⁸ Clay	6.27 \pm 0.99	10.07 \pm 2.10	10.43 \pm 1.17
⁹ Texture	Loamy sand	Sandy loam	Sandy loam

¹ Wet digestion (Walkley–Black); ² Kjeldahl method; ³ potentiometry; ⁴ ammonium lactate; ⁵ ammonium acetate; ⁶ potassium chloride; ⁷ cation exchange capacity; ⁸ Robinson pipette method; ⁹ USDA, United States Department of Agriculture (refer to Section 2.4 for further details on the analytical procedures).

2.2. Experimental Designs and Fertilizing Materials

The Valongo and Marmelos Prodi trials were arranged using the same experimental design. They are organized in a factorial design with two factors: inoculant, with two levels of application (yes and no), and mineral N fertilization at three levels (0, 40, and 80 kg ha⁻¹). The Valongo trial had eight replicates, each consisting of an individual tree. The trial included 48 marked trees (2 inoculants \times 3 N rates \times 8 replicates). In the Marmelos Prodi trial, three replicates were included, each consisting of a group of three uniform trees. Thus, for this trial, 54 trees were marked (2 inoculants \times 3 N rates \times 3 replicates \times 3 trees). The Marmelos Bio trial was organized in a completely randomized design with three treatments: application of the inoculant (yes), application of a commercial seaweed extract as a foliar spray (foliar), and unfertilized control (no). In this trial, 3 replicates of three uniform trees were included, resulting in 27 marked trees.

As a N fertilizer for soil application, ammonium nitrate 27% N (50% NH₄⁺, 50% NO₃⁻) was used. The fertilizer was manually broadcast under the canopy projection in the last week of March. It was applied at rates of 40 and 80 kg ha⁻¹ of N, with a third treatment receiving no fertilizer. Each tree received a fraction of the fertilizer based on the number of trees per hectare (204) and the N concentration in the fertilizer (27%).

The inoculant used was a commercial product that contains 3×10^7 colony-forming units (CFU g⁻¹) of *M. symbiotic*. The foliar spray was prepared according to manufacturer specifications using 333 g ha⁻¹, diluted in 200 L of water, and applied once a year in the spring (3 June 2021; 20 May 2022; and 26 May 2023). Each tree received a fraction of the spray equivalent to 1/204, based on the number of trees per hectare.

As a foliar spray, a commercial algae extract was used. It is a pure extract (100% fresh matter) of seaweed (*Ascophyllum nodosum*) obtained through cold extraction. Three annual applications were performed, with the first coinciding with microbial inoculant application, followed by two more during the growing season, approximately one month apart. A concentration of 3 L ha⁻¹ was used as recommended by the manufacturer.

2.3. Crop Management

The farmers applied the foliar sprays using their own equipment. The groups of trees designated to receive the inoculant were positioned 40 m apart from other groups to minimize the risk of contamination. The sprays were applied in the early morning on days with low wind speed.

The soil management in the olive groves remained unchanged with the implementation of the experimental protocols. Therefore, in the Valongo olive grove, the soil was maintained with annual tillage, carried out in the first half of April. The soils in the Marmelos Prodi and Marmelos Bio trials were maintained with natural vegetation and shredding once a year in the first half of May.

During the experimental period, the marked trees were subjected to annual light pruning, removing less than 15% of the branch mass (assessed visually) during each pruning event.

2.4. Sampling and Laboratory Analyses

Before implementing the experimental designs, soil samples were taken for the initial characterization of the plots. In each plot, three composite samples (each consisting of 10 sub-samples) were randomly collected from the experimental area at a 0–0.20 m depth. These samples were oven-dried at 40 °C and sieved through a 2 mm mesh.

In the samples, the following analyses were conducted according to the methods compiled by van Reeuwijk [30]: soil separates using the pipette method (cap 3), $\text{pH}_{(\text{H}_2\text{O})}$ by potentiometry (cap 4), organic carbon (C) by the Walkley–Black method (cap 5), total N by the Kjeldahl method (cap 6), exchangeable bases by the ammonium acetate method (cap 9), and exchangeable acidity by the potassium chloride method (cap 11.1). Extractable P and K were determined by the Égner–Riehm method, with an extractant solution of 3.5 M ammonium lactate, pH 3.75 [31].

In July, during endocarp sclerification, and in December, during the resting period of the olive trees, leaf samples were collected for elemental composition analysis. Leaves were harvested from the middle third of one-year-old non-bearing shoots throughout the canopy. In the laboratory, leaves were oven-dried at 70 °C until they were at a constant weight, and they were then ground in a mill with a 1 mm mesh size. Ten minerals were analyzed. N concentration was determined using the Kjeldahl method [30]. P concentration was determined by colorimetry; K concentration was determined by atomic emission spectroscopy; and calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn) concentration were determined by atomic absorption spectroscopy [32]. Tissue samples (0.25 g) were digested using microwave digestion equipment with 10 mL of 65% (*w/v*) nitric acid at 180 °C for 30 min. Subsequently, distilled water was added to adjust the final volume to 50 mL. Boron (B) was determined by colorimetry after dry-ashing digestion in the presence of CaO [32].

The olives were harvested every year in November. Trunk-shaker machines with an attached inverted umbrella were used for harvesting. After each experimental unit (1 or 3 trees, depending on the experimental design) was harvested, the olives were discharged onto a tarp on the ground and then transferred into appropriately sized plastic containers for weighing.

2.5. Data Analysis

The data were first tested for normality and homogeneity of variances using the Shapiro–Wilk test and Bartlett’s test, respectively. The effects of the treatments were compared using one-way ANOVA (Marmelos Bio) and two-way ANOVA (Valongo and Marmelos Prodi) tests with the statistical software SPSS Statistics (version 25, IBM SPSS,

Armonk, NY, USA). In factors with more than 3 levels, means with significant differences were separated by the multiple range Tukey HSD test ($\alpha = 0.05$).

3. Results

3.1. Field Trial of Valongo Farm

In the 2021 Valongo trial, the average olive yield exceeded 20 kg tree⁻¹, with no significant differences observed between treatments (Figure 2). However, the olive yield in 2022 was minimal and below that of a normal year, and the yield in 2023 was also poor. Significant differences were observed between untreated trees and those treated with inoculant in both the 2022 and 2023 years, and in 2023, there were also differences when evaluating the effects of mineral N application. However, cumulative total olive yields of the three years showed no significant variation with either inoculant application or N fertilization.

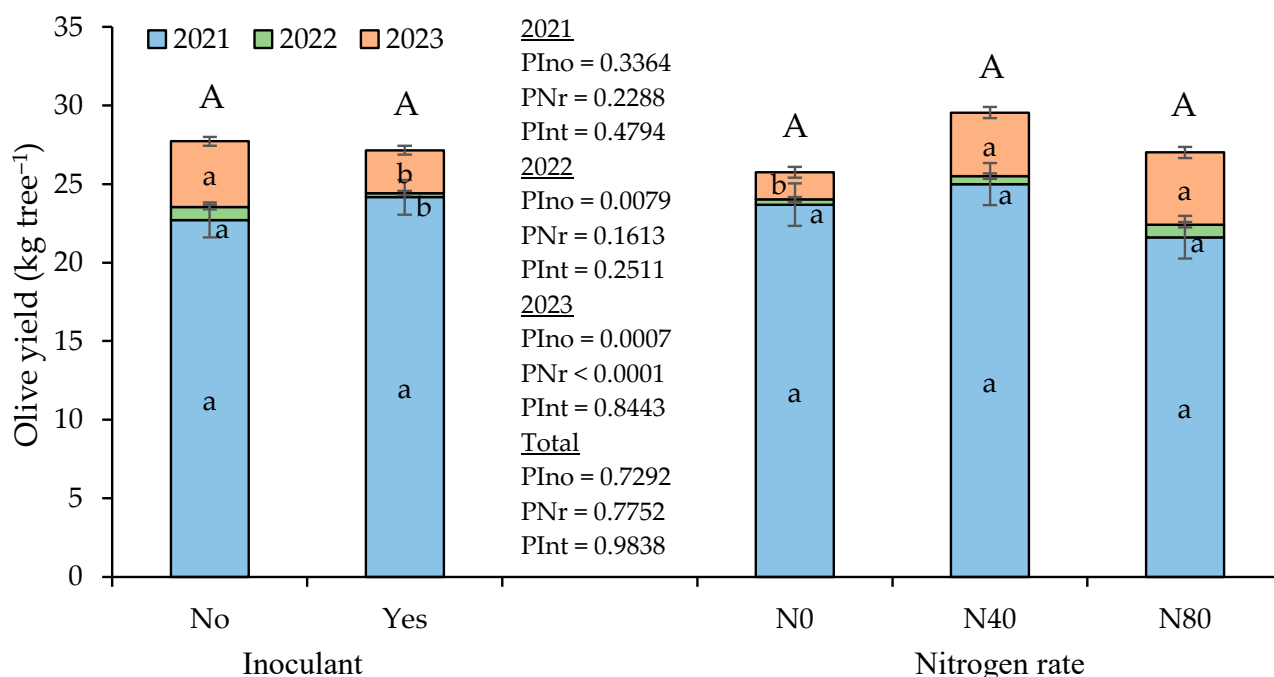


Figure 2. Olive yield in the Valongo olive grove according to the factorial design of inoculant (not applied (No) and applied (Yes)) and nitrogen rate (0 (N0), 40 (N40), and 80 (N80) kg ha⁻¹). Separated by inoculant and N rates, means followed by the same letter (lowercase for each year; uppercase for accumulated total) are not significantly different according to Tukey's USD test ($\alpha = 0.05$). The probability of interaction provided as PI_{Int}. Line segments above the bars represent the standard errors.

The leaf N concentration exhibited a limited response to inoculant application, with average values not being significantly different across the first four samplings (Figure 3a). However, in January 2023, trees treated with the inoculant exhibited significantly higher values. In contrast, applying mineral N to the soil strongly influenced leaf N concentration, with significant treatment differences being noted at four out of five sampling dates (Figure 3b). The N0 treatment consistently showed lower values compared with fertilized treatments.

The concentration of other minerals in the leaves, namely P, K, Ca, Mg, B, Fe, Mn, Cu, and Zn, did not vary significantly and/or consistently with inoculant application or mineral N application to the soil.

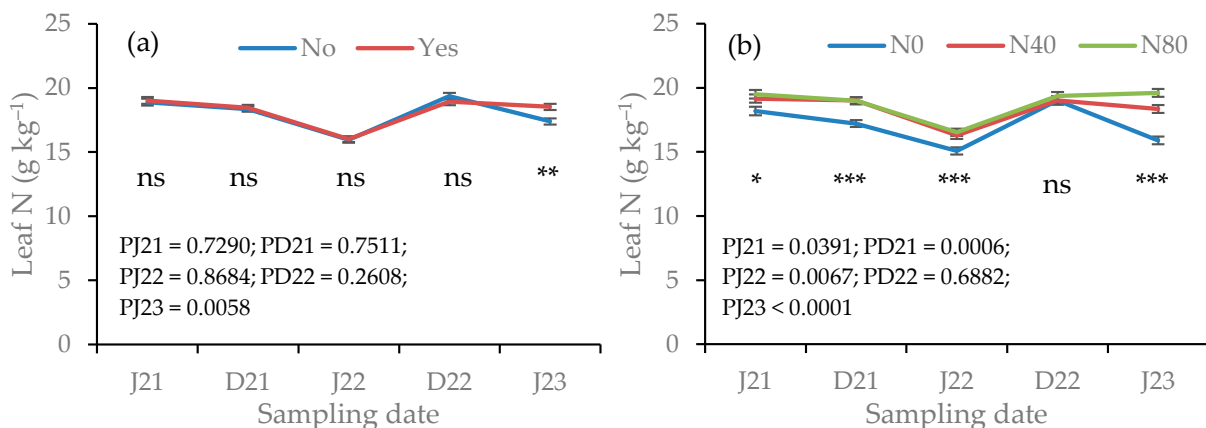


Figure 3. Leaf nitrogen (N) concentration in the Valongo olive grove according to the factorial design: (a) Inoculant (not applied (No) and applied (Yes)), (b) N rate (0 (N0), 40 (N40), and 80 (N80) kg ha⁻¹) across five samplings performed in January (J) and December (D) of 2021, 2022, and 2023. Significance levels are denoted as ns (not significant), * (significant at $p < 0.05$), ** (significant at $p < 0.01$), and *** (significant at $p < 0.001$). Error bars represent the standard errors.

3.2. Field Trial of Marmelos Prodi

Applying inoculant had a limited effect on olive yield (Figure 4). In 2023, the most productive year, olive yield did not vary significantly between treatments, nor did the accumulated total production. However, in 2022, a year characterized by low productivity, trees treated with inoculant exhibited a significantly higher average yield than untreated trees. In contrast, mineral N application to the soil pronouncedly affected olive yield. Significant differences emerged in 2022, which intensified in 2023 and strongly influenced the accumulated total production. Trees in the N80 treatment yielded more olives than those in the N40 treatment, and trees in the N40 treatment yielded more than those in the N0 treatment.

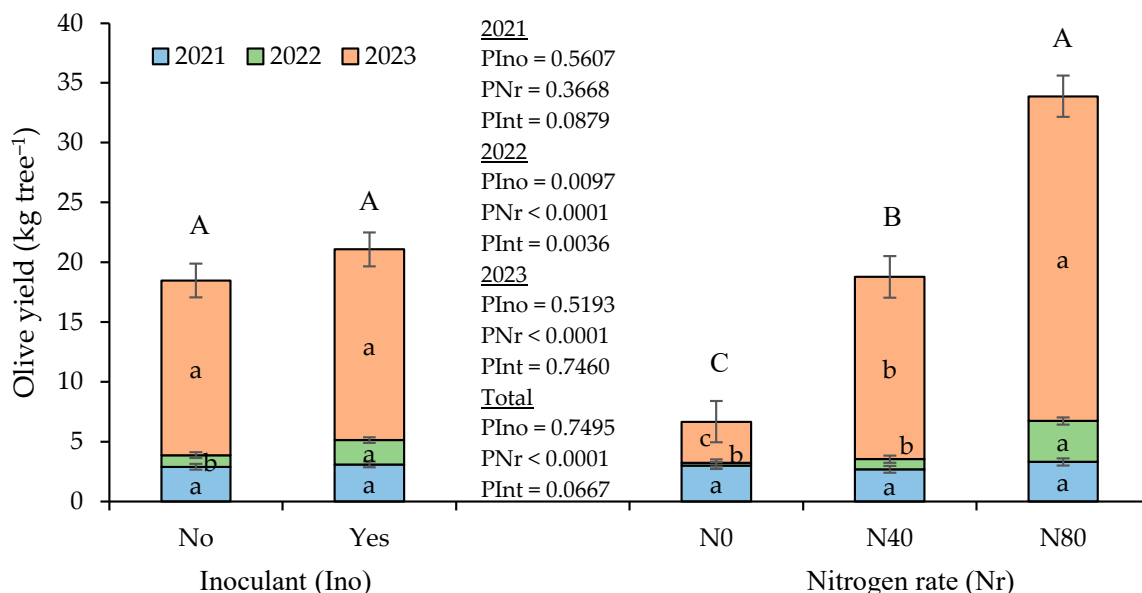


Figure 4. Olive yield in the Marmelos Prodi olive grove according to the factorial design of inoculant (not applied (No) and applied (Yes)) and nitrogen rate (0 (N0), 40 (N40), and 80 (N80) kg ha⁻¹). Separated by inoculant and N rates. Means followed by the same letter (lowercase for each year; uppercase for accumulated total) are not significantly different according to Tukey’s USD test ($\alpha = 0.05$). The probability of interaction provided as PInt. Line segments above the bars represent the standard errors.

The N concentration in the leaves tended to be slightly higher in trees that did not receive inoculant than those that were treated, with significant differences observed on one of the sampling dates (Figure 5a). The application of mineral N to the soil significantly impacted the N concentration in the leaves, with the values for the N0 treatment being significantly lower than those for the treatments that received N in the soil on all sampling dates (Figure 5b). The mean values for the N80 treatment tended to be higher than those for the N40 treatment. In the N0 treatment, the mean values were below 16 g kg^{-1} . There was also a clear trend towards higher values in the December samplings compared with summer ones.

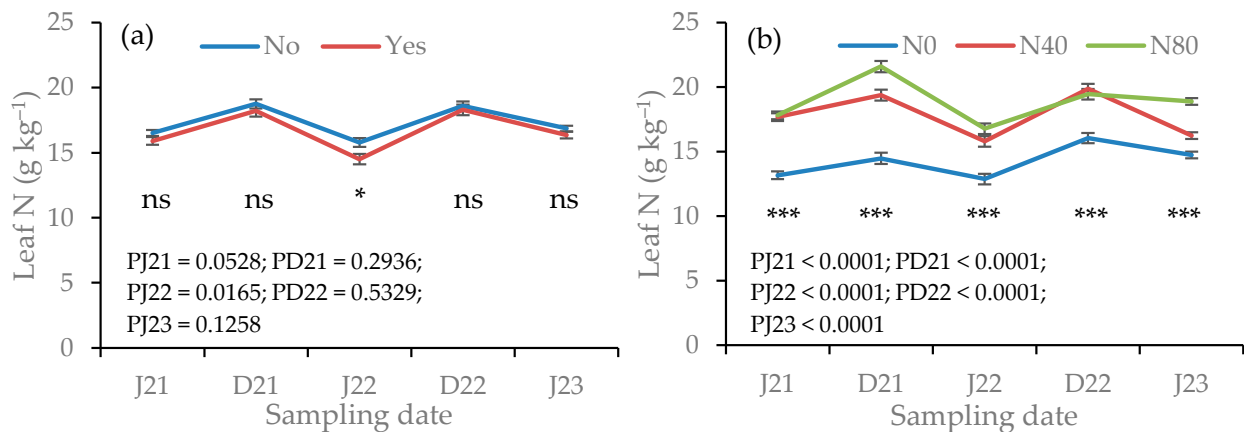


Figure 5. Leaf nitrogen (N) concentration in the Marmelos Prodi olive grove according to the factorial design: (a) inoculant (not applied (No) and applied (Yes)), (b) N rate (0 (N0), 40 (N40), and 80 (N80) kg ha^{-1}) across five samplings performed in January (J) and December (D) of 2021, 2022, and 2023. Significance levels are denoted as ns (not significant), * (significant at $p < 0.05$), and *** (significant at $p < 0.001$). Error bars represent the standard errors.

The P concentration in the leaves was not significantly affected by the application of the inoculant (Figure 6a). However, N mineral fertilization appears to have influenced the P concentration in the leaves (Figure 6b). The p levels in the N0 treatment tended to be higher than the fertilized treatments, with significant differences observed between treatments at two sampling dates. Overall, the mean values ranged from 1.2 to 1.9 g kg^{-1} .

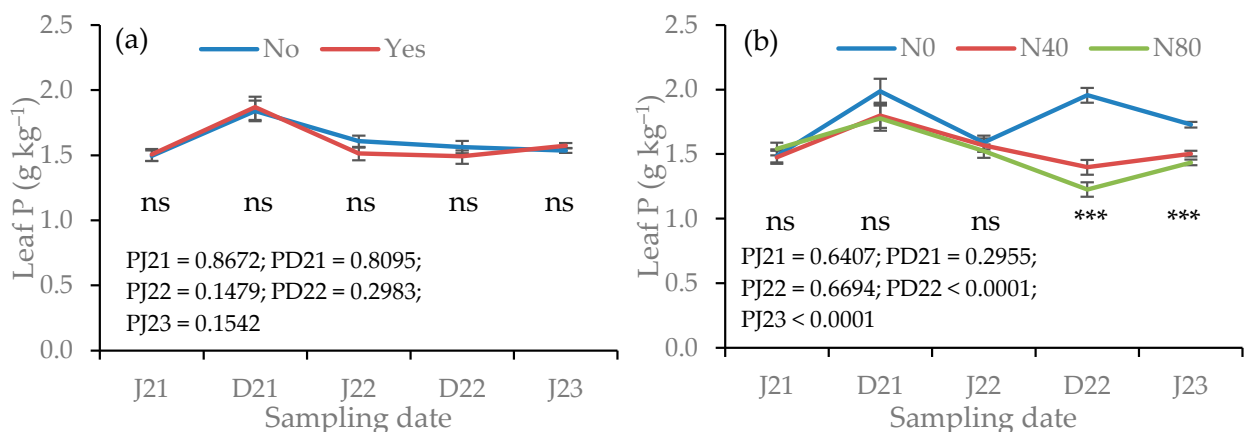


Figure 6. Leaf phosphorus (P) concentration in the Marmelos Prodi olive grove according to the factorial design: (a) inoculant (not applied (No) and applied (Yes)), (b) nitrogen rate (0 (N0), 40 (N40), and 80 (N80) kg ha^{-1}) across five samplings performed in January (J) and December (D) of 2021, 2022, and 2023. Significance levels are denoted as ns (not significant), and *** (significant at $p < 0.001$). Error bars represent the standard errors.

The K concentration in the leaves also did not vary significantly with the application of the inoculant (Figure 7a). However, the mean values of leaf K concentrations exhibited a slight tendency to be higher in the N0 treatment, with differences observed between the fertilized treatments at the final sampling date. The K levels showed a noticeable fluctuation with the sampling date, which was higher in the July samples than in the December samples. Furthermore, the mean values consistently remained above 8 g kg⁻¹.

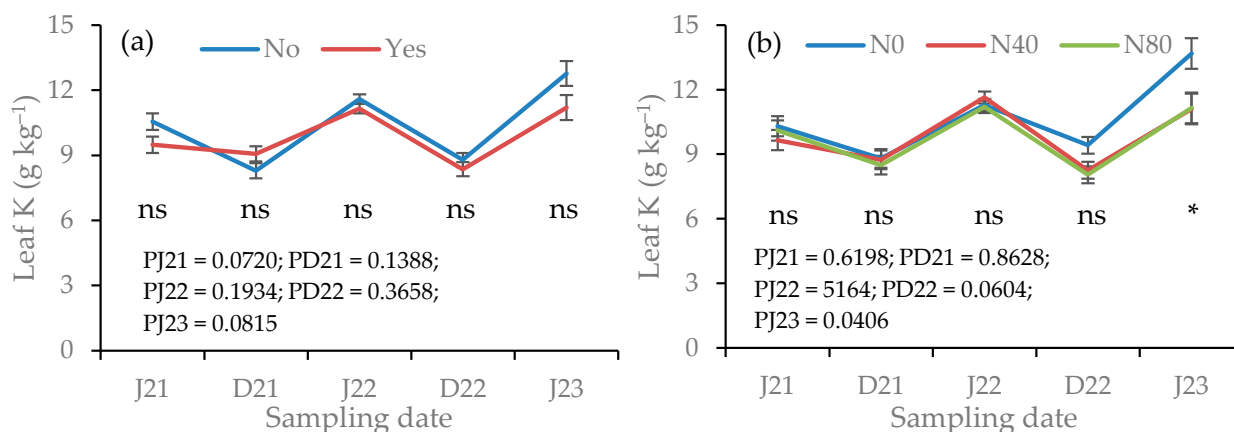


Figure 7. Leaf potassium (K) concentration in the Marmelos Prodi olive grove according to the factorial design: (a) inoculant (not applied (No) and applied (Yes)), (b) nitrogen rate (0 (N0), 40 (N40), and 80 (N80) kg ha⁻¹) across five samplings performed in January (J) and December (D) of 2021, 2022, and 2023. Significance levels are denoted as ns (not significant), and * (significant at $p < 0.05$). Error bars represent the standard errors.

The B concentration in the leaves showed no variation when comparing trees that received the inoculant with those that did not (Figure 8a). Conversely, applying mineral fertilizer to the soil appears to have influenced the B concentration in the leaves (Figure 8b). At two of the five sampling dates, B concentrations in the leaves were significantly higher in the N0 treatment compared with the treatments that received N mineral fertilizer. The B levels in the leaves consistently remained above 16 mg kg⁻¹ and exhibited considerable fluctuation throughout the year, being substantially higher in the summer than in the winter samples.

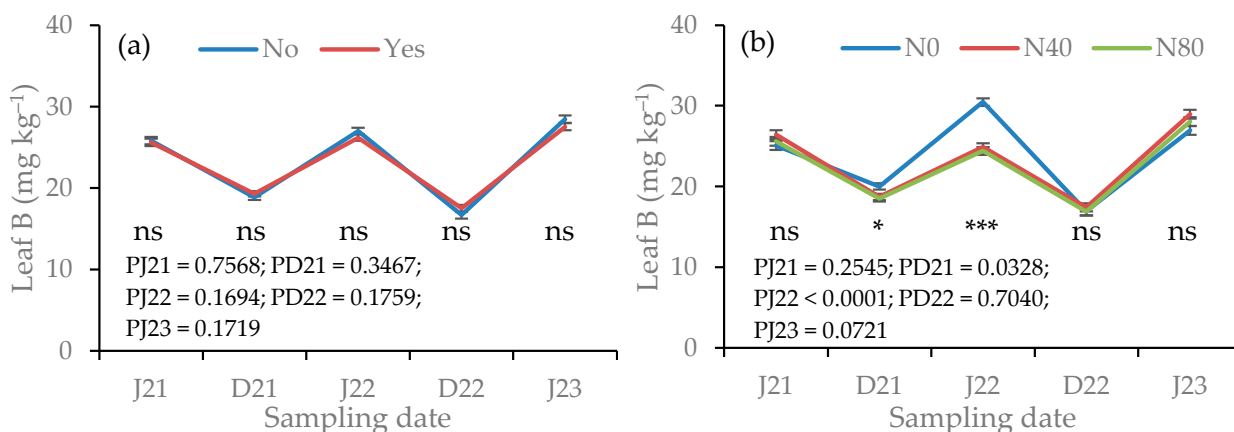


Figure 8. Leaf boron (B) concentration in the Marmelos Prodi olive grove according to the factorial design: (a) inoculant (not applied (No) and applied (Yes)), (b) nitrogen rate (0 (N0), 40 (N40), and 80 (N80) kg ha⁻¹) across five samplings performed in January (J) and December (D) of 2021, 2022, and 2023. Significance levels are denoted as ns (not significant), * (significant at $p < 0.05$), and *** (significant at $p < 0.001$). Error bars represent the standard errors.

Several other analyzed nutrients, such as Ca, Mg, Fe, Mn, Cu, and Zn, did not provide relevant information for the study, as no significant differences between treatments or notable trends were observed.

3.3. Field Trial of Marmelos Bio

In the Marmelos Bio trial, no significant differences between treatments were observed for any individual year or the total accumulated olive yield (Figure 9). The results indicated two years of acceptable yield (2021 and 2023) and one year of very low yield (2022), reflecting olive trees' typical alternate bearing pattern.

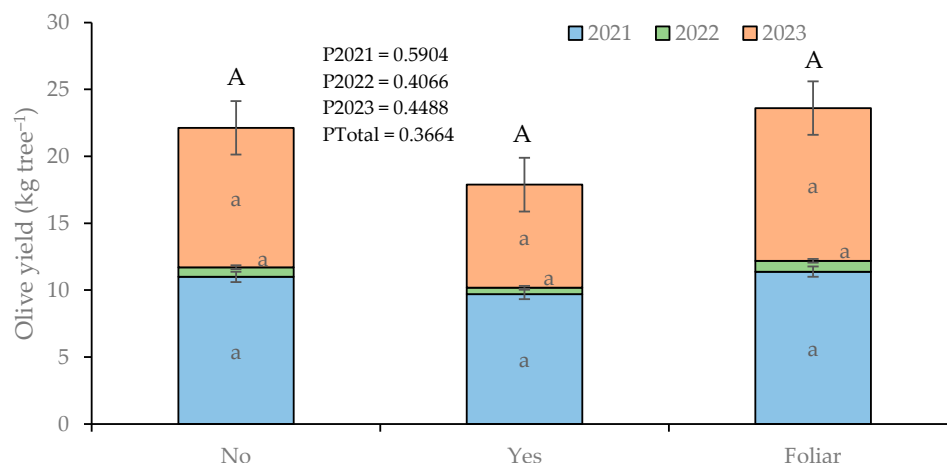


Figure 9. Olive yield in the Marmelos Bio orchard according to fertilization treatments, inoculant application (not applied (No) and applied (Yes)), and foliar spray application (foliar). Means followed by the same letter (lowercase for each year; uppercase for accumulated total) are not significantly different according to the Tukey HSD test ($\alpha = 0.05$). Line segments above the bars represent the standard errors.

The N concentration in the leaves also showed no significant differences between treatments (Figure 10) for any sampling dates. The mean values ranged from 11.8 to 17.1 g kg⁻¹. In this trial, the N concentration in the leaves tended to be higher in the December samples during the resting period of the olive period compared with the July samples. The results for the other nutrients also showed no significant variation with the treatments, nor any noteworthy trends.

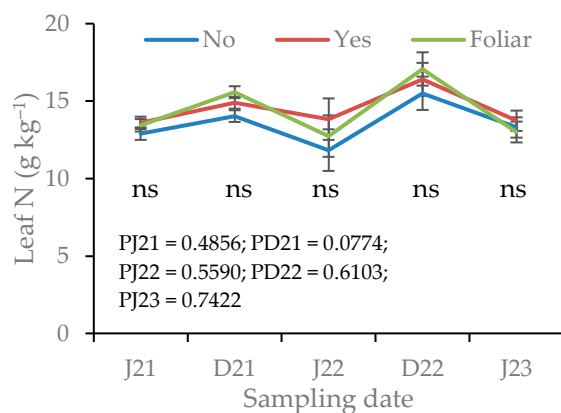


Figure 10. Leaf nitrogen (N) concentration in the Marmelos Bio olive orchard according to fertilization treatments, inoculant application (not applied (No) and applied (Yes)), and foliar spray application (foliar) in five samplings conducted in January (J) and December (D) in 2021, 2022, and 2023. Significance levels are denoted as ns (not significant) from the one-way ANOVA test. Line segments represent the standard errors.

4. Discussion

4.1. Effect of Mineral Fertilizer on Olive Yield and Nitrogen Nutritional Status of the Trees

The application of N to the soil did not significantly influence the total accumulated olive yield in the Valongo trial (Figure 2). However, in 2023, olive yield in the control treatment was significantly lower than in the fertilized treatments. This finding was somewhat obscured by the very low production in 2022 and the reduced production in 2023. Olive trees typically exhibit an alternate bearing cycle, where a year of abundant crop is invariably followed by a poor harvest [33–36]. Alternate bearing is attributed to the inhibition of floral bud induction by seeds [37–39] and/or the competition for resources between the current year's fruits and the new shoots essential for flowering in the subsequent year [34,38]. Thus, in years of abundant fruiting, the length of new shoots and potential sites for new flowers are compromised [34,40], a situation that worsens under marginal growing conditions [33,34]. In this study, alternate bearing may explain the very low production observed in 2022. Unfortunately, in 2023, olive yield was also low, making it challenging to assess the treatment effects in this study.

The N concentration in the leaves varied significantly among treatments (Figure 3b). Across the five sampling dates, values in the control treatment were significantly lower than those in the fertilized treatments. Soil N application is a primary method of crop fertilization, directly increasing nutrient concentration in plant tissues [41–43]. Under normal conditions, especially when N is limiting, its application typically enhances productivity [2,4]. However, as mentioned earlier, the effect of increased N concentration in leaf tissues on olive production may have been masked by a productivity failure in two consecutive years.

In the Marmelos Prodi trial, soil N application increased olive yield starting from the second year (2022), resulting in a significant disparity among the three treatments in 2023, with olive yield in the N80 treatment being nearly eight times higher than that observed in the N0 treatment. Additionally, N concentration in the leaves varied significantly among treatments, with highly significant differences ($p < 0.001$) observed at all sampling dates. This outcome highlights the strong effect of soil N application on nutrient concentration in tissues and olive yield, as commonly observed in field trials [41,42]. In this case, the plant's initial poor nutritional status further amplified the effect. In the N0 treatment, average N concentrations in the tissues consistently remained below the established sufficiency range for the crop [44].

4.2. Effect of the Inoculant on Olive Yield and Tree Nutritional Status

Applying the inoculant did not positively influence the accumulated olive yield in the Valongo trial. In fact, in 2022 and 2023, production was significantly higher in the treatment without inoculant. However, this result was somewhat obscured by the very low olive yield in 2022, which may be attributed to the biennial bearing pattern typical of olive trees [33–35,40], and further reduced in 2023 for reasons that are difficult to elucidate. In the Valongo trial, the N concentration in the leaves also offered limited insight. Significant differences between treatments were observed only at the last sampling date, with the highest average value appearing in the inoculated treatment (Figure 3a). This result contrasts with the olive yield findings and may be justified by the higher fruit production acting as a strong sink, thereby reducing N concentration in the leaves [43,45].

The olive yield in the Marmelos Prodi trial exhibited a distinct pattern, with low yields in the first two years and good productivity in 2023. These trees were in a very poor nutritional condition, only recovering productivity two years after regular N fertilization, reflecting the positive effect that N fertilization tends to have on crop productivity [2,40–42]. Regarding the effect of inoculant application, no significant differences were recorded for

the accumulated olive yield, although in 2022, the value was significantly higher in the inoculant-treated plot. N concentration in the leaves showed minimal variation with inoculant application, with four samplings showing no significant differences and one sampling showing significantly higher values in trees that did not receive the inoculant. Similar to the Valongo trial, higher olive yield appeared to correspond to lower N concentration in the leaves and vice versa, which can be explained by the increased sink effect that fruits display for the same amount of N available to the plant [43,45].

In the Marmelos Bio trial, no significant differences were observed in olive yield or N concentration in the leaves due to inoculant application. Overall, the results from the three experimental fields, which assessed olive yield over three years and N concentration in the leaves across five sampling dates, show that the inoculant did not significantly contribute to N supply to the trees. This is particularly evident when considering the remarkable effect of N applied to the soil. These findings somewhat align with recent studies demonstrating that the N-fixing capacity of *M. symbioticum* applied to lettuce [19], young olive trees grown in pots [20], and maize grown in field and pots [21] is modest. However, the topic is far from exhausted, as the importance of alternative strategies to industrial fertilizers remains significant, and further research is needed to identify the plant species and cropping conditions in which this kind of inoculant is most effective.

Biological N fixation is considered the second most important biological process on Earth after photosynthesis [1,46]. It has been widely promoted in agricultural ecosystems through the cultivation of legumes, whether in crop rotations [47,48], intercropping systems [7,49], or as cover crops in orchards and vineyards [50,51]. Inoculations with rhizobia strains are often employed to enhance fixation efficiency by ensuring the presence of more suitable strains [52,53]. Under favorable conditions, nodulated legumes can fix over 400 kg ha⁻¹ yr⁻¹ of N [1,54]. The high efficiency of these N-fixing systems is attributed to the microorganisms residing in specialized structures, where nitrogenase in bacteroids is protected from excess oxygen by leghemoglobin, while the plant ensures a regular supply of photosynthates through the phloem [1,3,54].

High N fixation capacity can also occur in other symbiotic systems. For example, in rice paddies, the aquatic fern *Azolla* forms a symbiotic relationship with the heterocyst-forming cyanobacterium *Anabaena azollae* [1,8,9]. Similarly, in sugarcane, N-fixing microorganisms such as *Gluconoacetobacter diazotrophicus* and *Azospirillum brasiliense* play a key role [8,10]. However, in systems that achieve high fixation capacity, microorganisms benefit from host protection and privileged access to photosynthates [16,18].

Free-living N₂ fixers, especially when they are not autotrophic, must compete for scarce resources in their habitat, resulting in a very low fixation capacity [11,12]. Although N fixation by free-living N-fixing organisms may be significant in natural ecosystems, its impact is diminished in agricultural fields due to the substantial amounts of N removed annually by crops.

Some species of the genus *Methylobacterium* are known to fix atmospheric N during interactions with plants and have been identified as putative endophytes [18]. However, the specificity relationships that ensure high rates of N fixation tend to be established between N-fixing microorganisms and hosts within restricted botanical groups [1,3,54]. The possibility of a microorganism establishing a specific relationship and benefiting from an unlimited host diversity is unparalleled in nature.

On the other hand, various microorganisms of the genus *Methylobacterium* are ubiquitous in nature and can be found in diverse environments such as soil, water, and plants [16,17]. If they possessed a high N fixation capacity, this would confer them a competitive advantage, potentially leading to their natural presence in plants without the need for application. However, even though these microorganisms, once established in the

phyllosphere, may have privileged access to plant-released products like methanol, soluble carbohydrates, or amino acids [16,18,55], this alone may not be enough to ensure significant N fixation rates for cultivated plants. This underscores the need for caution when recommending such products to farmers and highlights the necessity for stricter regulation before these products are introduced to the market, as emphasized by Giller et al. [56] in a recent literature review on the topic.

4.3. Application of Seaweed Extract

The application of the commercial seaweed extract also did not significantly influence olive yield or the concentration of N or any other nutrient in the leaves. Seaweed extracts now fall under a new category of production factors known as plant biostimulants [24]. Plant biostimulants encompass a variety of products, with the most relevant groups being seaweed and plant extracts, protein hydrolysates, humic and fulvic acids, chitosan and other biopolymers, inorganic compounds, and beneficial microorganisms [25,26]. In this classification, the microbial inoculant also falls into this category. Plant biostimulants have low nutrient content and are not expected to affect plant nutrition directly. They are primarily used for their potential to help plants cope with abiotic or biotic stresses [27,28].

They can also influence plant nutrition if they improve nutrient use efficiency [57] or if products with N-fixing microorganisms are used, such as rhizobia in legume seed inoculation [50,53]. However, as observed in this study, positive effects on plants from seaweed extract application in field conditions have not always been consistently observed [26,28,58,59]. This suggests that significant research efforts are still required to better define the conditions for their application, especially as farmers perceive them as external input factors with associated market and application costs, expecting a financial return through increased productivity and/or product quality.

4.4. The Effect of Treatments on the Concentration of Nutrients Other than Nitrogen

The inoculant application did not significantly affect the concentration of other nutrients in the leaves. However, N application to the soil significantly affected the leaf concentration of some non-applied nutrients, particularly in the Marmelos Prodi trial. P concentration in the leaves remained higher in the N0 treatment, with significant differences observed at two out of five sampling dates. Similarly, K and B concentrations in the leaves followed a similar trend as P, with higher values occurring in the N0 treatment. K values showed significant differences at one sampling date, while B showed differences at two sampling dates. These results suggest a concentration/dilution effect. In other words, by increasing shoot growth and olive yield, N application led to the dilution of P, K, and B nutrients, as their availability in the soil remained unchanged. The concentration/dilution effect is a common phenomenon in plant nutrition [43,60] and has been reported in various recent studies [20,61]. For the other nutrients analyzed, this phenomenon was not observed, likely due to their high availability in the soil, which mitigated the dilution effect caused by N application.

5. Conclusions

N applied to the soil significantly affected N concentration in the leaves and olive yield, especially in the Marmelos Prodi trial. This demonstrates the high efficacy of this form of fertilization in supplying N to the plants and highlights the nutrient's critical role in tree productivity. On the other hand, the inoculant containing the microorganism *Methylobacterium symbioticum* showed no significant effect on N concentration in the leaves or olive yield in any of the three olive groves where it was applied. Similarly, the seaweed

extract in the Marmelos Bio olive grove did not show a measurable positive effect on plant mineral nutrition or olive yield.

These results emphasize the need to better define the conditions under which commercial products can be more effective. In this on-farm study, where treatments were applied by the olive growers, the expected positive results were not prominent. Given that commercial products are costly to acquire and apply, ensuring that their use provides a clear economic return for farmers is crucial. It should also be emphasized that future work should monitor the inoculant's ability to establish in the phyllosphere for a better understanding of its efficacy, an aspect that could not be addressed in these studies.

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