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Effect of Foliar Application of Nitrogen-Fixing Microorganisms and Algae Extracts on Nutritional Status and Yield of Hazelnut and Walnut Trees

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Abstract: This study presents the results of two on-farm trials evaluating the efficacy of a nitrogen (N)-fixing inoculant (*Methylobacterium symbioticum*) applied as a foliar spray to provide N to hazelnut (*Corylus avellana* L.) and walnut (*Juglans regia* L.) trees. In the hazelnut trial, a factorial design was employed with soil N application at three levels [0 (N0), 40 (N40), and 80 (N80) kg ha^{-1}] and foliar application of the inoculant (Yes and No). The walnut trial was arranged as a completely randomized design with three treatments: the N-fixing microorganism, a seaweed extract, and a control. Soil N application significantly increased hazelnut yield in 2021 (1.99, 2.49, and 2.65 t ha^{-1} for N0, N40, and N80, respectively) but not in 2022 (average values ranging from 0.28 to 0.33 t ha^{-1}). The inoculant application did not significantly affect hazelnut yield. In the walnut trial, no significant differences were observed among the treatments in either year. The average yields ranged from 1.72 to 2.38 t ha^{-1} in 2021 and 0.66 to 0.84 t ha^{-1} in 2022. Soil N application in hazelnuts tended to increase leaf N concentration and significantly increased kernel N concentration. The inoculant increased leaf N concentration in one of the three sampling dates but did not affect kernel N concentration. In walnuts, the inoculant did not increase leaf N concentration but significantly increased kernel N concentration in one of the two years. The seaweed extract did not influence walnut yield or leaf N concentration. None of the treatments in either trial consistently affected the concentration of other macronutrients and micronutrients in the leaves. Therefore, while the inoculant showed some potential to improve the N nutritional status of the trees, it did not affect the yield. Overall, the results of the inoculant application were not sufficiently compelling, indicating the need for further studies on these species before the commercial product can be confidently recommended to farmers.

Keywords: *Methylobacterium symbioticum*; plant biostimulant; biological nitrogen fixation; *Juglans regia*; *Corylus avellana*; plant nutritional status; nut yield

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1. Introduction

Most legumes establish symbiotic relationships with N-fixing microorganisms, known collectively as rhizobia [1]. Numerous microorganisms capable of fixing N associated with legumes have already been identified. They have been classified into 238 species of 18 genera and distributed into three phylogenetic classes, α-, β- and γ-Proteobacteria [2]. Commercial inoculants, with specific strains of rhizobia, have been applied to legume seeds or the soil for many decades. This has considerably increased N fixation in agricultural fields [3,4]. Nodulated legumes can access all the N they need for their growth and leave a N-rich organic residue in the soil that can benefit other intercropping crops or crops that follow in rotation [5–7].

The symbiotic relationship established between the aquatic fern of the genus *Azolla* and the cyanobacterium *Anabaena azollae* has also been promoted by farmers in rice (*Oryza sativa* L.) paddies. Azolla is a floating aquatic fern that can obtain ammonia excreted by Anabaena, which inhabits cavities in its leaves [8,9]. Azolla is cultivated as a green manure before transplanting rice or being intercropped with rice [10,11]. It can provide more than 100 kg ha^{-1} yr^{-1} of N, representing more than half of the N required for rice growth [9,10].

Several other non-legume species establish endophytic relationships with N-fixing microorganisms. These relationships' high ecological and agronomic importance was initially observed in Brazil when non-nodule-producing and endophytic N-fixing bacteria were detected in sugarcane (*Saccharum officinarum* L.). The bacterium was initially classified as a new genus and species called *Saccharobacter nitrocaptans* [12] and later reclassified as *Gluconacetobacter diazotrophicus* [13]. Since then, endophytic N fixation has been observed in various plant species, and it is known that high amounts of N can be fixed. Sugarcane is capable of meeting more than 50% of its total nitrogen requirements through biological nitrogen fixation (BNF) [14,15].

There is also a large range of free-living microorganisms capable of fixing N without any relation to plants, which thrive in a diverse range of terrestrial and aquatic environments [16]. These microorganisms fix atmospheric N for their use, although plants can later use it. The amounts of fixed N in terrestrial ecosystems tend to be low (perhaps 3–5 kg ha^{-1} yr^{-1}), although it is considered highly important in some tropical and temperate forest ecosystems [8,17]. In agricultural fields, the importance of free-living N-fixing microorganisms is considered low due to the high amounts of N required by crops [18].

Orchards in the Mediterranean region are agro-systems in which biological N fixation tends to be low, with plant nutrition being highly dependent on commercial fertilizers [19,20]. One way to take advantage of biological N fixation is through cover cropping with legume species. Legumes can be grown in orchards and managed as green manures. Rodrigues et al. [5] showed that a cover of self-reseeding annual legumes provided more N to olive trees (*Olea europaea* L.) than the application of 60 kg ha^{-1} of N as a mineral fertilizer. However, installing and managing such covers is not an easy task. Seeds are costly, and it is tough to achieve their persistence due to false breaks (i.e., germination-inducing rainfall events followed by death from severe drought) and strong competition from weeds, especially when soil fertility and N availability begin to increase [21,22].

Recently, a new N-fixing microorganism was isolated from spores of *Glomus iranicum* var. *tenuihypharum* and classified as *Methylobacterium symbioticum* sp. nov. (strain SB0023/3T) [23]. Microorganisms of the genus *Methylobacterium* can be found dispersed in a wide variety of habitats due to their great phenotypic plasticity, including soil, water, air, and plants [24,25], and some can form nodules and fix N associated with the roots of legume species such as *M. nodulans* and *M. radiotolerans* [25]. *M. symbioticum* is unique because it can also thrive in the phyllosphere, invading stomata and supplying plants with N [23]. This particularity paved the way for the emergence of a commercial inoculant, BlueN® (Symborg Business Development S.L.U., Murcia, Spain) for foliar application that promises to provide N to a wide range of annual and perennial crops. The possibility of a plant biostimulant promoting plant growth and reducing dependence on mineral fertilizers opens up immense possibilities for use in conventional and organic agriculture. Thus, the objectives of this study were to test the effectiveness of the BlueN® inoculant in N fixation in two commercial orchards, one with hazelnut (integrated crop production, rainfed management) and the other with walnut (in organic and irrigated agriculture). The effectiveness of the product was tested in the hazelnut orchard under different fertilization regimes generated by the application of N fertilizers to the soil and in the walnut orchard, comparing the effect of the BlueN® inoculant with an algae extract (Fitoalgas Green®, Tradecorp Nutri-Performance, Lisbon, Portugal) another plant biostimulant applied as a foliar spray.

2. Materials and Methods

2.1. Experimental Conditions

Two field experiments were carried out over two years (2021 and 2022), one in a 20-year-old walnut orchard, cv. Franquette, spaced at 7 m × 7 m and managed as organic, according to European Union (EU) legislation [Regulation (EC) nº 834/2007], and the other in a 25-year-old hazelnut orchard, cv. Ennis, established in an inter- and intra-row spacing of 5.0 m × 3.5 m and managed under the EU Integrated Crop Management System [26]. The orchards are located in Macedo de Cavaleiros, northeast of Portugal, with the walnut orchard situated at coordinates 41°32′35.0″ N 6°53′15.6″ W and the hazelnut orchard at 41°32′00.9″ N 6°59′08.3″. The region's climate is warm and temperate and of the Mediterranean type Csb according to the Köppen–Geiger classification. The average air temperature is 12.8 °C, and the annual precipitation is 689 mm [27]. Rainfall is concentrated in the winter, with higher temperatures in the summer months (Figure 1).

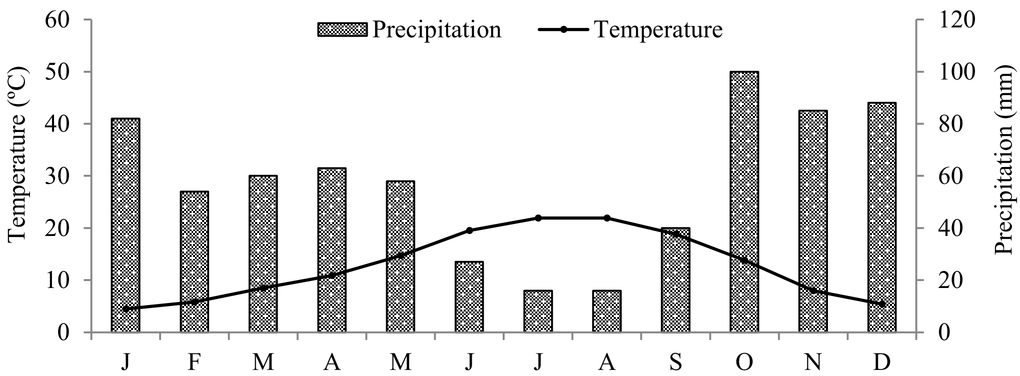

Figure 1. Mean average monthly temperature and precipitation in Macedo de Cavaleiros, Portugal.

The walnut orchard is established on a Solimovic Regosol [28] characterized by a sandy loam texture, while the hazelnut orchard is installed on a Solimovic Regosol with a loamy sand texture. Some soil properties determined when setting up the field experiments are shown in Table 1.

Table 1. Selected soil properties (average ± 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	Walnut Orchard	Hazelnut Orchard
[1] Organic carbon (g kg^{-1})	20.5 ± 2.22	8.9 ± 0.96
[2] pH (H_2O)	6.3 ± 0.07	5.4 ± 0.07
[3] Extract. phosphorus (mg kg^{-1}, P_2O_5)	85.0 ± 4.70	21.4 ± 3.90

Table 1. *Cont.*

Soil Properties	Walnut Orchard	Hazelnut Orchard
[3] Extract. potassium (mg kg^{-1}, K_2O)	406.1 ± 81.68	135.7 ± 3.21
[4] Exchang. calcium ($cmol_c$ kg^{-1})	15.8 ± 1.53	5.0 ± 0.17
[4] Exchang. magnesium ($cmol_c$ kg^{-1})	4.0 ± 0.37	0.9 ± 0.06
[4] Exchang. potassium ($cmol_c$ kg^{-1})	0.2 ± 0.02	0.3 ± 0.01
[4] Exchang. sodium ($cmol_c$ kg^{-1})	1.2 ± 0.02	0.3 ± 0.03
[5] Exchang. acidity ($cmol_c$ kg^{-1})	0.2 ± 0.01	0.1 ± 0.00
[6] CEC ($cmol_c$ kg^{-1})	21.2 ± 1.89	6.5 ± 0.21
[7] Sand	62.01 ± 1.68	78.8 ± 4.44
[7] Silt	21.56 ± 0.52	17.0 ± 4.11
[7] Clay	16.4 ± 1.17	4.2 ± 0.34
[8] Texture	Sandy loam	Loamy sand

[1] Wet digestion (Walkley–Black); [2] potentiometry; [3] ammonium lactate; [4] ammonium acetate; [5] potassium chloride; [6] cation exchange capacity; [7] Robinson pipette method; [8] USDA (The United States Department of Agriculture).

2.2. Experimental Designs and Crop Management

In the walnut tree, three treatments were established: BlueN® inoculant (inoculant), Fitoalgas Green® (algae), and non-fertilized control (control). Three groups (three replicates) of two trees (experimental units) were selected, totaling six trees per treatment and eighteen trees in total for the trial. It was ensured that the groups of trees treated with BlueN were more than 50 m away from the trees marked for the other treatments to avoid the risk of contamination with the microorganism. BlueN contains 3×10^7 colony-forming units (CFU g^{-1}) of *M. symbioticum*. The leaf spray was prepared at the concentration recommended by the manufacturer, 333 g ha^{-1}, diluted in 200 L of water, and applied once a year in phenological state 32 (shoots about 30% of the final length) [29]. Each tree received a fraction of the inoculant corresponding to its representativeness in one hectare. Fitoalgas Green® is a pure extract (100% fresh matter) of seaweed (*Ascophyllum nodosum* (L.) Le Jolis), obtained by cold extraction. Three annual sprays were applied, the first coinciding with the BlueN application and then two more during the growing season, separated by approximately one month. A concentration of 3 L ha^{-1} was used, as recommended by the manufacturer. The orchard floor was maintained with natural vegetation cover, which was mowed twice a year to reduce competition between weeds and trees. The experiment was drip-irrigated between June and September. During the growing season, no other cropping practices were carried out (Table 2).

Table 2. Important dates for the establishment of experimental protocols and sampling.

	2021		2022	
	Walnut	**Hazelnut**	**Walnut**	**Hazelnut**
Pruning	Feb 16	Feb 17	Feb 17	Feb 10
Mowing	Mar 25/May 18	Mar 24/Apr 28	Mar 22/May 27	Mar 21/Apr 26
Irrigation	Jun 2–Sep 10		Jun 4–Sep 9	
Soil-applied N		Mar 29		Mar 27
Inoculant	May 31	May 31	May 27	May 27
Algae extract	May 31, Jun 20, Jul 20		May 27, Jun 17, Jul 20	
Leaf sampling	Jun 20, Jul 20	Jun 20, Jul 20	Jul 20	Jul 20
Soil sampling	Feb 16	Feb 17		
Harvest	Oct 19	Sep20/Oct 1	Oct 28	Sep 26/Oct 11

The hazelnut experiment was arranged as a factorial: N fertilization, with three N rates [0 (N0), 40 (N40), and 80 (N80) kg ha^{-1}] applied to the soil and the application of the inoculant BlueN (Yes and No). Three groups (three replicates) of three trees (experimental units) were selected, totaling nine trees per treatment and fifty-four trees in the trial. It was ensured that the groups of trees that received BlueN were more than 50 m away from the groups that did not receive BlueN. The BlueN inoculant was applied under the conditions mentioned for the walnut tree. N fertilizer was applied to the soil as ammonium nitrate 20.5% (50% NH_4^+, 50% NO_3^-) at the above-mentioned rates. The application was performed in the last week of March of each year in the projection zone of the canopy. The fertilizer applied to the soil was not incorporated, as the orchard was managed with a natural vegetation cover, which was mowed twice in spring. The orchard was managed in rainfed conditions and was pruned very lightly during the winter resting period, mainly to eliminate the suckers and water sprouts that tend to appear in these trees. No more practices were carried out in the orchard.

2.3. Field Sampling and Laboratory Analysis

At the beginning of the trials, three composite soil samples (10 cores per sample) were randomly taken from the 0–0.20 m soil layer to characterize the field plots. In the laboratory, soil samples were air-dried and sieved (2 mm). Twice a year, during the summer growing season, twenty (walnut) and sixty (hazelnut) young fully developed leaves were detached from the middle of the shoots developing in the ongoing growing season from all quadrants. For walnuts, the trees were harvested by a trunk shaker head, which detached the fruits. Sheets spread on the floor allowed the fruits to be recovered. They were then cleaned of leaves, and the mesocarp was removed. In the hazelnut trial, the harvest was carried out after the fruits had fallen naturally to the ground. Fruits were manually harvested from the ground in two passes during autumn due to staggered fruit drop. The kernel/shell ratio was evaluated in the laboratory, and a separate elemental chemical analysis was carried out. All plant tissues were dried in an oven at 70 °C at constant weight and ground (1 mm mesh).

Soil samples were submitted for the determination of soil separates (clay, silt and sand) by the Robinson pipette method, pH (H_2O and KCl) by potentiometry, organic carbon by the Walkley–Black method, cation exchange capacity by extraction with ammonium acetate at pH 7.0, extractable phosphorus (P) and potassium (K) by the Egner–Riehm method, and extractable boron (B) by the hot water and azomethine-H method. The reader is referred to a study by Van Reeuwijk [30] for more detail on the analytical procedures. Elemental analyses of tissue samples (leaves and kernels) were performed by the Kjeldahl (N), colorimetry (B and P), and atomic absorption spectrophotometry [K, calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn)] methods [31] after tissue samples had been digested with nitric acid in a microwave.

2.4. Data Analysis

Data were first tested for normality and homogeneity of variance using the Shapiro–Wilk test and Bartlett's test, respectively. The treatments' effects were compared by one-way (walnut) and two-way (hazelnut) ANOVA. When significant differences were found ($p < 0.05$), the means were separated by the multiple-range Tukey HSD test ($\alpha = 0.05$).

3. Results

3.1. Hazelnut and Walnut Yields

The hazelnut yield did not vary significantly with the application of inoculant in any of the trial years and nor did the cumulative total (Figure 2). In 2021, the hazelnut yield ranged from 2.58 to 2.17 t ha^{-1} in the No and Yes treatments, respectively, and in 2022, it ranged from 0.25 to 0.36 t ha^{-1}. The application of N significantly influenced the hazelnut yield in 2021 and the cumulative total. In 2021, the average hazelnut yield was 1.99, 2.49, and 2.65 t ha^{-1} for N0, N40, and N80, respectively. In 2022, the average hazelnut yield did not differ significantly between the treatments, ranging from 0.28 to 0.33 t ha^{-1}.

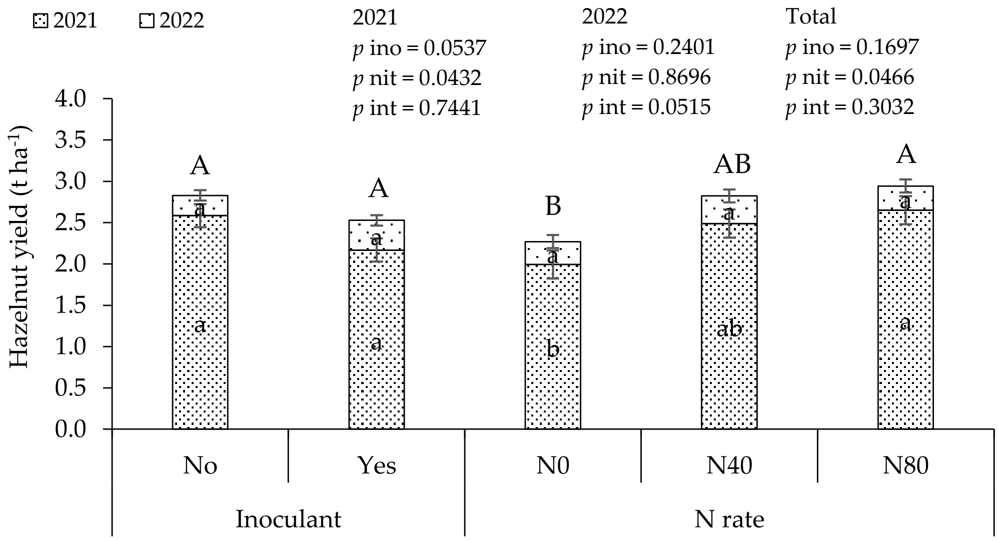

Figure 2. Hazelnut yield over two consecutive years in response to the application of inoculant (No, Yes) and nitrogen (N) fertilization (0 (N0), 40 (N40), and 80 (N80) kg ha^{-1}). p, probability associated with the factors inoculant (ino), N fertilization (nit), and interaction (int) after applying two-way ANOVA. Lowercase and uppercase letters indicate significant differences for each year and cumulative total, respectively. Error bars represent the standard error.

The walnut yield did not vary significantly between the treatments in 2021 or 2022 and nor did the cumulative total (Figure 3). Like the hazelnut yield, the 2021 yield was significantly higher than that in 2022. In 2021, the average walnut yield ranged from 1.72 to 2.38 t ha^{-1}; in 2022, it ranged from 0.66 to 0.84 t ha^{-1}.

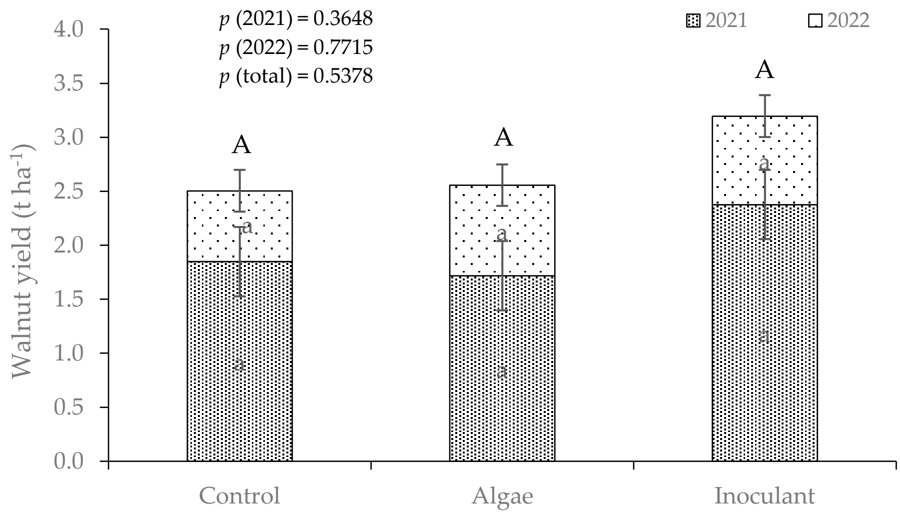

Figure 3. Walnut yield over two consecutive years in response to treatments: control, algae extract (algae), and inoculant BlueN (inoculant). p, the probability associated with each year of the trial and the total walnut yield after applying one-way ANOVA. Lowercase and uppercase letters indicate significant differences for each year and cumulative total, respectively. Error bars represent the standard error.

3.2. Indicators of Trees' Access to Nitrogen

Applying the inoculant did not significantly influence the N concentration in hazelnut leaves in the June and July 2021 samples but resulted in significantly higher values in the 2022 samples (Table 3). The application of N to the soil appears to have consistently increased the N concentrations in the leaves, with the highest average values observed in the N80 treatment compared to the N0 treatment in all samples, though these differences were not significant. The N content in the kernel did not vary significantly with the application of the inoculant in either of the trial years, nor were the average values higher in the treatment with inoculant application. Conversely, applying N to the soil significantly affected the N concentration in the kernel in both trial years. A consistent gradient with the N rate was observed, with average values of 28.7, 35.6, and 45.3 g kg^{-1} in 2021 and 34.4, 39.2, and 46.2 g kg^{-1} in 2022 for the N0, N40, and N80 treatments, respectively.

Table 3. Nitrogen (N) concentration in hazelnut leaves and kernels in response to the application of inoculant (No, Yes) and N fertilization (0 (N0), 40 (N40), and 80 (N80) kg ha^{-1}). p, probability associated with the factors inoculant (ino), N fertilization (nit), and interaction (int) after the application of two-way ANOVA.

	Leaf N (g kg^{-1})			**Kernel N (g kg^{-1})**	
	20 June 2021	**20 July 2021**	**20 July 2022**	**2021**	**2022**
Micro					
No	20.8 a	17.0 a	22.9 b	36.9 a	40.3 a
Yes	21.2 a	17.1 a	23.8 a	36.2 a	39.6 a
N rate					
N0	20.6 a	16.6 a	23.1 a	28.7 c	34.4 b
N40	21.0 a	17.0 a	23.4 a	35.6 b	39.2 b
N80	21.4 a	17.4 a	23.4 a	45.3 a	46.2 a
p ino	0.3928	0.7805	0.0145	0.6244	0.6831
p nit	0.3140	0.3446	0.7253	0.0002	0.0019
p int	0.8056	0.7558	0.0161	0.2648	0.2401

In the columns, separated by the factor (inoculant or nitrogen fertilization), means followed by the same letter are not significantly different according to Tukey's HSD test (α = 0.05).

In the walnut trial, the N concentration in the leaves did not differ significantly between the treatments in any of the three samplings (Table 4). No apparent trend was observed among the treatments across the three samplings. In June and July 2021 and July 2022, the mean values ranged between 22.2 and 24.7 g kg^{-1}, 24.0 and 25.1 g kg^{-1}, and 22.1 and 23.6 g kg^{-1}, respectively. On the other hand, the N concentration in the kernel varied significantly between treatments in 2021, with the mean values in the BlueN treatment (32.2 g kg^{-1}) being significantly higher than in the algae (29.1 g kg^{-1}) and control (29.0 g kg^{-1}) treatments. In 2022, the mean values did not differ significantly between the treatments, ranging from 28.9 to 32.2 g kg^{-1}.

Table 4. Nitrogen (N) concentration in walnut leaves and kernel in response to treatments: control, algae extract (algae), and inoculant BlueN (inoculant).

	Leaf N (g kg^{-1})			Kernel N (g kg^{-1})	
	20 June 2021	**20 July 2021**	**20 July 2022**	**2021**	**2022**
Control	24.8 a	25.1 a	22.3 a	29.0 b	28.9 a
Algae	24.1 a	25.0 a	23.6 a	29.1 b	32.2 a
Inoculant	22.2 a	24.0 a	22.1 a	32.2 a	31.7 a
Probability	0.0535	0.1412	0.1938	0.0191	0.1070

In the columns, means followed by the same letter are not significantly different according to Tukey's HSD test (α = 0.05).

3.3. Effect of Treatments on the Concentration of Other Macronutrients and Micronutrients in the Leaves

In the hazelnut orchard, the P levels in the leaves did not vary significantly with the application of the inoculant or N fertilization on any of the sampling dates (Table 5). The mean values ranged between 1.2 and 1.5 g kg^{-1}. In the walnut orchard, significant differences between treatments were observed in the first sampling of 2021 and the sampling of 2022. However, the results did not show consistency in the relative positions of the treatments, with BlueN, for example, showing the lowest average value in the June 2021 sampling and the highest average value in the 2022 sampling.

Table 5. Phosphorus concentration (g kg^{-1}) in hazelnut leaves in response to inoculant application (No, Yes) and nitrogen (N) fertilization (0 (N0), 40 (N40), and 80 (N80) kg N ha^{-1}) and in walnut leaves in response to control, algae extract (algae), and inoculant BlueN (inoculant) treatments. Probability is associated with the inoculant (ino), N rate (nit), and interaction (int) in the hazelnut trial and with the sampling date in the walnut trial.

	Inoculant		N Rate			Probability		
Hazelnut	**No**	**Yes**	**N0**	**N40**	**N80**	**ino**	**nit**	**int**
20 June 2021	1.4 a	1.3 a	1.4 a	1.4 a	1.4 a	0.1003	0.5911	0.4110
20 July 2021	1.3 a	1.2 a	1.2 a	1.2 a	1.3 a	0.0639	0.7839	0.7257
20 July 2022	1.5 a	1.4 a	1.5 a	1.5 a	1.4 a	0.2602	0.7492	0.5592

Walnut	Control	Algae	Inoculant	Probability
20 June 2021	1.1 a	1.1 a	0.9 b	0.0246
20 July 2021	1.2 a	0.8 a	1.0 a	0.2314
20 July 2022	1.0 b	0.9 b	1.3 a	0.0163

In the columns, and for each experiment, means followed by the same letter are not significantly different according to Tukey's HSD test (α = 0.05).

In the hazelnut orchard, the K concentrations in the tissues did not vary significantly among the treatments in the first sampling of 2021 and in the 2022 sampling, whether with inoculant application or N fertilization (Table 6). However, in the July 2021 sampling, the average K concentration in the leaves was significantly lower in the plants without inoculant compared to those with inoculant. Across all the treatments and sampling dates, the average values ranged from 4.8 to 6.0 g kg^{-1}. In the walnut orchard, no significant differences were observed among the treatments in the two samplings of 2021. However, in 2022, the K values in the BlueN plot were significantly higher than in the other treatments, showing the opposite effect of inoculant application compared to what was observed in the second sampling of 2021. Overall, the average values ranged from 4.8 to 9.4 g kg^{-1}.

Table 6. Potassium concentration (g kg^{-1}) in hazelnut leaves in response to inoculant application (No, Yes) and nitrogen (N) fertilization (0 (N0), 40 (N40), and 80 (N80) kg N ha^{-1}) and in walnut leaves in response to control, algae extract (algae), and inoculant BlueN (inoculant) treatments. Probability is associated with the inoculant (ino), N rate (nit), and interaction (int) in the hazelnut trial and with the sampling date in the walnut trial.

	Inoculant		**N Rate**			**Probability**		
Hazelnut	**No**	**Yes**	**N0**	**N40**	**N80**	**ino**	**nit**	**int**
20 June 2021	5.6 a	5.8 a	6.0 a	5.2 a	5.8 a	0.6177	0.2477	0.0005
20 July 2021	5.8 a	4.8 b	5.4 a	5.4 a	5.2 a	0.0132	0.8874	0.4719
20 June 2022	5.3 a	5.0 a	5.1 a	5.3 a	5.1 a	0.3278	0.7517	0.1298
Walnut	Control		Algae		Inoculant		Probability	
20 June 2021	5.3 a		5.6 a		6.4 a		0.3920	
20 July 2021	4.8 a		6.2 a		7.6 a		0.4385	
20 July 2022	7.2 b		5.7 b		9.4 a		0.0044	

In the columns, and for each experiment, means followed by the same letter are not significantly different according to Tukey's HSD test (α = 0.05).

The Ca concentration in the hazelnut leaves did not vary significantly with inoculant application or nitrogen fertilization across the three sampling dates (Table 7). The mean values ranged from 14.2 to 17.9 g kg^{-1}. In the walnut leaves, the Ca concentration varied significantly among the treatments in the first sampling of 2021, with the mean values in the BlueN treatment being significantly higher than those in the algae treatment. However, this trend of higher values in the BlueN treatment did not persist in the 2022 sampling.

Table 7. Calcium concentration (g kg^{-1}) in hazelnut leaves in response to inoculant application (No, Yes) and nitrogen (N) fertilization (0 (N0), 40 (N40), and 80 (N80) kg N ha^{-1}) and in walnut leaves in response to control, algae extract (algae), and inoculant BlueN (inoculant) treatments. Probability is associated with the inoculant (ino), N rate (nit), and interaction (int) in the hazelnut trial and with the sampling date in the walnut trial.

	Inoculant		**N Rate**			**Probability**		
Hazelnut	**No**	**Yes**	**N0**	**N40**	**N80**	**ino**	**nit**	**int**
20 June 2021	16.3 a	16.2 a	16.6 a	16.4 a	15.7 a	0.8461	0.4299	0.0176
20 July 2021	17.1 a	17.9 a	17.3 a	17.5 a	17.6 a	0.1945	0.9321	0.2835
20 July 2022	14.2 a	14.6 a	14.2 a	14.6 a	14.4 a	0.3761	0.7696	0.2211
Walnut	Control		Algae		Inoculant		Probability	
20 June 2021	9.4 ab		7.8 b		12.0 a		0.0089	
20 July 2021	13.1 a		14.3 a		16.1 a		0.3157	
20 July 2022	14.3 a		15.7 a		13.7 a		0.2884	

In the columns, and for each experiment, means followed by the same letter are not significantly different according to Tukey's HSD test (α = 0.05).

The Mg concentration in the hazelnut leaves did not vary significantly with inoculant application across the samplings (Table 8). N fertilization significantly influenced the Mg concentration in the leaves during two samplings: the second in 2021 and that in 2022. In these cases, there was a consistent trend towards higher average values in the N0 treatment. In the walnut orchard, significant differences among treatments were observed in the first sampling of 2021, with the BlueN treatment showing the highest average value.

Table 8. Magnesium concentration (g kg^{-1}) in hazelnut leaves in response to inoculant application (No, Yes) and nitrogen (N) fertilization (0 (N0), 40 (N40), and 80 (N80) kg N ha^{-1}) and in walnut leaves in response to control, algae extract (algae), and inoculant BlueN (inoculant) treatments. Probability is associated with the inoculant (ino), N rate (nit), and interaction (int) in the hazelnut trial and with the sampling date in the walnut trial.

	Inoculant		**N Rate**			**Probability**		
Hazelnut	**No**	**Yes**	**N0**	**N40**	**N80**	**ino**	**nit**	**int**
20 June 2021	6.2 a	5.8 a	6.3 a	5.8 a	5.9 a	0.0984	0.1698	0.4643
20 July 2021	5.5 a	5.8 a	5.9 a	5.2 b	5.9 a	0.2725	0.0483	0.7316
20 July 2022	5.6 a	5.5 a	6.4 a	5.9 ab	4.4 b	0.7822	0.0298	0.8066
Walnut	Control		Algae		Inoculant	Probability		
20 June 2021	3.6 ab		3.0 b		4.5 a	0.0491		
20 July 2021	4.8 a		4.6 a		5.9 a	0.4608		
20 July 2022	5.8 a		6.2 a		5.6 a	0.3730		

In the columns, and for each experiment, means followed by the same letter are not significantly different according to Tukey's HSD test (α = 0.05).

The hazelnut trees that received the inoculant tended to exhibit lower leaf B levels than those that did not, with significant differences observed in the second sampling of 2021 (Table 9). N application did not influence the B concentration in the hazelnut leaves in the two samplings of 2021, but in 2022, the average values were significantly lower in the N0 treatment compared to the N40 treatment. Overall, the average values ranged from 33.3 to 70.0 mg kg^{-1}. In the walnut leaves, the B concentration did not vary among the treatments in the two samplings of 2021, but it was significantly higher in the BlueN treatment compared to the other treatments in the 2022 sampling. In the walnuts, the average leaf B levels ranged from 29.2 to 59.9 mg kg^{-1}.

Table 9. Boron concentration (mg kg^{-1}) in hazelnut leaves in response to inoculant application (No, Yes) and nitrogen (N) fertilization (0 (N0), 40 (N40), and 80 (N80) kg N ha^{-1}) and in walnut leaves in response to control, algae extract (algae), and inoculant BlueN (inoculant) treatments. Probability is associated with the inoculant (ino), N rate (nit), and interaction (int) in the hazelnut trial and with the sampling date in the walnut trial.

	Inoculant		**N Rate**			**Probability**		
Hazelnut	**No**	**Yes**	**N0**	**N40**	**N80**	**ino**	**nit**	**int**
20 June 2021	41.2 a	35.9 a	37.8 a	38.1 a	39.8 a	0.0803	0.8367	0.6556
20 July 2021	42.7 a	33.3 b	35.3 a	39.7 a	38.9 a	0.0034	0.3724	0.5558
20 July 2022	66.6 a	64.3 a	59.2 b	70.0 a	67.0 ab	0.4976	0.0492	0.0012
Walnut	Control		Algae		Inoculant	Probability		
20 June 2021	35.9 a		37.1 a		50.9 a	0.1288		
20 July 2021	50.4 a		49.9 a		59.9 a	0.1689		
20 July 2022	33.9 b		29.2 b		48.2 a	0.0053		

In the columns, and for each experiment, means followed by the same letter are not significantly different according to Tukey's HSD test (α = 0.05).

The concentration of Fe in the hazelnut leaves varied significantly with inoculant application in two samplings but did not vary significantly with the N rate (Table 10). The average values ranged from 122.8 to 254.1 mg kg^{-1}. In the walnut trees, the leaf Fe levels varied significantly among the treatments in the second sampling of 2021. The average values ranged from 77.5 to 146.0 mg kg^{-1}.

Table 10. Iron concentration (mg kg^{-1}) in hazelnut leaves in response to inoculant application (No, Yes) and nitrogen (N) fertilization (0 (N0), 40 (N40), and 80 (N80) kg N ha^{-1}) and in walnut leaves in response to control, algae extract (algae), and inoculant BlueN (inoculant) treatments. Probability is associated with the inoculant (ino), N rate (nit), and interaction (int) in the hazelnut trial and with the sampling date in the walnut trial.

	Inoculant		**N Rate**			**Probability**		
Hazelnut	**No**	**Yes**	**N0**	**N40**	**N80**	**ino**	**nit**	**int**
20 June 2021	123.9 b	145.8 a	122.8 a	143.7 a	138.2 a	0.0073	0.0684	0.0006
20 July 2021	241.2 a	214.5 b	209.7 a	242.4 a	231.4 a	0.0173	0.0579	0.6065
20 July 2022	249.7 a	242.4 a	253.2 a	254.1 a	230.9 a	0.4260	0.0962	0.3712

Walnut	Control	Algae	Inoculant	Probability
20 June 2021	115.8 a	93.3 a	127.8 a	0.1028
20 July 2021	127.7 a	146.0 a	77.5 b	0.0041
20 July 2022	109.2 a	111.4 a	101.8 a	0.4085

In the columns, and for each experiment, means followed by the same letter are not significantly different according to Tukey's HSD test ($\alpha = 0.05$).

The Mn levels in the hazelnut leaves were significantly higher in the trees that received the inoculant than those that did not across all three samplings (Table 11). N application also significantly influenced the Mn concentration in the tissues in two of the three samplings, with the average values tending to decrease with higher N doses. Across all the treatments and sampling dates, the average values ranged from 406.1 to 677.7 mg kg^{-1}. No significant differences were observed among the treatments in the walnut orchard on any sampling dates. The average values were considerably lower, ranging from 75.9 to 111.5 mg kg^{-1}.

Table 11. Manganese concentration (mg kg^{-1}) in hazelnut leaves in response to inoculant application (No, Yes) and nitrogen (N) fertilization (0 (N0), 40 (N40), and 80 (N80) kg N ha^{-1}) and in walnut leaves in response to control, algae extract (algae), and inoculant BlueN (inoculant) treatments. Probability is associated with the inoculant (ino), N rate (nit), and interaction (int) in the hazelnut trial and with the sampling date in the walnut trial.

	Inoculant		**N Rate**			**Probability**		
Hazelnut	**No**	**Yes**	**N0**	**N40**	**N80**	**ino**	**nit**	**int**
20 June 2021	465.6 b	581.5 a	616.4 a	536.2 b	418.0 c	<0.0001	<0.0001	0.0266
20 July 2021	488.0 b	677.7 a	593.6 a	627.7 a	527.3 a	0.0003	0.1251	0.2693
20 July 2022	391.8 b	533.5 a	524.7 a	457.2 ab	406.1 b	0.0003	0.0173	0.0001

Walnut	Control	Algae	Inoculant	Probability
20 June 2021	82.8 a	94.8 a	86.2 a	0.6917
20 July 2021	86.2 a	111.5 a	80.2 a	0.1151
20 July 2022	77.1 a	97.5 a	75.9 a	0.0955

In the columns, and for each experiment, means followed by the same letter are not significantly different according to Tukey's HSD test ($\alpha = 0.05$).

The Zn concentrations in the hazelnut leaves did not vary significantly with inoculant application or N fertilization across the samplings (Table 12). Across all the treatments and sampling dates, the average values ranged from 16.8 to 20.7 mg kg^{-1}. In the walnut trees, significant differences among treatments were observed in the 2022 sampling, with the highest average value observed in the BlueN treatment and the lowest in the algae treatment. Overall, the average Zn levels ranged from 26.6 to 44.5 mg kg^{-1}.

Table 12. Zinc concentration (mg kg^{-1}) in hazelnut leaves in response to inoculant application (No, Yes) and nitrogen (N) fertilization (0 (N0), 40 (N40), and 80 (N80) kg N ha^{-1}) and in walnut leaves in response to control, algae extract (algae), and inoculant BlueN (inoculant) treatments. Probability is associated with the inoculant (ino), N rate (nit), and interaction (int) in the hazelnut trial and with the sampling date in the walnut trial.

	Inoculant		N Rate			Probability		
Hazelnut	**No**	**Yes**	**N0**	**N40**	**N80**	**ino**	**nit**	**int**
20 June 2021	18.7 a	18.7 a	20.0 a	17.9 a	18.4 a	0.9938	0.0635	0.6240
20 July 2021	17.1 a	17.5 a	18.2 a	16.8 a	17.0 a	0.5738	0.1872	0.6628
20 July 2022	19.2 a	19.9 a	18.9 a	19.0 a	20.7 a	0.3103	0.0546	0.4899

Walnut	Control	Algae	Inoculant	Probability
20 June 2021	34.7 a	31.3 a	28.7 a	0.6917
20 July 2021	44.5 a	26.6 a	29.2 a	0.2343
20 July 2022	27.0 ab	26.9 b	34.7 a	0.0414

In the columns, and for each experiment, means followed by the same letter are not significantly different according to Tukey’s HSD test (α = 0.05).

The Cu concentration in the hazelnut leaves was significantly higher in the inoculant-treated group compared to the non-inoculant group in the first sampling of 2021 (Table 13). However, in subsequent samplings, no significant differences were observed in Cu levels between the plants that received the inoculant and those that did not. N fertilization did not significantly influence the Cu concentration in the hazelnut leaves across the sampling dates. Across all the treatments and samplings, the average values ranged from 2.4 to 4.8 mg kg^{-1}. No significant differences in the leaf Cu levels were observed across the walnut trees’ sampling dates. The average values ranged from 5.2 to 9.1 mg kg^{-1}.

Table 13. Copper concentration (mg kg^{-1}) in hazelnut leaves in response to inoculant application (No, Yes) and nitrogen (N) fertilization (0 (N0), 40 (N40), and 80 (N80) kg N ha^{-1}) and in walnut leaves in response to control, algae extract (algae), and inoculant BlueN (inoculant) treatments. Probability is associated with the inoculant (ino), N rate (nit), and interaction (int) in the hazelnut trial and with the sampling date in the walnut trial.

	Inoculant		N Rate			Probability		
Hazelnut	**No**	**Yes**	**N0**	**N40**	**N80**	**ino**	**nit**	**int**
20 June 2021	3.1 b	4.2 a	2.4 a	3.6 a	3.4 a	0.0062	0.3693	0.5815
20 July 2021	2.4 a	2.6 a	2.6 a	2.6 a	2.4 a	0.6307	0.8441	0.8525
20 July 2022	4.3 a	4.8 a	4.8 a	4.3 a	4.4 a	0.1487	0.4435	0.3900

Walnut	Control	Algae	Inoculant	Probability
20 June 2021	8.4 a	8.3 a	6.9 a	0.3601
20 July 2021	7.1 a	5.2 a	7.4 a	0.0521
20 July 2022	7.2 a	8.3 a	9.1 a	0.3618

In the columns, and for each experiment, means followed by the same letter are not significantly different according to Tukey’s HSD test (α = 0.05).

4. Discussion

4.1. Fruit Yield

The poor yields in the second year might have obscured the effects of the treatments on the hazelnut and walnut yields. However, this is a common pattern in Mediterranean fruit species, which are prone to alternate bearing, where a year of high yield is invariably followed by a poor crop [32,33]. Alternate bearing has been attributed to the hormonal influence the growing seeds exert on the induction of buds that will produce fruit in the following year [34,35]. Another explanation is the competition for photosynthates, where many fruits, being the primary sink of the tree for photosynthates, reduce vegetative growth and shoot development in the current year. This, in turn, affects the potential for a sufficient quantity and quality of flowers to be formed for fruiting in the following year [33,36,37]. Additionally, it has been observed that alternate bearing is more pronounced under poor growing conditions, which limit the photosynthetic capacity of plants [33,37]. In this study, the alternate bearing in the hazelnuts was likely due to dry farming. In the walnuts, this might have been due to organic farming practices since there had been no soil fertilization in recent years, including in the trial period.

In the hazelnut trees, significant differences were observed due to the application of N to the soil in the first year, which also affected the cumulative hazelnut yield over the two years. This response to applying N to the soil is common in agricultural experimentation [38,39]. This can be explained by the fact that natural nutrient cycling processes cannot replenish the soil N at the same rate as it is removed in crops [18,40]. N is essential for various cellular constituents, such as proteins, nucleic acids, chlorophylls, and numerous secondary metabolites [41]. It is typically found in high concentrations in plant tissues [40,42], explaining crops' frequent positive response to N application.

The application of the inoculant, on the other hand, did not significantly affect the hazelnut yield compared to the untreated trees. Similarly, in the walnut trees, the inoculant application showed no significant effect compared to the application of the seaweed extract and the control treatment. The expected effect of this inoculant application was primarily due to its N-fixing ability, which could influence crop productivity. The N application to the soil only significantly affected the hazelnut trial during the year with the higher yield. The poor effect of N on the hazelnut yield might explain why no significant effect of inoculant application was observed. The main reasons for this likely include the alternate bearing in the second year, which may have obscured the N's impact on productivity, and the fact that in trees, due to the large volume of their canopy, it is more difficult to obtain a response to fertilizer application [43–45]. It should also be noted that in other studies, difficulties have been observed in obtaining plant responses to the application of this inoculant [46–48], suggesting that it may not be sufficiently effective, at least not for all crops, contrary to what has been suggested by the manufacturer [49,50]. The generalized recommendation of commercial products from the plant biostimulant group, without their effects being sufficiently clarified, has faced increasing criticism [46,47,51], particularly due to the negative impact on farmers' activities.

The seaweed extract, used only in the walnut trial, did not positively affect crop productivity. However, in other studies, using seaweed extracts has frequently positively affected crop growth and yield [52,53]. Even positive effects are often difficult to explain due to the complex and heterogeneous composition of seaweed extracts, which complicates the elucidation of their mode of action [54,55] and, consequently, the establishment of optimal application conditions. As a result, failures are also common, where no positive effects on crop yield are observed with the application of commercial products prepared from seaweed extracts, especially when studies are conducted under field conditions [56,57].

4.2. Trees' Access to Nitrogen

In the hazelnuts, applying N to the soil did not significantly increase the N concentration in the leaves. However, the average values were higher than those of the control in all the samplings, indicating a consistent trend. On the other hand, the N content in the kernels significantly increased in both harvests. These results unequivocally demonstrate that the trees fertilized with soil-applied N had access to higher amounts of the nutrient. As more N becomes available to plants, its concentration tends to increase in their tissues. This modest increase in leaf N might also be due to a dilution effect, as N availability enhances vegetative growth, leading to a dilution effect of the nutrient [42,58]. However, there was a significant increase in the N concentration in the kernels. Growing fruits are priority sinks for photosynthates and nutrients [59]. Some seeds accumulate a significant portion of their reserves in the form of protein [60,61]. Storage protein ensures seed germination and provides high nutritional value, as seen in hazelnuts [62] and walnuts [63]. Thus, it is unsurprising that the N concentration in the kernel increases as nutrient availability to the plant increases, and this occurs more proportionally than with its concentration in the leaves.

In the hazelnuts, the application of BlueN resulted in a significant increase in the leaf N concentration on one of the three sampling dates. In contrast, no significant differences were observed in the kernel. Regarding the walnuts, no significant differences were found in the leaf N concentration, but significant differences were observed in the kernel N concentration in one of the samplings. Although the average values from the samplings where no significant differences were found do not support this trend, it seems plausible that the application of the inoculant may have contributed to some improvement in the N nutritional status of the trees. Depending on the N-fixing system, biological N fixation can provide substantial amounts of this nutrient to plants. The legume/rhizobia symbiotic relationship, which is the most studied and promoted relationship in agricultural fields with a high potential for fixation, can exceed 400 kg N ha^{-1} yr^{-1} [9]. However, legume/rhizobia relationships are endophytic, where the high fixation capacity is due to the protection that leghemoglobin provides to nitrogenase from excess oxygen and the regular supply of photosynthates from the phloem [9]. In the aquatic fern Azolla filiculoides, N fixation can exceed 100 kg ha^{-1} yr^{-1}, facilitated by numerous cavities along its surface that house the cyanobiont *Anabaena azollae*. This symbiotic relationship allows for efficient nutrient exchange between the fern and the cyanobacteria, resulting from a 50-million-year coevolutionary process [10,17,64]. Similarly, some tropical grasses, such as sugarcane, access substantial amounts of atmospheric N through endophytic relationships with N-fixing microorganisms like *Gluconacetobacter diazotrophicus* and *Azospirillum brasilense*, which reside within their tissues [15,17,25]. All these N-fixing systems exhibit high specificity between the host plant and the microorganism responsible for N fixation.

The phyllosphere is known to harbor a diverse microbiome, which can include N-fixing microorganisms, particularly free-living fixers, though their N-fixation capacity is often limited by substrate availability [8,25,64]. Some microorganisms residing in the phyllosphere can access compounds released by plants, such as methanol, amino acids, or soluble carbohydrates [25,65]. The potential for a specific microorganism, such as *M. symbioticum*, to competitively inhabit the phyllosphere across different cultivated species and environmental conditions and to effectively fix and transfer N to the plant challenges conventional understanding. The results of this study, however, are inconclusive, though they suggest the possibility that the treated plants might have derived some N benefit from the presence of such microorganisms.

The application of algae to the walnut trees did not result in any observable effect on the yield or N nutrition of the plants. Various studies have reported positive outcomes from applying algae extracts for some physiological processes of plants, sometimes leading to increased productivity and/or quality of the products, as previously mentioned. Particularly positive results have been noted when studies involve natural or induced abiotic or biotic stresses [66–68]. In this study, the walnut trees were managed organically and had not received soil fertilization in recent years.

The leaf nutrient levels indicated a clear situation of nutritional stress, with low N levels in particular. In this study, the average N concentration in the leaves across the three samplings ranged from 22.2 to 25.1 g kg^{-1} (Table 4), while the sufficiency range is typically between 25.0 and 32.5 g kg^{-1}, as proposed by Bryson et al. [40]. The application of the algae extract did not have a significant measurable effect on alleviating N nutritional stress, suggesting that this type of stress may be less responsive to the application of algae extracts.

4.3. Effect of Treatments on Nutrients Other than Nitrogen

The effect of the soil N application and inoculant on the concentration of nutrients other than N in the hazelnuts was inconsistent. Significant differences occasionally appeared, but this trend did not persist across other sampling dates. When observing the results for the walnuts, a similar pattern emerged, with occasional significant differences that were inconsistent across other sampling dates. Inconsistencies also arose when comparing the two species, where one might show a trend of higher values with a given treatment, while the opposite occurred in the other species. For example, the B levels in the hazelnuts tended to be lower with inoculant application, whereas in the walnuts, they tended to be higher. In the factorial trial with hazelnuts, significant interaction sometimes appeared. However, once again, there was a lack of consistency between sampling dates. The results suggest that the effects of the treatments did not surpass the experimental variability, which is always challenging to control in field experiments [69].

Furthermore, it was shown that the application of soil N, the inoculant, and the algae extract had a modest effect on the concentrations of nutrients in the leaves, likely due to the relatively large size of the trees, which always makes it more difficult to observe a response to fertilizers [43–45].

5. Conclusions

The application of the inoculant did not affect the productivity of hazelnuts or walnuts, but it had a slight tendency to improve the N nutritional status of the trees. The algae extract did not affect the crop productivity or elemental composition of the walnut leaves. Considering the growing interest in studying plant biostimulants and the rapid introduction of new commercial products into the market, much remains to be understood about the conditions under which these products are truly effective. It is essential to remember that from a farmer's perspective, these inputs incur acquisition and application costs, and therefore, they should provide an economic return in terms of increased productivity and/or quality of agricultural products or reduced costs associated with other fertilizing materials.

The results of this study are not entirely conclusive, and further field evaluations of these products with these specific species are warranted. Efforts to reduce the use of commercial fertilizers should be a priority for all stakeholders, including scholars and industry professionals. However, this goal must be pursued without undermining profitability for farmers, which remains a cornerstone of the sustainability of agricultural processes.

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