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Nitrogen Assimilation, Biomass, and Yield in Response to Application of Algal Extracts, *Rhizobium* sp., and *Trichoderma asperellum* as Biofertilizers in Hybrid Maize

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Abstract: Nitrogen is essential for plants' growth, yield, and crop quality, and its deficiency limits food production worldwide. In addition, excessive fertilization and inefficient use of N can increase production costs and cause environmental problems. A possible solution to this problem is the application of biofertilizers, which improve N assimilation and increase biomass and yield. Therefore, the objective of this research was to evaluate the impact of the application of a combination of green and red algae (*Ulva lactuca* and *Solieria* spp.), *Rhizobium* sp., *Trichoderma asperellum*, and the combination of the above three biofertilizers on N assimilation. A completely randomized design was performed, with 10 plants per treatment and five treatments: T1 = control; T2 = algal extracts; T3 = *Rhizobium* sp.; T4 = *T. asperellum*; T5 = T2 + T3 + T4. Our analyses showed that the biofertilizers' application was better than the control. The application of *Rhizobium* sp. had the best performance amongst all of the biofertilizers, with the highest nitrate reductase activity in maize leaves, which enhanced photosynthesis, increasing biomass and yield. The use of *Rhizobium* sp. showed increases in biomass (13.4%) and yield (11.82%) compared to the control. This research shows that biofertilizers can be a key component for sustainable agricultural practices.

Keywords: algal extracts; biofertilizers; nitrogen assimilation; *Rhizobium* sp.; *Trichoderma asperellum*; *Zea mays* L.

1. Introduction

Maize (*Zea mays* L.) is the most used cereal for human consumption in Mexico, being a fundamental food item in the daily diet [1]. The largest maize producer in the world is the USA, with 348.75 million tons, while Mexico ranks sixth with 26.63 million tons [2]. To achieve high yields, chemical nitrogen (N) fertilizers are widely used in intensive maize production systems, due to the crop's high N demand [3,4].

Nitrogen (N) is essential for plants' growth, yield, and crop quality, and N deficiency constrains food production worldwide [5]. N is crucial for the synthesis of amino acids, proteins, and nucleic acids and is a key element in many physiological processes [6].

N assimilation is a complex process that begins with the uptake of nitrate (NO_3^-) and ammonium (NH_4^+), the main inorganic forms that plants can absorb. Once accumulated inside the roots, the nitrate reductase enzyme (NR) reduces NO_3^- to NO_2^-, and then the nitrite reductase enzyme (NiR) reduces NO_2^- to NH_4^+, which is then converted into organic N compounds [7,8]. Among all of the enzymes that participate in N assimilation, NR is the key player in regulating N's incorporation in plants [9]. Thus, improving N uptake and assimilation can boost yields and crop quality. However, excessive N fertilization and low N-use efficiency can lead to increased production costs and environmental concerns [10].

A potential solution to this issue is the application of biofertilizers, which improve soil fertility [11] and enhance plants' growth and yield [12]. Biofertilizers contain microorganisms or natural substances [13] that enhance soil properties, influence plant growth, and increase productivity both under optimal conditions and under stress, without harming the environment [14]. As examples of these biofertilizers, Plant Growth-Promoting Fungi (PGPF) and Plant Growth-Promoting Bacteria (PGPB) are beneficial microorganisms that are often used as biofertilizers to aid in plant growth [15] and can work as biological control agents [16–18]. Previous studies have demonstrated the effective use of biofertilizers as a sustainable strategy to improve plants' N uptake [19–21]. PGPB can facilitate the absorption of crucial nutrients such as N, phosphorous (P), and iron (Fe) or regulate plant hormones including auxin and cytokinin to promote plant growth [22]. Some of the most important genera of PGPB are *Rhizobium*, *Azotobacter*, *Bacillus*, and *Azospirillum* [23,24].

Algae, essential photosynthetic microorganisms abundant in N and potassium, are present in all terrestrial environments. They are used in agriculture as bio-based fertilizers to enhance soil nutrients, thereby promoting plant growth and increasing yields [25,26]. The levels (2, 4, and 6 kg L^{-1} of water) of seaweed extract (brown seaweed *Ecklonia maxima*) significantly affected all growth traits (plant height, stem diameter, number of cobs per plant, cob seeds' weight) and the yield of maize in both the spring and autumn seasons [27]. Several authors have reported the use of seaweed extracts (*Gracilaria edulis*, *Laminaria* sp., *A. nodosum*, and *K. alvarezii*) in maize crops, with increases in seed germination percentage and rate, seedling vigor, shoot and root growth, net carbon assimilation, and total grain yield [28–30]. When applied to drought-stressed green bean plants, *Ulva rigida* extracts improved vegetative growth by increasing chlorophyll contents, enhancing antioxidant systems, and reducing lipid peroxidation damage [31]. In a similar way, *Solieria chordalis* extracts promoted the height, weight, and chlorophyll contents of in vitro radish seedlings [32]. Different micronutrients (Ca, Mg, Mn, B, Zn, Cu) and plant regulators (abscisic acid, auxins, cytokinins, gibberellins, jasmonates) have been found in these extracts, which stimulate plants' metabolism and growth [33,34].

Several species of the genus *Rhizobium* produce nodules on the roots of legumes, as well as endophytes that can reside within plant tissues [35]. Under certain conditions, these bacteria act as phosphate solubilizers, fix N, and produce hormones in association with non-legume crops [36]. According to Widodo et al. [37], *Rhizobium* can enhance plants' growth directly by regulating the aforementioned plant hormones, which modulate root architecture and growth. Indeed, N absorption and utilization are dependent on root length, root surface area, and root biomass [36]. The inoculation of maize crops with *Rhizobium* sp. from various rhizospheres (rice rhizosphere isolates, maize rhizosphere isolates, soybean rhizosphere isolates, peanut rhizosphere isolates, and edamame rhizosphere isolates) showed a significant effect on plant height, stem diameter, husked ear weight, and dry ear weight compared to controls [37]. Additionally, significant increases in maize grain yield were reported when the crop was inoculated with several species of PGPB (*Azospirillum brasilense*, *Rhizobium tropici*, *Bradyrhizobium* sp., and *Rhizobium* sp.) [38,39].

Species of the genus *Trichoderma* are commonly found across the globe [40]. These microorganisms inhabit nearly all soil types and frequently function as plant mycoparasites, saprotrophs, and symbionts [41,42]. In a similar way to PGPF, *Trichoderma* inoculation improves N accessibility [43] by releasing organic acids in the soil. It has been observed that certain *Trichoderma* species provide benefits to crops, including enhanced plant growth, better seedling emergence, and improved seed germination [44]. As plant growth regulators, *Trichoderma* spp. can produce phytohormones [45,46] such as gibberellic acid [47] and indole-3-acetic acid [48], which are essential for plant growth. These phytohormones increase flowering, photosynthesis efficiency, and yield quality and improve root condition and structure, enhancing seed germination and viability [49]. Earlier studies on maize utilized *T. asperellum* and *Bacillus amyloliquefaciens* to treat seeds and plants in greenhouses, resulting in increased plant growth (shoot, root, and seedling length) [50]. In another experiment with maize, the authors used *T. harzianum* and *B. subtilis* to treat seeds and plants, observing increased plant growth and yield [25].

Incorporating biofertilizers into agricultural systems can address the growing food demand while minimizing environmental concerns, promoting environmental sustainability. These benefits make them a key component of sustainable agricultural initiatives. However, there is scarce literature on the use of biofertilizers based on algal extracts (*Ulva lactuca* and *Solieria* spp.), *Rhizobium* sp., and *Trichoderma asperellum* in hybrid maize. Therefore, the objective of the present study was to evaluate the impact of the application of a green and red algal mixture (*Ulva lactuca* and *Solieria* spp.), *Rhizobium* sp., *Trichoderma asperellum*, and the combination of the above three biofertilizers on N assimilation, biomass, and yield in hybrid maize.

2. Materials and Methods

2.1. Crop Management

The maize crop was grown in an open field. The location was in Lomas del Consuelo, Meoqui, Chihuahua, Mexico, from August to December 2022. Hybrid maize seeds (ASGROU A 75-73) were sown in loam soil, in 80 cm furrows, with 3 seeds per linear meter (33.3 cm between plants). Three fertilizations were applied, the first two a month apart and the third 22 days later. The first fertilization was 300 kg ha^{-1} of urea and 50 kg ha^{-1} of commercial MAP® 11-52-00, the second was 20 L ha^{-1} of commercial Nitro-Sul®, and the third was 150 kg ha^{-1} of urea dissolved in water and applied in irrigation. The plants were irrigated every 15 days using a rolling irrigation system.

2.2. Experimental Design and Treatments

A completely randomized design was implemented, with five treatments—T1 = control; T2 = algal extracts; T3 = *Rhizobium* sp.; T4 = *Trichoderma asperellum*; T5 = T2 + T3 + T4—using 10 replications per treatment (Figure 1).

Treatment 1 was the control; it was not under the effects of any biofertilizer; it only received the commercial fertilizers.

Treatment 2 was a combination of three commercial green and red algal products: Algomel Push® (10 mL L^{-1} of water), Algomel Proact® (5 mL L^{-1} of water), and Oceamax® (10 mL L^{-1} of water), which were applied to the leaves every 15 days, after the appearance of the first pair of true leaves.

For Treatment 3, the bacterium (*Rhizobium* sp.) was cultured in potato extract and 1% glucose [51] for 72 h to six days, and it was used at 10^9 colony-forming units (CFU) mL^{-1}. These products were provided by Dr. Micah Royan Isaac of the Olmix Company. *Rhizobium* sp. strains were isolated from the root nodules of bean plants (*Phaseolus vulgaris* L.) collected in Delicias, Chihuahua, México. The disinfection of nodules and the bacterium culture were carried out following the methodology of [52]. The bacterium isolate was grown in liquid medium for 72 h at 30 °C, and it was applied at a density of 10^6–10^7 CFU mL^{-1}.

For Treatment 4, *T. asperellum* biofertilizer was used as described by [53], and the fungus concentration used was 1×10^{10} UFM mL^{-1}. The *T. asperellum* strain used in this work is part of the Faculty of Agricultural and Forestry Sciences of Chihuahua, Mexico strain collection and is registered in GenBank under the accession number MN950427.

Treatment 5 was a combination of T2 + T3 + T4; the *Trichoderma asperellum* and *Rhizobium* sp. were applied to the soil near the rhizosphere, immediately followed by foliar application of the algal extracts.

In all treatments except the control, *Trichoderma asperellum* and *Rhizobium* sp. were applied to the soil near the roots of the plants at a rate of 20 L ha^{-1} every 15 days.

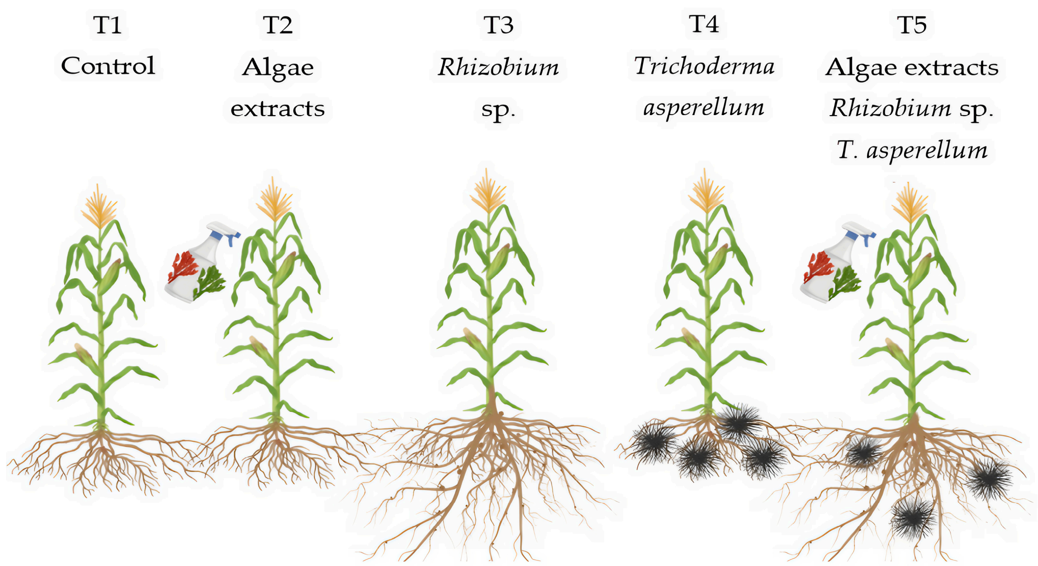

Figure 1. Application of biofertilizers in maize plants. T1 = control: plants without biofertilizers applied. T2 = algae extracts: foliar application of algae extracts to maize plants. T3 = *Rhizobium* sp.: root inoculation of maize plants with bacteria from the genus *Rhizobium* sp. T4 = *Trichoderma asperellum*: root inoculation of maize plants with the fungus *Trichoderma asperellum*. T5 = combination of algae extracts, *Rhizobium* sp., and *Trichoderma asperellum*: combined application of the three aforementioned biofertilizers.

2.3. Plant Sampling

At physiological maturity of the plants, 100 days after sowing, plant samples were collected and the roots, aerial parts, and cobs were separated from the 10 plants of each treatment and then carefully washed with distilled water. The fresh plant material was used for analysis of biomass, yield, nitrate reductase enzyme activity, and photosynthetic pigments.

2.4. Plant Analysis

2.4.1. Total Biomass and Yield

After 24 h of washing with distilled water, the roots, aerial parts, and cob of each plant were weighed on a precision balance (Luna LBL 34001e Precision Balance, Milton Keynes, UK). Biomass was expressed as the sum of all plant organs (g $plant^{-1}$ f.w.). Yield was expressed as the fresh weight of ears per plant (g $plant^{-1}$ f.w.).

2.4.2. Nitrate Reductase Enzyme Activity (NR Activity) "In Vivo"

The nitrate reductase enzyme (NR, EC 1.7.1.1) activity was determined by the methodology suggested in [54], where 0.1 g of fresh 7 mm diameter leaf discs was weighed and placed in 10 mL of incubation buffer (100 mM K-phosphate buffer, pH 7.5 and 1% (*v*/*v*) propanol). The samples were infiltrated at a pressure of 0.8 bars (NAPCO 5851 vacuum oven, Winchester, VA, USA) and they were incubated at 30 °C in the dark for 1 h (WIG-50 digital incubator, DAIHAN SCIENTIFIC, Seoul, Republic of Korea), after which they were placed in a FELISA FE-371 boiling water bath (Zapopan, Jalisco, Mexico) for 15 min to stop NR activity. Then, 1 mL of enzyme extract was taken, and 2 mL of 1% (*w*/*v*) sulfanilamide in 1.5 M HCl and 2 mL of 0.02% (*w*/*v*) N-(1-naphthyl)-ethylenediamine dichlorohydrate were added (20 mg of NNEDA dissolved in 100 mL of distilled water). Finally, the resulting nitrite concentration was determined spectrophotometrically (Thermo Fisher Scientific, GENESYS™ 10S, Madison, WI, USA) at 540 nm against a NO_2^- standard curve. The results were expressed as µmol of NO_2^- formed g^{-1} f.w. h^{-1}.

2.4.3. SPAD Values

SPAD (Soil Plant Analysis Development) index values were determined using the SPAD-502 portable chlorophyllometer (Konica Minolta Sensing, Inc., Osaka, Japan), which provides a quantitative assessment of leaves' green intensity [55]. The measurements were conducted around midday (during peak light hours), with three random readings taken per plant. Two readings were made with the SPAD-502 m: one on 15 October 2022, before the grain-filling stage, and the second one on 10 November 2022, before the maturation stage.

2.4.4. Photosynthetic Pigments

The methodology of [56] was used for photosynthetic pigment analysis. Leaves sample stalks of 7 mm in diameter, with approximately 0.125 g of weight from each treatment, were placed in test tubes, and 10 mL of methanol was added to each sample. The samples were left to rest for 24 h in the dark. After this time, the samples were read at wavelengths of 470, 653, and 666 nm with a Genesis 10S UV-VIS spectrophotometer (Thermo Fisher Scientific, GENESYS™ 10S, Madison, WI, USA). The results were expressed in μg cm^2 of fresh weight, and the following formulae were used (Equations (1)–(4)):

$$\text{Chl } a^* = [15.65(\text{A666}) - 7.34(\text{A653})] \quad (1)$$

$$\text{Chl } a = (\text{Chl } a^* \times \text{Vf} \times \text{W}_1)/(\text{W}_2 \times \pi \times \text{r}^2 \times n)$$

$$\text{Chl } b^* = [27.05(\text{A653}) - 11.21(\text{A666})] \quad (2)$$

$$\text{Chl } b = (\text{Chl } b^* \times \text{Vf} \times \text{W}_1)/(\text{W}_2 \times \pi \times \text{r}^2 \times n)$$

$$\text{Carotenoids}^* = [(1000^*\text{A470}) - 2.86(\text{Chl } a) - 129.2(\text{Chl } b)]/(221) \quad (3)$$

$$\text{Carotenoids} = (\text{Carotenoids}^* \times \text{V} \times \text{W}_1)/(\text{W}_2 \times \pi \times \text{r}^2 \times n)$$

$$\text{Total Chlorophyll} = \text{Chl} = \text{Chl } a + \text{Chl } b \quad (4)$$

where Vf: final volume; W_1: weight per leaf disc; W_2: total weight of leaf discs; r: radius of the leaf discs; *n*: number of leaf discs.

2.4.5. Number of Leaves and Plant Height

The number of leaves was determined by manual counting. The plant height refers to the measurement taken from the highest point to the base of naturally growing plants, and it was determined with a measuring tape. The results were expressed as cm. Both variables were measured before flowering.

2.5. Statistical Analysis

The normal distribution of the data was examined using the Shapiro–Wilk test ($p \leq 0.05$), and the equality of variances among the analyzed variables was assessed via Levene's test, considering significance at $p \leq 0.05$. Following this, ANOVA was conducted at a significance level of 0.05. The variables were then subjected to Duncan's test to compare the means of the two factor levels (dose and application time) at a significance level of $p \leq 0.05$. The statistical program SAS version 9.0 was used.

3. Results and Discussion

3.1. Biomass and Yield

Biomass is a crucial indicator for crops, as it reflects the total amount of organic matter accumulated by plants, reflecting crop yield potential [57]. In this experiment, all treatments displayed significant differences in biomass compared to the control. The highest biomass occurred in T3 (*Rhizobium* sp.), followed by T2 (algal extracts), T4 (*T. asperellum*), and T5 (combination of the three biofertilizers) (Figure 2). The application of the bacterium *Rhizobium* sp. induced an increment of 13.4% in the results compared to the control. The other treatments (algal extracts, *T. asperellum*, and the combination of all biofertilizers) had increases of 11.39%, 11.18%, and 11.52%, compared to the control, respectively. Several researchers have reported the application of *Rhizobium* sp. as PGPR (Plant Growth-Promoting Rhizobacteria) in other crops [24,58,59], because *Rhizobium* sp., like other rhizobacteria, effectively colonizes the roots of non-leguminous plants. It can solubilize phosphorus, produces siderophores and plant hormones, and also shows antagonistic effects against plant pathogens [60,61]. In the present study, the application of *Rhizobium* sp. improved the biomass. In physiological terms, this phenomenon may arise in maize because, by receiving an additional supply of N through biological N fixation, it can increase its rate of photosynthesis and its capacity to produce more plant matter. The combined treatment (T5) also showed high biomass, suggesting that synergistic interactions between the biofertilizers were optimizing the uptake of N and other nutrients, thus maximizing biomass production. As reported in [62–64], the use of biofertilizers promotes biological N fixation, making a more efficient use of N and increasing nutrient uptake, generating more biomass.

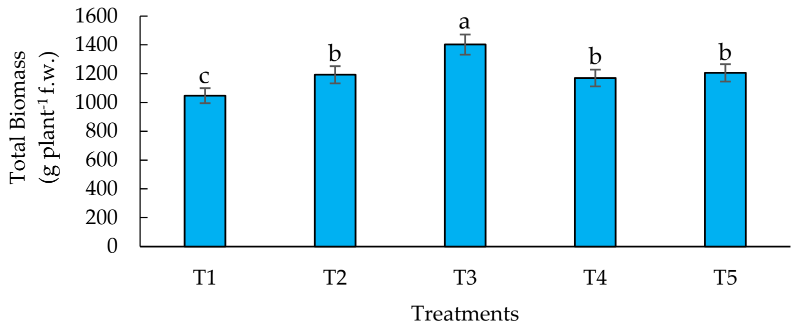

Figure 2. Effects of biofertilizers' application on total biomass: T1 = control; T2 = algal extracts; T3 = *Rhizobium* sp.; T4 = *T. asperellum*; T5 = T2 + T3 + T4. Means with equal letters do not differ according to Duncan's multiple range test ($p \leq 0.05$).

Crop yields are widely recognized as a key indicator for global food security [65]. In this experiment, the highest yields were obtained with *Rhizobium* sp. (T3) and the combination of algal extracts, *Rhizobium* sp., and *T. asperellum* (T5) as a management practice for hybrid maize cultivation. These treatments were significantly different from the rest. Treatments 2 (algal extracts) and 4 (*T. asperellum*) were not different to the control (Figure 3). Additionally, when applying *Rhizobium* sp. (T3) on the hybrid maize crop, an increase in yield of 11.82% was obtained, and when combining the three biofertilizers the increase was 11.60% compared to the control. In the case of maize, which has a high N demand, the presence of *Rhizobium* sp. could be a relevant factor that allows plants to avoid N deficiencies and favor fruit filling [66]. Furthermore, according to [67], the application of *Rhizobium* sp. reduces the internal competition for resources between vegetative and reproductive growth, optimizing both processes and favoring the final yield in terms of grain production. According to [68,69], the use of biofertilizers and their synergy can promote nutrient uptake and, thus, improve yield.

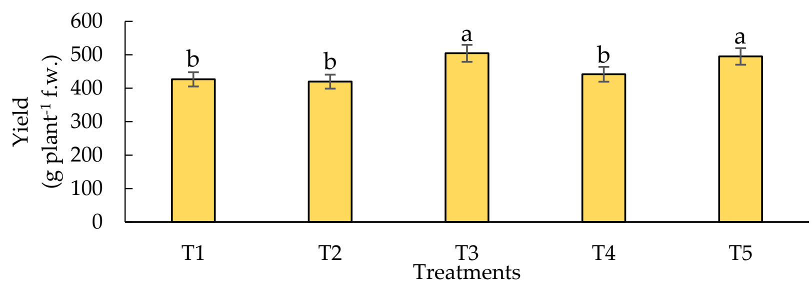

Figure 3. Effects of biofertilizers' application on yield: T1 = control; T2 = algal extracts; T3 = *Rhizobium* sp.; T4 = *T. asperellum*; T5 = T2 + T3 + T4. Means with equal letters do not differ according to Duncan's multiple range test ($p \leq 0.05$).

3.2. Nitrate Reductase Enzyme Activity (NR Activity) "In Vivo"

The NR enzyme is essential for nitrate assimilation in higher plants, so its activity is often a limiting process for N assimilation in plants [70]. In this experiment, the control (T1), algal extracts (T2), and *Rhizobium* sp.(T3) treatments showed no significant differences between them (Figure 4). However, T3 demonstrated the highest NR activity among these treatments. Moreover, there was a 20% increase in activity when compared to T. *asperellum* (T4) and the combination of the three biofertilizers (T5).

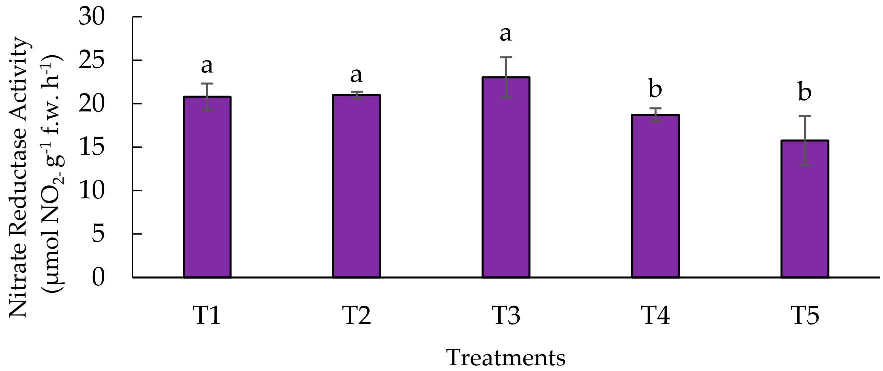

Figure 4. Effects of biofertilizers' application on nitrate reductase activity "In Vivo": T1 = control; T2 = algal extracts; T3 = *Rhizobium* sp.; T4 = *T. asperellum*; T5 = T2+T3+T4. Means with equal letters do not differ according to Duncan's multiple range test ($p \leq 0.05$).

It was observed that as NR activity decreased (particularly in T5), biomass and yield were not compromised; in fact, significant improvements in these parameters were observed (Figures 2 and 3). This suggests that T4 and T5 facilitated inorganic N uptake, making the process more efficient, where NR plays an important role. This is consistent with the findings reported by Shoresh et al. [71], where *Trichoderma* spp. applied to *Sebacinales* spp. and their interaction with other biofertilizers was able to improve N uptake in plants. This may be related to how microorganisms modify the rhizosphere environment and nitrogen assimilation pathways in plants. *T. asperellum* is known for its ability to promote plant growth and improve nutrient uptake efficiency through phosphate solubilization and providing organic N through the degradation of organic matter [72]. The availability of organic forms of nitrogen, such as amino acids or peptides, may reduce the need for inorganic nitrate as the main source of N, which would explain the lower activity of NR in this treatment [73]. In addition, the combination of algal extracts, *Rhizobium* sp., and *T. asperellum* (T5) could increase the availability of organic N and promote direct assimilation of ammonium or reduced forms of N, decreasing the need for the plant to use nitrate and, therefore, reducing NR activity. This synergy between biofertilizers may favor alternative metabolic pathways that are more efficient in nitrogen incorporation, optimizing biomass production without relying so much on nitrate reduction [74]. In the case of algal extracts, Kaushal et al. [75] noted that they provide not only alternative sources of N but also bioactive compounds that improve N assimilation efficiency and promote growth and yield in crops, such as maize, by overexpression of N transporter genes. Regarding T3 (*Rhizobium* sp.), it was observed that N assimilation was favored due to increased NR activity. *Rhizobium* sp. can fix atmospheric N and also produce auxins, which are essential for root growth. According to what was reported in [76], auxins promote cell elongation and the formation of lateral roots, increasing the nutrient uptake area in plants. On the other hand, there are factors that also affect nitrogen assimilation, such as the light/darkness cycle, CO_2 levels, temperature, nitrate and ammonium ions, sugars and sucrose, and genetic variability [55]. These findings reinforce the results obtained in this experiment, where inoculation with *Rhizobium* sp. led to a significant increase in NR activity. Similarly, previous research has shown that different *Rhizobium* species, such as *Mesorhizobium ciceri* and *Rhizobium phaseoli*, promote the growth of legumes and non-leguminous crops such as maize. These studies show that improved nutrient uptake and plant yield are related to the ability of these bacteria to stimulate root growth mechanisms and the assimilation of inorganic N [77–79].

3.3. SPAD Values

The chlorophyll index is a fast and non-destructive way to detect chlorophyll levels in plants and, thus, form an idea of their nutritional situation [80]. The index of chlorophyll in hybrid maize (ASGROU A 75-73) was affected by the application of the biofertilizers used (Figure 5). This variable allows us to know the levels of chlorophyll in the leaves and estimate the efficiency of the photosynthetic process.

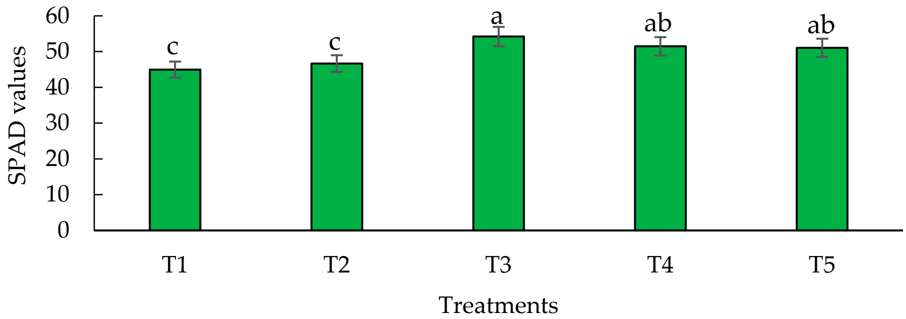

Figure 5. Effects of biofertilizers' application on SPAD values: T1 = control; T2 = algal extracts; T3 = *Rhizobium* sp.; T4 = *T. asperellum*; T5 = T2 + T3 + T4. Means with equal letters do not differ according to Duncan's multiple range test ($p \leq 0.05$).

The highest result was observed with the application of *Rhizobium* sp. compared to the other treatments, with a significant increase of 12% over the control. The treatments with *T. asperellum* and the combination of all biofertilizers did not show significant differences between them, although they were significantly different to the control, showing increases of 11.45% (T4) and 11.35% (T5) over it, respectively. The lowest SPAD values were observed in the control and the algal extracts. Previous research has documented increases in chlorophyll content (SPAD values), photosynthetic pigments, biomass, and yield after the application of biofertilizers to maize crops [81–83]. Referring to the chlorophyll content SPAD values, the highest values in this research were obtained when applying *Rhizobium* sp.; likewise, in terms of total chlorophyll and yield, the values were similar when applying the bacterium and the combination of the three biofertilizers. In this context, [59] inoculated *Rhizobium* sp. in maize crops, resulting in an increase in shoots' relative chlorophyll content, dry weight, photosynthetic rate, leaf area, and grain yield. Hussain et al. [59] reported a significant increase in chlorophyll levels when a rhizobial strain identified as *Rhizobium phaseoli* was used in maize under drought conditions induced by polyethylene glycol. Comparable results were described by Egamberdieva et al. [84] in fodder maize inoculated with rhizobia in field conditions, noting significant increases in chlorophyll content, photosynthesis, and transpiration rate.

3.4. Photosynthetic Pigments

Photosynthetic pigments are molecules that capture light and transfer it to the reaction centers to be converted into chemical energy, enabling photosynthesis [85]. These pigments can be divided into total Chl (Chl a and Chl b) and total carotenoids. Chl is the main pigment for light absorption, while carotenoids play a supporting role. N is essential for the synthesis of photosynthetic pigments; thus, a continuous and adequate supply of N is necessary [86]. In this experiment, the application of *Rhizobium* sp. in maize plants and the combination of the three biofertilizers induced higher Chl contents, with significant differences compared to the other treatments. Likewise, the highest values for carotenes were obtained with *Rhizobium* sp. In both cases, the lowest pigment contents occurred in the control (Figure 6). The increase in Chl content observed in the *Rhizobium* sp. treatment compared to the control was 18.4%, and the treatment (T5) with the combination of biofertilizers had an increase of 15.98% compared to the control. Regarding carotenes, treatment with *Rhizobium* sp. and the combination of biofertilizers (T5) had the highest significant values compared to the control (13.6%). Photosynthesis is one of the most important processes in plants, determining biomass and yield [81,82]. This increase could be attributed to improved NR activity (Figure 4) but also to increased N fixation and uptake. It is also possible that siderophores (iron-chelating molecules) released by *Rhizobium* sp. increased Fe's accessibility for plants [87]. Fe is also a key component for Chl synthesis, redox reactions, and several biochemical reactions, e.g., N fixation. Algal effects on crops include increased N availability from soil and improved photosynthetic pigment content due to the presence of phytohormones that share similar regulatory roles in higher plants [12,88]. *T. asperellum* could upregulate the photosynthetic pigment concentration by the upregulation of genes modulating Chl and carotenoid biosynthesis or the response against reactive oxygen species [89,90]. The secondary metabolites released by *Trichoderma* sp. may influence plants' nutrient uptake and growth by activating phytohormones consisting of indole acetic acid or auxin [91].

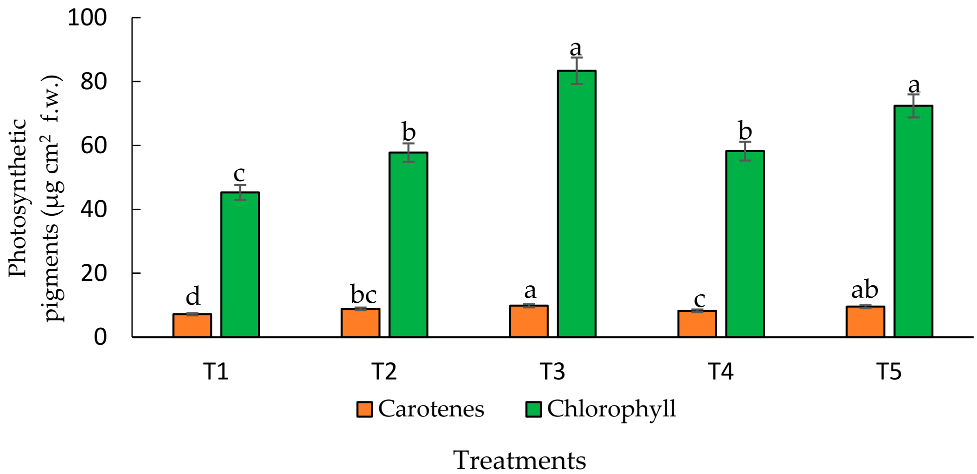

Figure 6. Effects of the application of biofertilizers on photosynthetic pigment activity: T1 = control; T2 = algal extracts; T3 = *Rhizobium* sp.; T4 = *T. asperellum*; T5 = T2 + T3 + T4. Means with equal letters do not differ according to Duncan's multiple range test ($p \leq 0.05$).

3.5. Number of Leaves and Plant Height

Leaves play key roles in photosynthesis, carbon fixation, respiration, and transpiration [92]. The number of leaves in hybrid maize plants (ASGROU A 75-73) was higher with the application of *Rhizobium* sp., with significant differences compared to the other treatments (Figure 7).

The hybrid maize plants increased their leaf numbers by 11.4% compared to the control when *Rhizobium* sp. was used (T3). The combination of the three biofertilizers (T5) increased this variable by 11.1%, while the application of *T. asperellum* (T4) showed a 10% increase. Regarding plant height, the results did not show significant differences when using *Rhizobium* sp. (T3) and the three combined biofertilizers (T5) (Figure 8), obtaining increases of 11.27% and 11.21% when compared to the control, respectively. The lowest value was obtained with the algal extracts (T2). On the other hand, the control (T1) and the plants treated with *T. asperellum* (T4) were statistically similar.

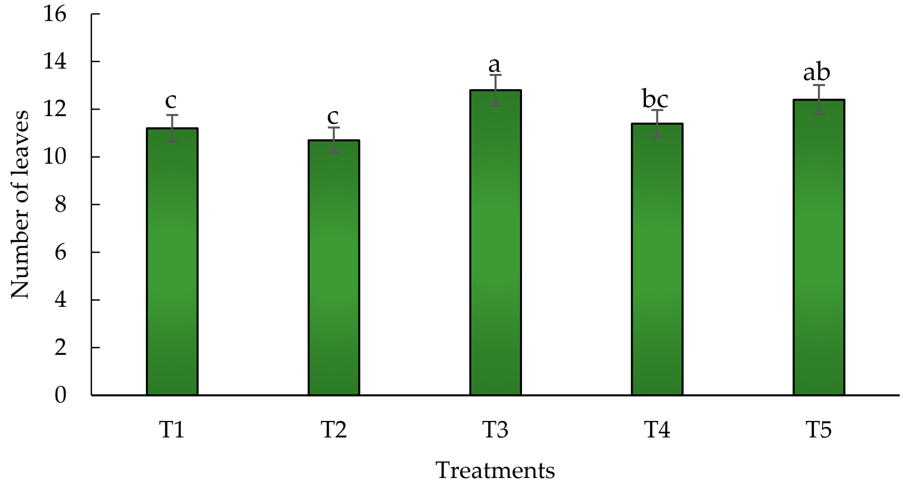

Figure 7. Effects of biofertilizers' application on leaf numbers: T1 = control; T2 = algal extracts; T3 = *Rhizobium* sp.; T4 = *T. asperellum*; T5 = T2 + T3 + T4. Means with equal letters do not differ according to Duncan's multiple range test ($p \leq 0.05$).

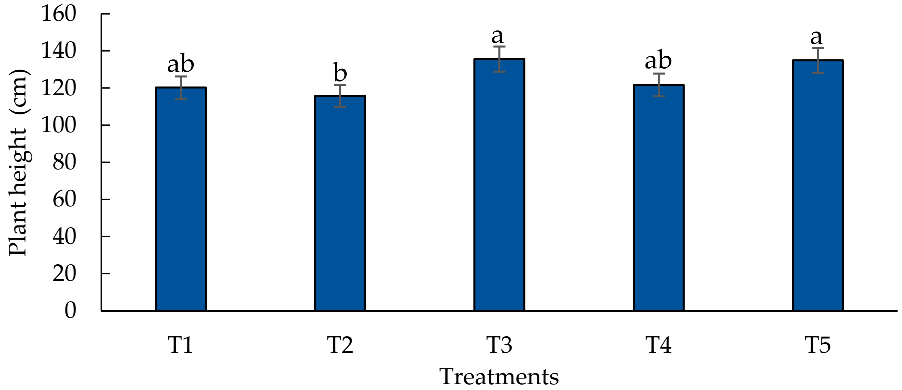

Figure 8. Effects of biofertilizers' application on plant height: T1 = control; T2 = algal extracts; T3 = *Rhizobium* sp.; T4 = *T. asperellum*; T5 = T2 + T3 + T4. Means with equal letters do not differ according to Duncan's multiple range test ($p \leq 0.05$).

In this investigation, the application of *Rhizobium* sp. to the soil increased the number of leaves and the height of the maize plants, most likely because *Rhizobium* has different mechanisms to promote plant growth. These include direct mechanisms such as N fixation, phosphate solubilization, and the production of phytohormones [85]. An increase in N fixation due to *Rhizobium* can improve plant growth, leading to more biomass accumulation, as noted in alfalfa [93]. Another trait of *Rhizobium* is the capacity to transform soluble phosphate from its insoluble form via the release of different organic acids (acetic acid, oxalic acid, and gluconic acid) or by proton (H^+) extrusion. P is not only essential for the formation of organic compounds (DNA, RNA, ATP), it is also involved in cell division and energy transfer processes [94]. The production of phytohormones by various bacteria is also an important trait, and these comprise gibberellins, indole acetic acid, auxins, cytokinins, and abscisic acid, which are crucial for plant and leaf growth and stem elongation in plants [84]. Apart from phytohormones, enzymes including phytase and protease can also be synthesized, enhancing nutrient uptake [95]. Moreover, *Rhizobium* can stimulate root growth, facilitating the absorption of crucial elements such as Fe and P [22]. Similar results were found in [96], where the application of *Rhizobium* (*R. etli* bv. *Phaseoli*, *R. leguminosarum* bv. *Trifolii*) and *Sinorhizobium* sp. improved the plant height, corn growth, and grain yield of maize in several areas. In another study, the inoculation of maize with several *Rhizobium* species (Cp3, Lt2, and Br3) boosted the plant height from 101.0 to 104.0 cm [97]. In addition, Yalçın et al. [33] reported an increase in plant height, stem diameter, ear dry weight, and ear weight without husks upon the application of *Rhizobium* sp. in maize. In the present experiment, the maximum height of the plants ranged from 135 to 134 cm (T3–T5).

3.6. Correlation Analysis and Heatmap

The Pearson heatmap and correlation (Figure 9) allowed us to observe the dependence or interdependence relationships between the variables analyzed. The color scale indicates that the shades closer to red are strong positive correlations (close to +1), while the bluish shades show negative correlations (close to −1) and white or light color suggests no or very low correlation.

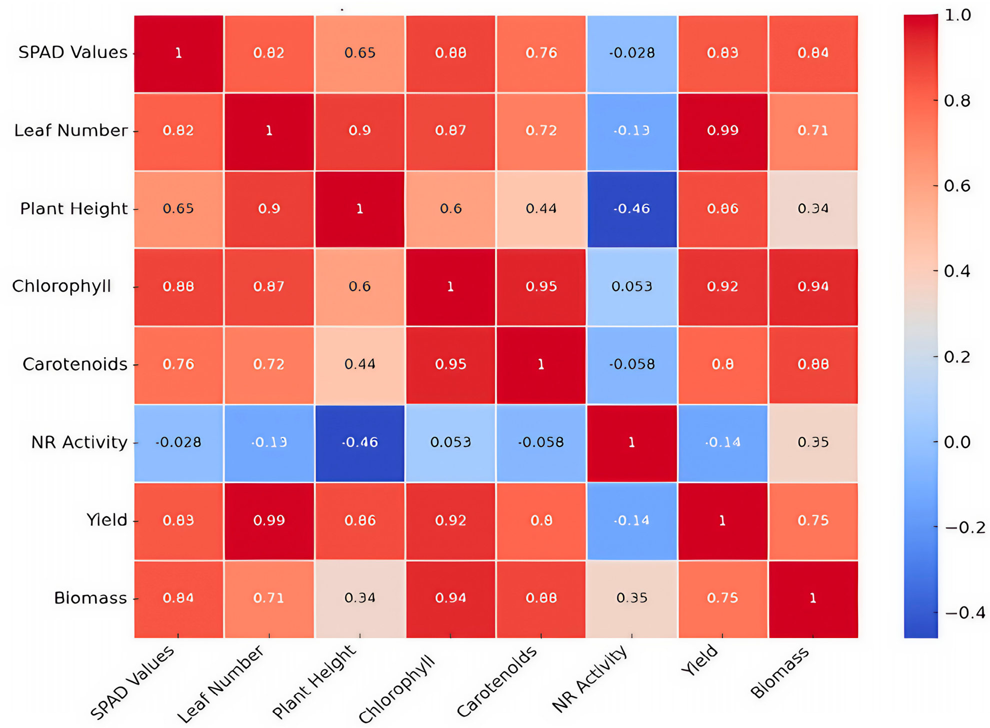

Figure 9. The figure shows the Pearson correlation analysis between the variables of biomass, yield, nitrate reductase activity, SPAD values, photosynthetic pigments, number of leaves, and plant height, evaluating the effects of the biofertilizers. The colors in the heatmap represent the magnitude and direction of the correlations, with red tones indicating strong positive correlations and blue tones indicating negative or weak correlations. The numerical values within each cell indicate the correlation coefficient between pairs of variables, providing a clear view of the interdependent relationships among the measured variables.

The map shows that leaf number and yield have an almost perfect correlation (0.99), indicating that a higher the number of leaves increases the yield significantly. Total Chl and carotenoids have a very high correlation (0.95), suggesting that the presence of one of these pigments is closely related to that of the other. Total Chl and biomass also have a strong correlation (0.94), indicating that biomass accumulation is directly related to the amount of Chl in plants.

The number of leaves and plant height (0.9) are strongly correlated, indicating that the higher the number of leaves, the greater the plant height. Plant height and yield (0.86) and plant height and number of leaves (0.9) have strong positive correlations, suggesting that taller plants tend to generate higher yields and have more leaves. Biomass is also strongly correlated with yield (0.75), indicating that an increase in biomass is related to higher plant yields. NR activity had weak negative correlations with variables such as plant height (−0.46), number of leaves (−0.13), yield (−0.14), and biomass (0.35), suggesting that an increase in the activity of this enzyme may be slightly related to a decrease in these variables, probably due to the effects of the biofertilizers applied.

3.7. Venn and Interaction Diagram

Figure 10 is a clear and concise representation of how different biofertilizer treatments (algal extracts, *Rhizobium* sp., *Trichoderma asperellum*, and their combination) directly impact the growth, physiology, and yield of hybrid maize.

Part (a): Venn diagram

This diagram illustrates the synergy between the different biological treatments and how, by combining them, outstanding effects on critical variables in plant growth can be obtained. The intersections of the diagram show that T2 is particularly associated with an increase in NR activity, which is crucial for nitrogen assimilation and, thus, for plant growth. *Rhizobium* sp. stands out in terms of the increase in SPAD values, which are related to the amount of Chl in the leaves and, therefore, to photosynthetic efficiency. *Trichoderma asperellum* favors the production of photosynthetic pigments, which are essential for light absorption and efficiency in the photosynthetic process. The central zone of the diagram, which combines the three treatments (T5), shows improvements in biomass, number of leaves, plant height, and yield, suggesting that the joint application of these microorganisms and plant extracts could be more effective than the individual treatments. This combination not only optimizes resource use but also tends to maximize plant growth and productivity.

Part (b): Bar chart

This bar chart quantifies the impact of each treatment (T1 to T5) on key variables. Analyzing biomass, treatment T3 generated the greatest increase, implying that the application of *Rhizobium* sp. on hybrid corn significantly enhances overall plant growth. In yield, as with biomass, the T3 treatment offered the best results, representing a crucial contribution to sustainable agricultural productivity. In NR activity, a significant increase was observed with the T2 and T3 treatments, indicating that these biofertilizers improve the efficiency of nitrogen assimilation, an essential process for plant metabolism. In SPAD values, T3 and T5 presented the greatest increases, suggesting an improvement in photosynthetic efficiency, crucial to biomass production and yield. For the variable photosynthetic pigments, the treatments with *Rhizobium* sp. (T3) and the combination of all biofertilizers (T5) promoted the production of essential pigments for photosynthesis, which also contributed to better growth and yield. As for the number of leaves and plant height, all biofertilizers except for T2 increased these variables considerably.

(**a**)

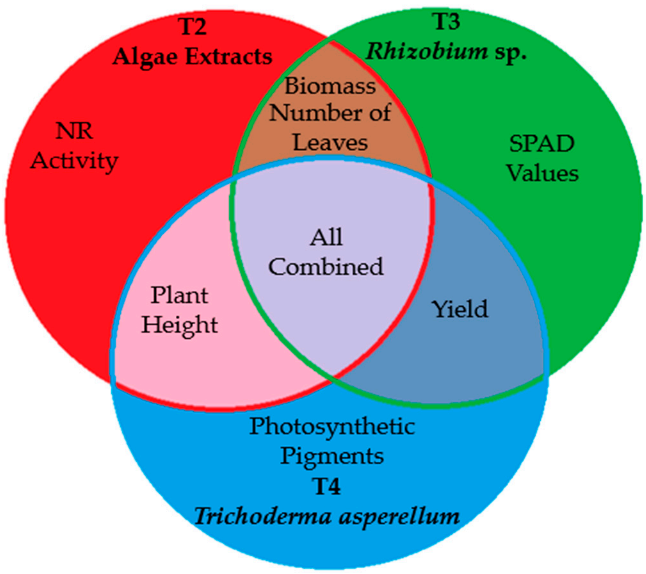

Figure 10. *Cont.*

(**b**)

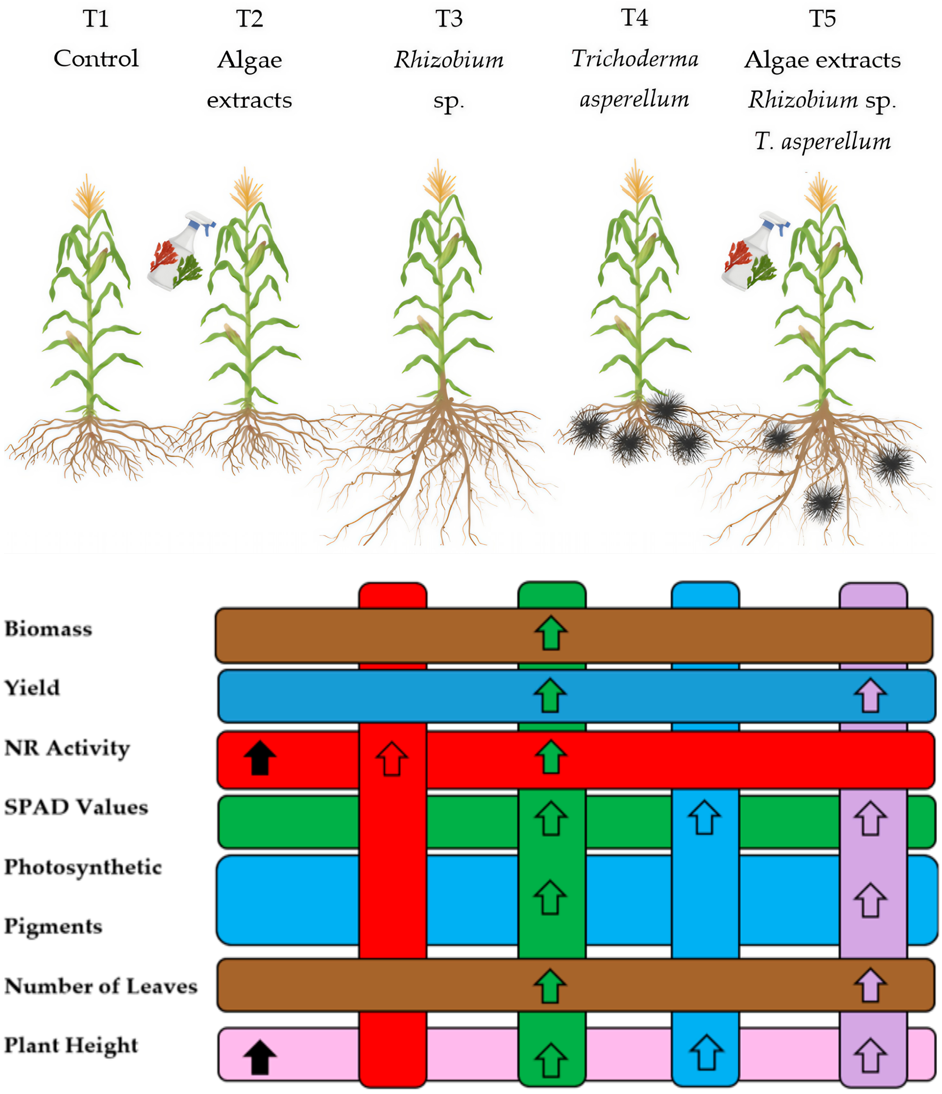

Figure 10. (**a**) Venn diagram representing the effects of different treatments on plant growth and physiological variables. (**b**) Interaction diagram: The bar chart shows how the different variables (biomass, yield, nitrate reductase activity, SPAD values, photosynthetic pigments, number of leaves, and plant height) respond to each treatment (T1 to T5). The arrows indicate an increase in these variables compared to the control (T1).

4. Conclusions

In this experiment, the application of *Rhizobium* sp. to the rhizosphere in hybrid maize plants had a greater effect on nitrogen assimilation, thanks to the symbiotic relationship generated with the maize. It is important to mention that treatment T5, which was the combination of algal extracts, *Rhizobium* sp., and *Trichoderma asperellum*, also had very positive effects on nitrogen assimilation. However, more studies are needed on the synergistic relationship that these biofertilizers form in terms of nitrogen assimilation and nitrogen-use efficiency. Finally, the use of *Rhizobium* sp. and the combination of algal extracts, *Rhizobium* sp., and *Trichoderma asperellum*, in relation to N assimilation and N-use efficiency, has not been sufficiently explored; thus, this research adds a new dimension to the field of study by demonstrating that these biofertilizers produce a very effective synergistic effect on nitrogen assimilation, which promises to be a key mechanism for sustainable agricultural practices in the future.

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