Effect of L-amino acid-based biostimulants on nitrogen use efficiency (NUE) in lettuce plants

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Abstract

BACKGROUND: Biostimulants are increasingly integrated into production systems with the goal of modifying physiological processes in plants to optimize productivity. Specifically, L-α-amino acid-based biostimulants enhance plant productivity through improved photosynthesis and increased assimilation of essential nutrients such as nitrogen (N). This element is a major component of fertilizers, which usually are applied in excess. Thus, the inefficient use of N fertilizers has generated a serious environmental pollution issue. The use of biostimulants has the potential to address problems related to N fertilization. Therefore, the objective of this study is to analyze whether two biostimulants based on L-α-amino acid (Terra Sorb® radicular and Terramin® Pro) designed by Bioiberica, S.A.U company can compensate deficient N fertilization and test its effect on lettuce plants.

RESULTS: Results showed that regardless of N fertilization, the use of both biostimulants, especially Terramin® Pro, increased biomass production. Moreover, both biostimulants enhanced photosynthetic, NO3− and total N accumulations as well as NUE parameters.

CONCLUSION: Therefore, Terra Sorb® radicular and Terramin® Pro constitute a useful tool for crops development in N-limiting areas, and in intensive agricultural areas without N deficiency allowing the reduction of N inputs without impairing crop yields and reducing environmental impact.

Keywords: biostimulants; chlorophyll; L-amino acid; nitrogen; nitrogen use efficiency; lettuce

INTRODUCTION

The regulation of plant growth along with the mitigation of the negative effects of environmental stresses during ontogenesis are important factors that determine the productivity of different agricultural crops. Current understanding of the mechanisms involved and the strategies to mitigate stress effects is still limited. Abiotic stress can be prevented by optimizing plant growth conditions by providing water, nutrients, and growth regulators. In addition to these traditional approaches, biostimulants are increasingly integrated into production systems with the goal of modifying physiological processes in plants to optimize productivity. Plant biostimulants based on natural materials have received considerable attention from both the scientific community and commercial companies, especially in the last two and a half decades. Thus, biostimulants have a potentially novel approach by intervening in the regulation/modification of physiological processes in plants to stimulate growth, mitigate stress-induced constraints, and increase yield.

To be considered as a biostimulant, a product should work at low doses, be ecologically benign, and have reproducible benefits in the cultivation of crops. Biostimulants may be based on beneficial microorganisms, algae extracts, or organic plant material. Another type of widely used biostimulant is those based on hydrolyzed proteins and amino acids (AAs) either of animal or vegetable origin. The mechanisms of action of biostimulants are very diverse and may include activation of nitrogen (N) metabolism or phosphorus (P) release from soils, generic stimulation of soil microbial activity, and root growth enhancement. Besides, biostimulants enhance plant growth by promoting physiological processes such as germination, photosynthesis, and nutrient uptake from the soil. Specifically, AA-based biostimulants increase plant productivity-improving ion transport, photosynthesis, and stress responses, and increase assimilation of essential nutrients such as N, carbon (C), and sulfur (S).
Considering N, this element is one of the main essential nutrients and a basic component in proteins and nucleic acids, AAs, NO₃⁻, and NH₄⁺ and is essential in the biochemistry of many non-protein compounds such as coenzymes, photosynthetic pigments, secondary metabolites, and polyamines. Nitrogen is a major component of fertilizers due to its importance in the plant as well as its high requirements. In addition, the importance of N is currently magnified due to the fact that current agriculture is dominated by production because of the growing worldwide food demand. This increment must be done without expanding the land under cultivation since agriculture currently occupies most of the fertile land and uses a large part of the resources such as water and fertilizers needed for its maintenance. The current situation has generated a dynamic in which the excessive application of fertilizers has become a common agronomic practice, especially in the case of nitrogenous fertilizers. Thus, the expected world demand for synthetic N fertilizers in 2022 is 111 591 thousand tons.

Besides, the inefficient use of N fertilizers has also increased in recent decades generating a serious environmental pollution issue. The most frequent impact produced by misuse of nitrogenous fertilizers is NO₃⁻ leaching to subterranean aquifers, which causes eutrophication of freshwater and marine ecosystems. Moreover, the generated gaseous NOxides can reach the troposphere and react with ozone to produce toxic ammonia emissions. Likewise, NO₃⁻ also poses a risk to human health since once ingested is rapidly transformed into nitrite and N-nitroso compounds. These compounds are toxic and can generate serious diseases such as methemoglobinemia and increase the risk of cancer by transforming nitrites into nitrosamines in the human body. These risks are remarkable considering that vegetables are only capable of converting 30–40% of the applied N into products destined for human consumption, producing later NO₃⁻ accumulation mainly into leaves, affecting the nutritional quality of the so-called ‘leafy vegetables’, such as lettuce and spinach.

Another problem with nitrogenous fertilizers is that their efficiency in agriculture is generally relatively low since a large part of the N provided in these fertilizers is not used to increase production. Nitrogen use efficiency (NUE) is defined as biomass production per unit of available N. This parameter may be divided into two processes: the ability of the plant to absorb N from the soil or N uptake efficiency (NUEp) and the capability of the plant to transfer and utilize this element in the biomass production of the different plant organs or N utilization efficiency (NUTE). Therefore, higher NUE could improve crop yield and quality, reducing economic costs, and decreasing environmental degradation caused by N fertilizer application.

Considering all the problems associated with N fertilizers, it is essential to use agronomic techniques to increase NUE by plants. Thus, the use of biostimulants has the potential to address all the problems related to N fertilization. In the present study, we analyzed two L-α-AA-based biostimulants obtained through enzymatic hydrolysis and designed and produced by Bioiberica, an S.A.U company. Therefore, the objective of this study is to test the efficacy of these biostimulants on the growth of lettuce plants and to analyze whether they can compensate for deficient N fertilization. The main hypothesis to be tested is that biostimulants will be able to offset the N requirements of lettuce plants.

**MATERIAL AND METHODS**

**Plant material and growing conditions**

Lettuce plants (*Lactuca sativa* cv. Isasa) were used for this project. The seeds of these plants were germinated and grown for 45 days in a tray with cells (cell size, 3 cm × 3 cm × 10 cm). Afterwards, the seedlings were transferred to a culture chamber with environmental-controlled conditions: relative humidity 60–80%, temperature 25 °C/15 °C (day/night), and 16 h/8 h of photoperiod with a PPFD (photosynthetic photon-flux density) of 350 μmol m⁻² s⁻¹ (measured with a sensor SB quantum 190, LI-COR Inc., Lincoln, NE, USA).

Under these conditions, the plants were grown in individual pots (13 cm upper diameter, 10 cm lower diameter, 12.5 cm high, and a volume of 2 L) filled with perlite: a vermiculite mixture. The fertilization consisted of a complete Hoagland type nutritive solution, with small modifications for the lettuce culture, composed of 4 mmol L⁻¹ KCl, 4 mmol L⁻¹ CaCl₂, 2 mmol L⁻¹ MgSO₄, 1 mmol L⁻¹ KH₂PO₄, 1 mmol L⁻¹ NaH₂PO₄, 2 mmol L⁻¹ MnCl₂, 1 mmol L⁻¹ ZnSO₄, 0.25 mmol L⁻¹ CuSO₄, 0.1 mmol L⁻¹ Na₂MoO₄, 125 mmol L⁻¹ Fe-EDDHA, and 50 μmol L⁻¹ H₃BO₃, with a pH of 5.8. The amount of the nutritive solution applied each day was approximately 50 mL per pot, not exceeding, in any case, the drainage volume by 10%.

**Treatment description and experimental design**

In the experiment, two different factors were considered, N fertilization and biostimulant application. The three different N fertilizations were: 8 mmol L⁻¹ NaNO₃ (N-100%), 4.8 mmol L⁻¹ NaNO₃ (N-60%), and 2.4 mmol L⁻¹ NaNO₃ (N-30%). Biostimulants were applied at a dose of 2 mL L⁻¹ in combination with the nutritive solution. Biostimulant 1 (BS-1) is Terra Sorb® radicular, whose composition is: 6% L-α-AA, 3% total N, 1.2% organic N, 8% organic matter (w/w). Biostimulant 2 (BS-2) is Terramin® Pro, whose composition is: 18% L-α-AA, 6% total N, 6% organic N, 40% organic matter (w/w). The organic matter comes exclusively from the hydrolyzed protein, which means from the free L-α-amino acid and peptides with a molecular weight <10 KDa. The specific AA profiles are detailed in the following table.

| Table 1. Free amino acid (AA) profiles for Terra Sorb® radicular and Terramin® Pro |
|-----------------------------|-----------------------------|
| Terra Sorb® radicular       | Terramin® Pro               |
| Asp                         | 0.43                        | 1.58                        |
| Glu                         | 0.59                        | 2.98                        |
| Ala                         | 0.39                        | 1.54                        |
| Arg                         | 0.32                        | 0.89                        |
| Cys                         | 0.02                        | 0.16                        |
| Phe                         | 0.29                        | 0.70                        |
| Gly                         | 0.58                        | 1.64                        |
| Hyp                         | —                           | 0.08                        |
| His                         | 0.17                        | 0.50                        |
| Ile                         | 0.30                        | 0.35                        |
| Leu                         | 0.53                        | 0.52                        |
| Lys                         | 0.39                        | 1.95                        |
| Met                         | 0.38                        | 0.09                        |
| Pro                         | 0.28                        | 1.02                        |
| Ser                         | 0.32                        | 1.08                        |
| Tyr                         | 0.36                        | 0.26                        |
| Thr                         | 0.36                        | 1.45                        |
| Trp                         | 0.05                        | 0.04                        |
| Val                         | 0.40                        | 1.18                        |

L-α-AA concentrations are expressed as % (w/w). aspartic acid (Asp), glutamic acid (Glu), alanine (Ala), arginine (Arg), cysteine (Cys), phenylalanine (Phe), glycine (Gly), hydroxyproline (Hyp), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), proline (Pro), serine (Ser), tyrosine (Tyr), threonine (Thr), tryptophan (Trp), valine (Val).
in Table 1. Both biostimulants were obtained through exclusive enzymatic hydrolysis (Enzyneer®). Both sodium nitrate (NaNO₃) and biostimulants were applied together with the nutritive solution. BS-1 provides 1.25 mmol L⁻¹ of N and BS-2 provides 2.5 mmol L⁻¹ of N. Thus, the total N supplied to the plants per treatment was: 8 mmol L⁻¹ (N-100%), 9.25 mmol L⁻¹ (N-100% + BS-1), 10.5 mmol L⁻¹ (N-100% + BS-2), 4.8 mmol L⁻¹ (N-60%), 6.05 mmol L⁻¹ (N-60% + BS-1), 7.3 mmol L⁻¹ (N-60% + BS-2), 2.4 mmol L⁻¹ (N-30%), 3.65 mmol L⁻¹ (N-30% + BS-1), 4.9 mmol L⁻¹ (N-30% + BS-1).

The application of the different treatments started 55 days after germination and the biostimulants were applied six times with a periodicity of 7 days between each time. The experimental design consisted of a complete randomized block with nine treatments, eight plants per treatment arranged in individual pots with the treatments randomly distributed in the culture chamber.

Plant sampling
The shoot of the plants was separated from the root. The plant material was washed with distilled water, dried on filter paper and then weighed to obtain the fresh weight (FW). A part of the leaves was frozen at −40 °C and was used for the analysis of chlorophyll and NO₃⁻ concentrations. The other part of the plant material, after drying in a forced-air oven was used to determine the dry weight (DW), as well as the total N concentration.

Chlorophyll (Chl) concentration
The concentration of photosynthetic pigments was analyzed by Wellburn's method with slight modifications. The plant material (0.1 g) was homogenized in 1 mL of methanol. Subsequently, it was centrifuged for 5 min at 5000 × g. Absorbance was measured at three different wavelengths: 666 nm, 653 nm, and 470 nm, and the following calculations were made based on the following equations:

\[
\text{Chlorophyll a (Chl a)} = 15.65 \times A_{666\text{nm}} - 7.34 \times A_{653\text{nm}}.
\]
\[
\text{Chlorophyll b (Chl b)} = 27.05 \times A_{653\text{nm}} - 11.21 \times A_{470\text{nm}}.
\]

Total Chl was calculated as the sum of Chl a + Chl b.

Analysis of chlorophyll a fluorescence
The plants were adapted to 30 min of darkness before taking the measurements using a special leaf clip that was placed on each of

Figure 1. Photography showing the effects of Terra Sorb® radicular (BS-1) and Terramin® Pro (BS-2) and 100% N supply (A), 60% N supply (B), and 30% N (C) supply on lettuce plants 90 days after germination.
the leaves. The kinetics of Chl a fluorescence was determined using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd, King’s Lynn, UK). Chl a fluorescence was induced by red light (650 nm) with a light intensity of 3000 μmol photons m⁻² s⁻¹. The fluorescence was analyzed by the JIP test. Measurements were made on fully developed leaves at the mean plant position of six plants per treatment. The following parameters obtained were made on fully developed leaves at the mean plant position of six plants per treatment. The following parameters obtained from the JIP test were used to study energy fluxes and photosynthetic activity: Fv/Fm and P_/ABS.²¹

Nitrate ion (NO₃⁻) and total nitrogen (N) concentration
Nitrate ion (NO₃⁻) was determined from aqueous extraction of 0.1 g of dry leaves in 10 mL of Milli-Q filtered water. A 100 μL aliquot was taken and added to 10% (w/v) salicylic acid in 96% sulfuric acid, determining the NO₃⁻ concentration by spectrophotometry as described by Cataldo et al.²²

For the total N analysis, a sample of 0.1 g of dry leaves was digested with sulfuric acid and hydrogen peroxide. After dilution with deionized water, a 1-mL aliquot of the digest was added to a reaction medium containing sodium silicate/sodium nitroprusside, sodium hydroxide, and sodium dichloroisocyanurate. Samples were incubated at 37 °C for 15 min, and total reduced N was determined using a spectrophotometer (Infinite 200 Nanoquant, Tecan, Switzerland) according to Krom.²³

Nitrogen use efficiency (NUE) parameters
The equations described by Xu et al.²⁴ were used for the calculation of NUE, N, NUpE, NUE, and apparent N fertilizer recovery.

| Table 2. Effect of Terra Sorb® radicular (BS-1) and Terramin® Pro (BS-2) on leaf and root biomass of lettuce plants grown under three different nitrogen (N) supplies |
|------------------------|------------------------|------------------------|------------------------|------------------------|
|                        | Leaf FW (g⁻¹ plant)    | Leaf DW (g⁻¹ plant)    | Root FW (g⁻¹ plant)    | Root DW (g⁻¹ plant)    |
| N-100%                 | 40.41 ± 5.38c          | 2.84 ± 0.44c           | 13.46 ± 2.45b          | 0.28 ± 0.03a           |
| N-100% + BS-1          | 46.23 ± 3.17b          | 3.48 ± 0.36b           | 13.37 ± 1.64b          | 0.31 ± 0.05a           |
| N-100% + BS-2          | 54.61 ± 5.59a          | 4.40 ± 0.50a           | 16.39 ± 2.20a          | 0.33 ± 0.04a           |
| P-Value                | ***                    | ***                    | *                      | NS                     |
| N-100%                 | 40.41 ± 5.38b          | 2.84 ± 0.44c           | 13.46 ± 2.45a          | 0.28 ± 0.03b           |
| N-60%                  | 35.66 ± 2.92c          | 2.42 ± 0.17d           | 10.22 ± 1.47c          | 0.28 ± 0.04b           |
| N-60% + BS-1           | 46.82 ± 3.91a          | 3.69 ± 0.34b           | 11.57 ± 1.33b          | 0.39 ± 0.07a           |
| N-60% + BS-2           | 46.59 ± 5.96a          | 4.17 ± 0.32a           | 11.58 ± 1.88b          | 0.37 ± 0.04a           |
| P-Value                | ***                    | ***                    | ***                    | **                     |
| N-100%                 | 40.41 ± 5.38c          | 2.84 ± 0.44b           | 13.46 ± 2.45c          | 0.28 ± 0.03b           |
| N-30%                  | 27.89 ± 5.96d          | 2.00 ± 0.24c           | 9.18 ± 0.81d           | 0.17 ± 0.06c           |
| N-30% + BS-1           | 47.58 ± 6.12b          | 3.93 ± 0.52a           | 14.95 ± 2.08b          | 0.38 ± 0.01a           |
| N-30% + BS-2           | 49.20 ± 6.92a          | 4.38 ± 0.56a           | 14.02 ± 1.67a          | 0.37 ± 0.07a           |
| P-Value                | ***                    | ***                    | ***                    | ***                    |

Values are means ± standard error (n = 9) and differences between means were compared by Fisher’s least-significance difference (LSD) test (P = 0.05). Values with different letters indicate significant differences. The levels of significance were represented by P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***)..

| Table 3. Effect of Terra Sorb® radicular (BS-1) and Terramin® Pro (BS-2) on chlorophyll (Chl) concentration of lettuce plants grown under three different nitrogen (N) supplies |
|------------------------|------------------------|------------------------|------------------------|
|                        | Chl a (mg g⁻¹ FW)      | Chl b (mg g⁻¹ FW)      | Total Chl (mg g⁻¹ FW)  |
| N-100%                 | 17.48 ± 0.17b          | 10.02 ± 0.20b          | 27.50 ± 0.20b          |
| N-100% + BS-1          | 20.14 ± 0.80a          | 10.41 ± 0.43a          | 31.05 ± 0.78a          |
| N-100% + BS-2          | 21.39 ± 0.25a          | 10.44 ± 0.25a          | 31.83 ± 0.45a          |
| P-Value                | ***                    | ***                    | ***                    |
| N-100%                 | 17.48 ± 0.17b          | 10.02 ± 0.20b          | 27.50 ± 0.20b          |
| N-60%                  | 18.34 ± 0.36b          | 9.01 ± 0.17c           | 27.35 ± 0.48b          |
| N-60% + BS-1           | 24.49 ± 0.91a          | 11.24 ± 0.39a          | 35.73 ± 1.27a          |
| N-60% + BS-2           | 24.47 ± 0.82a          | 10.12 ± 0.26b          | 34.59 ± 1.04a          |
| P-Value                | **                     | ***                    | ***                    |
| N-100%                 | 17.48 ± 0.17b          | 10.02 ± 0.20b          | 27.50 ± 0.20b          |
| N-30%                  | 15.16 ± 0.70c          | 8.42 ± 0.17c           | 23.58 ± 0.86c          |
| N-30% + BS-1           | 25.62 ± 0.68a          | 10.98 ± 0.33a          | 36.60 ± 0.95a          |
| N-30% + BS-2           | 23.30 ± 0.94a          | 11.07 ± 0.26a          | 34.36 ± 1.12a          |
| P-Value                | ***                    | **                     | ***                    |

Values are means ± standard error (n = 9) and differences between means were compared by Fisher’s least-significance difference (LSD) test (P = 0.05). Values with different letters indicate significant differences. The levels of significance were represented by P < 0.01 (**), P < 0.001 (***)..
(ANR). Total N accumulation (TNA) was obtained for NUpE and ANR calculations. The parameters were calculated as follows:
TNA was calculated as: total N concentration multiplied by leaf DW (mg N).
NUpE was calculated as: TNA divided by root DW (mg N g\(^{-1}\) DW).
NUE was calculated as leaf DW divided by N concentration (g\(^2\) DW mg\(^{-1}\) N).
ANR was calculated using the formula: \(\text{ANR} = \frac{[\text{TNA}_B - \text{TNA}_C]}{\text{ND}} \times 100\%\),
where \(\text{TNA}_B\) represents the total N accumulation of plants supplied with biostimulants; \(\text{TNA}_C\) total N accumulation of plants not supplied with biostimulants; ND, NaNO\(_3\) dose.

Statistical analysis
The mean and standard error of each treatment was calculated from nine individual data of each parameter analyzed. Data were subjected to a one-way analysis of variance (ANOVA) with 95% confidence, using the Statgraphics Centurion 16.1.03 software. Means were compared by Fisher’s least significant differences (LSDs). The significance levels for both analyses were expressed as *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\), or NS (not significant).

RESULTS

Plant biomass
The reduction in N-fertilization decreased lettuce growth, whereas both biostimulants enhanced plant growth, especially in plants under 30%-N fertilization (Fig. 1). Thus, the application of both biostimulants to 100% N-fertilized plants increased leaf FW and leaf DW. Terramin\(^\text{®}\) Pro (BS-2) also enhanced root FW values and produced higher increments in biomass parameters in comparison to Terra Sorb\(^\text{®}\) radicular (BS-1). The plants with reduced N fertilization (60% and 30%) showed lower leaf and root biomass values except for root DW that was similar in plants grown under N-60% and N-30%. However, biostimulants application increased both leaf and root weights. Although, Terramin\(^\text{®}\) Pro (BS-2) enhanced leaf DW under N-60% and leaf FW and root FW under N-30% to a greater extent than Terra Sorb\(^\text{®}\) radicular (BS-1) (Table 2).

Chlorophyll (Chl) concentration and fluorescence parameters
Both applications of biostimulants enhanced Chl concentration regardless of the N fertilization. Comparing N supplies, N-60% plants showed lower Chl \(b\) levels and N-30% plants registered a lower concentration of both Chl forms in comparison to N-100% plants. Nevertheless, both biostimulants restored similar Chl levels except in plants grown under N-60% that showed lower Chl \(b\) when Terramin\(^\text{®}\) Pro (BS-2) was applied (Table 3).

Regarding fluorescence parameters, no significant effect was observed on Fv/Fm by the two factors involved in the experiment. However, both biostimulants enhanced the ratio of active reaction centres (RC/ABS) and PIABS parameters, except for RC/ABS in plants supplied with N-60% and Terra Sorb\(^\text{®}\) radicular (BS-1). Indeed, plants grown under 30%-N showed a remarkable reduction in RC/ABS, PIABS, and \(\Psi_{\text{Eo}}\) parameters that were restored.

Table 4. Effect of Terra Sorb\(^\text{®}\) radicular (BS-1) and Terramin\(^\text{®}\) Pro (BS-2) on chlorophyll \(a\) (Chl \(a\)) fluorescence parameters of lettuce plants grown under three different nitrogen (N) supplies

|          | Fv/Fm          | RC/ABS         | PIABS         | \(\Psi_{\text{Eo}}\)  |
|----------|----------------|----------------|---------------|-----------------------|
| N-100%   | 0.848 ± 0.004a | 0.61 ± 0.04b   | 5.77 ± 0.68b  | 0.60 ± 0.02a          |
| N-100% + BS-1 | 0.850 ± 0.049a | 0.70 ± 0.04a   | 7.68 ± 1.59a  | 0.64 ± 0.03a          |
| N-100% + BS-2 | 0.850 ± 0.005a | 0.69 ± 0.03a   | 7.25 ± 0.47a  | 0.62 ± 0.00a          |
| P-Value  | NS             | **             | **            | NS                    |
| N-100%   | 0.848 ± 0.004a | 0.61 ± 0.04b   | 5.77 ± 0.68c  | 0.60 ± 0.02a          |
| N-60%    | 0.848 ± 0.004a | 0.64 ± 0.04b   | 5.73 ± 0.83c  | 0.61 ± 0.02a          |
| N-60% + BS-1 | 0.848 ± 0.001a | 0.68 ± 0.04b   | 6.41 ± 0.68b  | 0.66 ± 0.02a          |
| N-60% + BS-2 | 0.845 ± 0.003a | 0.80 ± 0.10a   | 7.79 ± 1.07a  | 0.64 ± 0.01a          |
| P-Value  | NS             | *              | ***           | NS                    |
| N-100%   | 0.848 ± 0.004a | 0.61 ± 0.04b   | 5.77 ± 0.68b  | 0.60 ± 0.02a          |
| N-30%    | 0.843 ± 0.007a | 0.44 ± 0.02c   | 3.56 ± 0.57c  | 0.55 ± 0.02b          |
| N-30% + BS-1 | 0.842 ± 0.004a | 0.78 ± 0.05a   | 6.95 ± 1.29a  | 0.62 ± 0.03a          |
| N-30% + BS-2 | 0.843 ± 0.005a | 0.83 ± 0.07a   | 7.54 ± 0.44a  | 0.63 ± 0.00a          |
| P-Value  | NS             | ***            | ***           | *                     |

Values are means ± standard error (\(n = 9\)) and differences between means were compared by Fisher’s least-significance difference (LSD) test (\(P = 0.05\)). Values with different letters indicate significant differences. The levels of significance were represented by \(P < 0.05\) (*), \(P < 0.01\) (**), and \(P < 0.001\) (***).
This lower biomass in plants, indeed, sub-optimal N fertilization did not have a significant effect on N-100% plants. In addition, Terra Sorb® radicular (BS-1) (BS-2) induced the highest increments in total N concentration, and plants treated with this biostimulant showed the highest values. However, Terra Sorb® radicular (BS-1) application slightly reduced the NO₃⁻ concentration of N-100% plants, increased NO₃⁻ in N-60% plants, and has no influence on N-30% plants (Fig. 2).

Unsurprisingly, the total N concentration of lettuce plants also decreased as the N fertilization was reduced. Again, Terramin® Pro (BS-2) induced the highest increments in total N concentration regardless of the N supply. In addition, Terra Sorb® radicular (BS-1) increased N accumulation of N-60% and N-30% lettuce plants but it did not have a significant effect on N-100% plants (Fig. 3).

Nitrate ion (NO₃⁻) and total nitrogen (N) concentration

As expected, the NO₃⁻ concentration of lettuce plants decreased as the N supply was reduced, showing N-30% plants the lowest levels. Nevertheless, Terramin® Pro (BS-2) application increased NO₃⁻ accumulation, and plants treated with this biostimulant showed the highest values. However, Terra Sorb® radicular (BS-1) application slightly reduced the NO₃⁻ concentration of N-100% plants, increased NO₃⁻ in N-60% plants, and has no influence on N-30% plants (Fig. 2).

Unsurprisingly, the total N concentration of lettuce plants also decreased as the N fertilization was reduced. Again, Terramin® Pro (BS-2) induced the highest increments in total N concentration regardless of the N supply. In addition, Terra Sorb® radicular (BS-1) increased N accumulation of N-60% and N-30% lettuce plants but it did not have a significant effect on N-100% plants (Fig. 3).

DISCUSSION

The biomass production showed by either fresh or dry biomass reliably defines the nutritional status of N in plants. Thus, these parameters are commonly used as stress indicators in the absence or excess of N in the medium. Indeed, sub-optimal N fertilization leads to lower plant growth. This lower biomass in plants grown under lower N fertilization was also observed in the current experiment with a 15% and 30% of biomass loss in N-60% and

![Figure 3](image1.png)

**Figure 3.** Effect of Terra Sorb® radicular (BS-1) and Terramin® Pro (BS-2) on total N concentration of lettuce plants grown under three different N supplies. Bars marked with different letters indicate statistical differences.

![Figure 4](image2.png)

**Figure 4.** Effect of Terra Sorb® radicular (BS-1) and Terramin® Pro (BS-2) on ANR percentage of lettuce plants grown under three different N supplies. Bars marked with different letters indicate statistical differences.

### Nitrogen use efficiency (NUE) parameters and apparent nitrogen fertilizer recovery (ANR)

Regarding N efficiency parameters, plants treated with either of the two biostimulants showed the highest NUpE, NutE, and NUE values. In all lettuce plants, Terramin® Pro (BS-2) caused the greatest increment in the three N efficiency parameters in comparison to plants without biostimulants. The reduction in N supply decreased NUpE and NUE values of lettuce plants, whereas it increased NUTE in plants supplied with N-30% treatment (Table 5).

Plant supplied with Terramin® Pro (BS-2) showed higher ANR percentage values compared to plants that received Terra Sorb® radicular (BS-1) regardless of the N fertilization. The most remarkable increments were observed for N-100% and N-30% doses (Fig. 4).

### Table 5. Effect of Terra Sorb® radicular (BS-1) and Terramin® Pro (BS-2) on nitrogen (N) efficiency parameters of lettuce plants grown under three different N supplies

|          | NUpE (mg N g⁻¹ DW) | NUTE (g² DW mg⁻¹ N) | NUE (g DW) |
|----------|--------------------|---------------------|------------|
| N-100%   | 380.85 ± 42.91c    | 0.077 ± 0.009b      | 28.85 ± 1.20c |
| N-100% + BS-1 | 425.78 ± 19.89b   | 0.092 ± 0.004a      | 39.25 ± 3.67b |
| N-100% + BS-2 | 568.05 ± 16.04a    | 0.091 ± 0.003a      | 51.44 ± 2.43a |
| P-Value  | ***                | ***                 | ***        |
| N-100%   | 380.85 ± 42.91c    | 0.077 ± 0.009b      | 28.85 ± 1.20c |
| N-60%    | 272.29 ± 6.34d     | 0.078 ± 0.002b      | 21.34 ± 0.86d |
| N-60% + BS-1 | 414.203 ± 23.58b   | 0.097 ± 0.006a      | 40.03 ± 1.34b |
| N-60% + BS-2 | 486.98 ± 23.29a    | 0.093 ± 0.005a      | 45.05 ± 0.36a |
| P-Value  | ***                | ***                 | ***        |
| N-100%   | 380.85 ± 42.91a    | 0.077 ± 0.009d      | 28.85 ± 1.20c |
| N-30%    | 141.30 ± 26.71d    | 0.110 ± 0.021c      | 15.23 ± 2.09d |
| N-30% + BS-1 | 199.77 ± 8.30c  | 0.161 ± 0.007a      | 32.18 ± 1.98b |
| N-30% + BS-2 | 311.15 ± 7.15b    | 0.131 ± 0.003b      | 40.84 ± 1.40a |
| P-Value  | ***                | ***                 | ***        |

Values are means ± standard error (n = 9) and differences between means were compared by Fisher’s least-significance difference (LSD) test (P = 0.05). Values with different letters indicate significant differences. The levels of significance were represented by P < 0.001 (***). NUpE, nitrogen uptake efficiency; NutE, nitrogen utilization efficiency; NUE, nitrogen use efficiency; DW, dry weight.
N-30% treatments, respectively. However, the application of the studied biostimulants offset this lower N fertilization and lettuce plants reached higher shoot and root biomass. Terramin® Pro (BS-2) produced a more remarkable positive effect because plants presented a 55%, 72%, and 120% more leaf DW depending on the N fertilization in contrast with the 40%, 52%, and 97% of Terra Sorb® radicular (BS-1). These results also prove that the biostimulants efficiencies are greater when plants are grown under low N fertilization. Moreover, the biostimulants application showed a fast and beneficial effect on plant growth, being significant with only two applications to the plants. Highlighting the effectivity of biostimulants, other studies also observed increments in plant growth, for instance using protein hydrolysates, seaweed, and other plant extracts applied to leafy vegetables.6 The enhancement in the photosynthesis process is a possible mechanism to explain the greater biomass attributed to biostimulants.6 The concentration of Chl a, b, and total Chl, as well as the Chl a fluorescence parameters, inform about photosynthesis performance in plants. Besides, they are also indicative of the N nutritional status in plants, and specifically of N utilization, since Chls synthesis depends on the availability of AAs such as glutamic acid (Glu).10 Thus, adequate N fertilization increased Chl content and photosystems efficiency of spinach plants6 and red clover. In the present study, we observed lower Chl concentration in plants grown under 30% N. However, plants supplied with both biostimulants, regardless of the N dose applied, showed the highest values of photosynthetic pigments. Hence, the application of Terra Sorb® radicular (BS-1) and Terramin® Pro (BS-2), especially under N deficient conditions, significantly induced the synthesis and foliar accumulation of Chls. Indeed, biostimulant-treated plants showed Chl a and total Chl values higher than those of control plants with 100% N fertilization. These results also prove that Terramin® Pro (BS-2) produced a more remarkable positive effect because plants reached higher shoot and root biomass. Terramin® Pro (BS-2) contains greater biostimulant formulation and dose.

The Chl a fluorescence reflects the photosynthetic state of plants and the changes produced under stress conditions. Thus, alterations in plant metabolism led to fluorescence emission to dissipate the excess of energy and avoid damage.21 Quantum yield of primary photosynthesis (Fv/Fm) is one of the parameters derived from the analysis of Chl a fluorescence, which is a good indicator of the photosynthetic yield of plants. We noted that the Fv/Fm value was similar in all treatments regardless of the biostimulant and N dose applied, which are expected results since a reduction in Fv/Fm index is usually produced as a consequence of environmental and non-nutritional stress.21 However, the rest of the analyzed fluorescence parameters indicated an impairment of photosynthesis performance caused by low N fertilization. Although, biostimulants application restored the normal photosynthesis performance at values that even exceed those of complete fertilization. Indeed, biostimulants’ positive effect was also observed under moderate and adequate N fertilization as showed by PABS and RC/ABS results. RC/ABS shows the proportion of active RC, PABS index indicates the functionality of the two photosystems, whereas Y in indicates the output of electrons from photosystem II.21 Plants supplied with biostimulants reached the highest values of these indices, indicating a greater photosynthetic efficiency, and therefore greater plant vitality. Therefore, a possible action mechanism of the biostimulants could be an improvement in photosynthetic efficiency that is clearly reflected in the parameters of Chl a fluorescence, and photosynthetic pigment content. Furthermore, the analysis of these parameters also reveals an increase in photosynthetic performance when N fertilization was 100% for both biostimulants analyzed. These results ultimately suggest that biostimulants application maintains and even improves the N nutritional status of plants given the close relationship between photosynthesis and N nutrition in plants.

Studying the NO3− status in leaves is an essential indicator of both N assimilation processes and the nutritional quality of the lettuce crop, especially when N fertilization is mainly based on NO3− as in this study. Generally, in plants under normal N fertilization, the increase in NO3− concentration in leaves is indicative of better N assimilation because NO3− is an inducing factor for important enzymes such as nitrate reductase (NR) and glutamine synthetase (GS).10,27,28 In this experiment, the reduction in N fertilization caused a decline in leaf NO3− concentration. Moreover, our results showed that under an optimal N fertilization dose the only treatment that enhanced leaf NO3− concentration was Terramin® Pro (BS-2). Likewise, under N deficient conditions this effect was greater for Terramin® Pro (BS-2), whereas Terra Sorb® radicular (BS-1) did not increase NO3− accumulation in plants with severe N restriction. Therefore, the application of Terramin® Pro (BS-2) due to their composition could be inducing NO3− assimilation processes, which would explain the higher biomass production.

From the nutritional quality perspective, the analysis of the NO3−/leaf concentration is essential in crops such as lettuce called ‘leafy vegetables’. High NO3− levels constitute a risk to human health because once ingested it is rapidly transformed into dangerous nitrite and N-nitrous compounds.15 Regarding NO3− accumulation of lettuce leaves, the application of Terra Sorb® radicular (BS-1) and Terramin® Pro (BS-2) did not modify this characteristic, since the obtained NO3− leaf concentrations obtained are not considered harmful to human health. Indeed, none of the analyzed plants exceeded the concentration of 2.5 mg NO3− g−1 FW considered the toxicity limit for human consumption.16 Other studies reported diverse results regarding NO3− accumulation. Thus, the application of protein hydrolysates coupled with high N fertilization increased NO3− accumulation in spinach,27 but the same type of biostimulant did not increase NO3− in baby lettuce24 and Tsouvaltzis et al.9 even observed a decrease in NO3− accumulation in lettuce after AA application. Therefore, the effect of AA-based biostimulants on NO3− accumulation depends on the species and the specific biostimulant formulation and dose.

The effectiveness of biostimulants application can also be tested by analyzing the total N accumulation, especially when the biostimulants are made up of AAs. The supply of higher N amounts to plants leads to a higher total N accumulation in their tissues.24,25 In addition, diverse studies proved that biostimulants application increased total N accumulation, e.g. in oilseed rape plants and wheat.29,30 In the present study, Terramin® Pro (BS-2) was particularly effective to increase total N accumulation, especially under low N fertilization. Likewise, Terra Sorb® radicular (BS-1) increased N accumulation but not under 100% N fertilization, which suggests a lower capacity to increase N accumulation once basal needs have been covered. The higher total N concentration in plants supplied with biostimulants could favor an enhancement of NR and GS activities to produce N assimilated compounds, which in turn would ease plant growth. Furthermore, the better efficiency of Terramin® Pro (BS-2) for stimulating N nutrition probably is due to this biostimulant having a higher percentage of N in the form of AAs [18% compared to 6% in Terra Sorb® radicular (BS-1)]. Also, Terramin® Pro (BS-2) contains greater...
percentages of key AAs for N metabolism such as Glu, aspartic acid (Asp), and glycine (Gly). \(^\text{31}\)

Besides total N accumulation, it is important to study the efficiency of which N is uptake and used in the plants. Thus, the cultivation of plants with higher NUE is basic to obtain high yield and sustainable crops. \(^\text{32}\) Several studies observed that biostimulants application enhanced the two NUE components. For instance, protein hydrolysates increased NUE in spinach, \(^\text{7}\) seaweed biostimulants incremented NUpE in oilseed rape and wheat, \(^\text{29,30}\) and \textit{Trichodema} biostimulants enhanced NUE and NUpE especially under low N application. \(^\text{32}\) We observed that both biostimulants studied here were effective to enhance all NUE parameters. However, the Terramin\(^\text{\textregistered}\) Pro (BS-2) application produced the highest NUpE, but regarding NUTE and NUE, both biostimulants produced similar increases in these indices. Moreover, NUTE increased as in N low conditions, which highlights the usefulness of both biostimulants. These data would explain the maximum foliar concentrations of NO\(_3^-\) and total N, and the higher growth that occurs in plants supplied with Terramin\(^\text{\textregistered}\) Pro (BS-2).

Finally, a key issue regarding N inputs, especially those based on NO\(_3^-\), is their relatively low efficiency in agriculture. ANR is a parameter used to check the effectiveness of N fertilizers, which indicates the proportion of fertilizer that is absorbed by the plant and thereby the wasted percentage. \(^\text{17}\) In our experiment, as fertilization decreased, the ANR value increased, probably because of the activation of uptake mechanisms by the plant. Terramin\(^\text{\textregistered}\) Pro (BS-2) was the most effective biostimulant to increase N recuperation regardless of the N fertilization and with a higher effect in low N fertilized plants. This better ANR produced by Terramin\(^\text{\textregistered}\) Pro (BS-2) would clearly explain the previous results described for this biostimulant: higher NUpE levels, higher leaf concentration of NO\(_3^-\) and total N, and higher NUE and crop biomass production. Therefore, the use of Terramin\(^\text{\textregistered}\) Pro (BS-2) might allow to diminish the use of N fertilizers without reducing crop yields, especially in N limiting areas, and to limit the N leakage in the environment. Although, these results should be confirmed in field studies.

CONCLUSIONS

Regardless of N fertilization conditions, the use of both biostimulants manufactured by the Bioiberica, an S.A.U. company, especially Terramin\(^\text{\textregistered}\) Pro (BS-2), clearly shows a beneficial effect on lettuce plants, increasing biomass production. The biostimulants effect on plant growth is positively influenced by induction of photosynthetic activity, higher NO\(_3^-\) and total N accumulations, and better efficiency in N use and uptake. Therefore, the use of Terramin\(^\text{\textregistered}\) Pro (BS-2) under optimal conditions of NO\(_3^-\) fertilization could be a very appropriate and effective strategy to increase the efficiency and recovery by plants of nitrogenous fertilizers in agriculture and improve crop yields. Besides, the use of Terra Sorb\(^\text{{\textregistered}}\) radicular (BS-1) and Terramin\(^\text{\textregistered}\) Pro (BS-2) could be a very useful tool for crops in N-limiting areas, to reduce the application of nitrogenous fertilizers and improve performance as well as reducing costs and environmental impact, all necessary at present for the development of sustainable agriculture.

ACKNOWLEDGEMENTS

This work was supported by the PAI program (Plan Andaluz de Investigación, Grupo de Investigación AGR282). Funding for open access charge: CBUA/Universidad de Granada.

REFERENCES

1. García-García AL, García-Machado FJ, Borges AA, Morales-Sierra S, Boto A and Jiménez-Arias D, Pure organic active compounds against abiotic stress: a biostimulant overview. \textit{Front Plant Sci} \textbf{11}:57829 (2020).
2. Bulgari R, Franzoni G and Ferrante A, Biostimulants application in horticultural crops under abiotic stress conditions. \textit{Agronomy} \textbf{9}:306 (2019).
3. Francesca S, Arena C, Hay Mele B, Schettini C, Ambrosino P, Barone A \textit{et al}, The use of a plant-based biostimulant improves plant performance and fruit quality in tomato plants grown at elevated temperatures. \textit{Agronomy} \textbf{10}:363 (2020).
4. Brown P and Saa S, Biostimulants in agriculture. \textit{Front Plant Sci} \textbf{6}:671 (2015).
5. Yakin OI, Lubyanov AA, Yakin IA and Brown PH, Biostimulants in plant science: a global perspective. \textit{Front Plant Sci} \textbf{7}:2049 (2017).
6. Paradkovic N, Vinkovic T, Vinkovic Vrček I, Zuntar I, Božić M and Medić-Šarić M, Effect of natural biostimulants on yield and nutritional quality; an example of sweet yellow pepper (\textit{Capsicum annuum} L.) plants. \textit{J Sci Food Agric} \textbf{91}:2146–2152 (2011).
7. Carillo P, Colla G, Fusco GM, Dell’Avvanesa E, El-Nakhl C, Giordano M \textit{et al}, Morphological and physiological responses induced by protein hydrolysate-based biostimulant and nitrogen rates in greenhouse spinach. \textit{Agronomy} \textbf{9}:450 (2019).
8. Di Mola I, Cazzolino E, Ottaiano L, Nocerino S, Rouphael Y, Colla G \textit{et al}, Nitrogen use and uptake efficiency and crop performance of baby spinach (\textit{Spinacia oleracea} L.) and Lamb’s lettuce (\textit{Valeriana locusta} L.) grown under variable sub-optimal N regimes combined with plant-based biostimulant application. \textit{Agronomy} \textbf{10}:278 (2020).
9. Tsouvaltsis P, Kasampilis DS, Aktsgolou D-C, Barbayiannis N and Siomos AS, Effect of reduced nitrogen and supplemented amino acids nutrient solution on the nutritional quality of baby green and red lettuce grown in a floating system. \textit{Agronomy} \textbf{10}:922 (2020).
10. Maathuis FJ, Physiological functions of mineral macronutrients. \textit{Curr Opin Plant Biol} \textbf{12}:250–258 (2009).
11. Shah F and Wu W, Soil and crop management strategies to ensure higher crop productivity within sustainable environments. \textit{Sustainability} \textbf{11}:1485 (2019).
12. Lea PJ and Azevedo RA, Nitrogen use efficiency. 1. Uptake of nitrogen from the soil. \textit{Ann Appl Biol} \textbf{149}:243–247 (2006).
13. FAO, \textit{World Fertilizer Trends and Outlook to 2022}. Rome (2019).
14. Sutton M, Howard C and Erisman J, \textit{The European Nitrogen Assessment: Sources, Effects and Policy Perspectives}. \textit{Cambridge Univ Press}, New York, p. 612 (2011).
15. Mensinga TT, Speijers GJA and Meulenberg J, Health implications of exposure to environmental nitrogenous compounds. \textit{Toxicol Rev} \textbf{22}:41–51 (2003).
16. Santamaria P, Nitrate in vegetables: toxicity, content, intake and EC regulation. \textit{J Sci Food Agric} \textbf{86}:10–17 (2006).
17. Xu G, Fan X and Miller AJ, Plant nitrogen assimilation and use efficiency. \textit{Annu Rev Plant Biol} \textbf{63}:153–182 (2012).
18. Lemaire G and Ciampitti I, Crop mass and N status as prerequisite covariables for unraveling nitrogen use efficiency across genotype-by-environment-by-management scenarios: a review. \textit{Plants} \textbf{9}:1309 (2020).
19. Rajabi Hamedani S, Rouphael Y, Colla G, Colantonii A and Cardarelli M, Biostimulants as a tool for improving environmental sustainability of greenhouse vegetable crops. \textit{Sustainability} \textbf{12}:5101 (2020).
20. Wellburn AR, The spectral determination of chlorophyll a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolutions. \textit{J Plant Physiol} \textbf{144}:307–313 (1994).
21. Strasser RJ, Tsimilli-Michael M and Srivastava A, Analysis of the Chlorophyll a Fluorescence Transient, in \textit{Chlorophyll a Fluorescence}. Springer Netherlands, Dordrecht, pp. 321–362 (2004).
22. Cataldo DA, Maroon M, Schrader LE and Youngs VL, Rapid colorimetric determination of nitrogen in plant tissue by nitrating of salicylic acid. \textit{Commun Soil Sci Plant Anal} \textbf{6}:71–80 (1975).
23. Krom MD, Spectrophotometric determination of ammonia: a study of a modified Berthelot reaction using salicylate and dichloroisocyanurate. \textit{Analyst} \textbf{105}:305–316 (1980).
24. Di Mola I, Cazzolino E, Ottaiano L, Giordano M, Rouphael Y, Colla G \textit{et al}, Effects of vegetal- and seaweed extract-based biostimulants on agronomic and leaf quality traits of plastic tunnel-grown baby lettuce under four regimes of nitrogen fertilization. \textit{Agronomy} \textbf{9}:571 (2019).

J Sci Food Agric 2022; \textbf{102}:7098–7106 © 2022 The Authors. wileyonlinelibrary.com/jsfa

\textit{Journal of The Science of Food and Agriculture} published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.
25 Di Mola I, Ottaiano L, Cozzolino E, Senatore M, Giordano M, El-Nakhel C et al., Plant-based biostimulants influence the agronomical, physiological, and qualitative responses of baby rocket leaves under diverse nitrogen conditions. *Plants* **8**:522 (2019).

26 Godlewska A and Ciepiela GA, Yield performance and content of selected organic compounds in *Trifolium pratense* treated with various biostimulants against the background of nitrogen fertilization. *Legum Res* **43**:850–855 (2020).

27 Ruiz JM, Rivero RM, Cervilla LM, Castellano R and Romero L, Grafting to improve nitrogen-use efficiency traits in tobacco plants. *J Sci Food Agric* **86**:1014–1021 (2006).

28 Vidal EA, Alvarez JM, Araus V, Riveras E, Brooks MD, Krouk G et al., Nitrate in 2020: thirty years from transport to signaling networks. *Plant Cell* **32**:2094–2119 (2020).

29 Siwik-Ziomek A and Szczepanek M, Soil extracellular enzyme activities and uptake of N by oilseed rape depending on fertilization and seaweed biostimulant application. *Agronomy* **9**:480 (2019).

30 Laurent E-A, Ahmed N, Durieu C, Grieu P and Lamaze T, Marine and fungal biostimulants improve grain yield, nitrogen absorption, and allocation in durum wheat plants. *J Agric Sci* **158**:279–287 (2020).

31 Ohyama T, Ohtake N, Sueyoshi K, Ono Y, Tsutsumi K, Ueno M et al., Amino Acid Metabolism and Transport in Soybean Plants, in *Amino Acid - New Insights and Roles in Plant and Animal*. InTech, Rijeka, Croatia (2017).

32 Fiorentino N, Ventorino V, Woo SL, Pepe O, De Rosa A, Gioia L et al., Trichoderma-based biostimulants modulate rhizosphere microbial populations and improve N uptake efficiency, yield, and nutritional quality of leafy vegetables. *Front Plant Sci* **9**:743 (2018).