Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Combined Protein Hydrolysate and Seaweed Extract Biostimulants Enhance Nodulation Dynamics and Nodule Development of Green Bean (Phaseolus vulgaris L.) under Semi-Arid Field Conditions

HASSANE TAHIRI
Université Ibn-Tofail
MONCEF BOUFELLOUS
moncef.boufellous@uit.ac.ma

Université Ibn-Tofail
HASSNA LEKNIZI
Université Ibn-Tofail
WIJDANE ZAIN
Université Ibn-Tofail
MOHAMED ELYACHIOUI
Université Ibn-Tofail
EL MAHJOUB AOUANE
Université Ibn-Tofail

Research Article

Keywords: Phaseolus vulgaris L, biostimulants, protein hydrolysates, seaweed extracts, nodulation, nodule biomass, symbiotic nitrogen fixation, semi-arid agriculture

Posted Date: October 29th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11232768/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: The authors declare no competing interests.

Combined Protein Hydrolysate and Seaweed Extract Biostimulants Enhance Nodulation Dynamics and Nodule Development of Green Bean (Phaseolus vulgaris L.) under Semi-Arid Field Conditions

Hassane Tahiri[1], Moncef Boufellous[1]*, Hassna Leknizi[1], Wijdane Zain[1], Mohamed Elyachioui[1], El Mahjoub Aouane[1]

[1]Natural Resources and Sustainable Development Laboratory, Ibn Tofail University, Faculty of Sciences Kenitra, Morocco

*Corresponding author's e-mail: moncef.boufellous@uit.ac.ma

Abstract

Biological nitrogen fixation is a key process for sustainable legume production, particularly under semi-arid environments where nitrogen availability and water resources frequently limit crop productivity. Although non-microbial biostimulants are increasingly recognized for improving plant growth and stress tolerance, their influence on the temporal dynamics of nodulation under field conditions remains insufficiently documented. The present study evaluated the effects of a combined protein hydrolysate- and seaweed extract-based biostimulant program on the development of nodules in green bean (Phaseolus vulgaris L.) cultivated under semi-arid conditions in central Morocco.

Field experiments were conducted using untreated and biostimulants-treated plants, and nodulation was monitored throughout seven phenological stages (T1–T7). At each stage, ten plants per treatment were sampled to determine nodule number and nodule dry biomass. The protein hydrolysate was applied by fertigation (5 L ha^{-1} at T1, T3 and T4), whereas the seaweed extract was applied as a foliar spray (500 mL hL^{-1} at T3, T4 and T6). Data were analyzed using Student's t-test after verification of variance homogeneity.

No nodules were observed during the first three developmental stages in either treatment. From the pre-flowering stage (T4), biostimulants-treated plants exhibited a marked increase in nodulation compared with untreated plants. Nodule number increased from 2.0 to 25.35 nodules $plant^{-1}$ at T4, 4.0 to 42.33 at T5, 11.0 to 71.25 at T6 and 13.0 to 119.33 nodules $plant^{-1}$ at T7, corresponding to relative gains ranging from 548% to 1167%. Nodule dry biomass also increased significantly throughout the reproductive period, indicating enhanced structural development of the symbiotic organs.

The stimulation of nodulation is interpreted as an indirect consequence of improved plant physiological performance resulting from the combined action of protein hydrolysates and seaweed extracts on root development, nutrient acquisition and photosynthetic activity. Although biological nitrogen fixation was not directly quantified, the simultaneous increase in nodule number and biomass demonstrates that the formulated biostimulants substantially promoted the establishment and development of the legume–rhizobium symbiosis under semi-arid field conditions.

These findings highlight the potential of combining agro-food-derived protein hydrolysates and seaweed extracts as sustainable biostimulants to improve nodulation in common bean while supporting environmentally friendly crop management strategies in water-limited environments.

Keywords: *Phaseolus vulgaris L*; biostimulants; protein hydrolysates; seaweed extracts; nodulation; nodule biomass; symbiotic nitrogen fixation; semi-arid agriculture.

1. Introduction

Biological nitrogen fixation represents one of the most important ecosystem services provided by legumes and constitutes a fundamental process for the transition towards sustainable agricultural systems. Through their symbiotic association with nitrogen-fixing rhizobia, legumes are able to reduce their dependence on synthetic nitrogen fertilizers while improving soil fertility, crop productivity and environmental sustainability (Lindström and Mousavi, 2020; Roy and al., 2020). Among cultivated legumes, common bean (Phaseolus vulgaris L.) is one of the most important grain and vegetable crops worldwide because of its high nutritional value and its contribution to food security, particularly in developing countries. However, despite its economic importance, common bean generally exhibits relatively low symbiotic efficiency compared with other grain legumes, making crop productivity highly dependent on environmental conditions that influence the establishment and functioning of the legume–rhizobium symbiosis (Graham, 1981; Reinprecht and al., 2020).

In semi-arid regions, irregular rainfall, high temperatures and limited soil fertility frequently reduce root growth, nutrient acquisition and biological nitrogen fixation, thereby constraining common bean productivity (Cramer and al., 2018; Lionello and Scarascia, 2018). Water deficit negatively affects rhizobial survival, infection thread formation, nodule initiation and nitrogenase activity, ultimately reducing the efficiency of biological nitrogen fixation (Serraj and al., 1999; Zahran, 1999). Improving the establishment and persistence of root nodules

under these environmental conditions therefore represents an important strategy for increasing crop resilience and reducing fertilizer inputs.

Nodule formation in P. vulgaris is a highly coordinated developmental process involving complex molecular communication between compatible rhizobia and host roots. The perception of rhizobial Nod factors by LysM receptor-like kinases activates the common symbiotic signaling pathway, inducing calcium oscillations and the expression of the transcription factor NODULE INCEPTION (NIN), which regulates infection thread formation, cortical cell division and nodule organogenesis (Roy and al., 2020; Shen and Feng, 2023). Once nodulation has been initiated, the Autoregulation of Nodulation (AON) pathway modulates nodule number through systemic signaling involving CLE peptides and shoot receptor kinases, thereby balancing the energetic cost of symbiosis with the nitrogen requirements of the host plant (Chaulagain and Frugoli, 2021). Successful nodulation therefore depends not only on rhizobial compatibility but also on root architecture, carbon allocation, nutrient availability and environmental conditions.

Because biological nitrogen fixation requires substantial amounts of carbon and mineral nutrients, strategies capable of improving plant physiological performance may indirectly enhance nodulation. Among non-microbial plant biostimulants, protein hydrolysates have received considerable attention owing to their high content of amino acids and bioactive peptides that stimulate root development, nutrient uptake, carbon metabolism and plant tolerance to abiotic stress (Jolayemi and al., 2022; Malécange and al., 2023a). Likewise, seaweed extracts contain polysaccharides, betaines, phenolic compounds, minerals and hormone-like substances that improve photosynthesis, antioxidant metabolism, water-use efficiency and nutrient-use efficiency, thereby enhancing plant adaptation to unfavorable environments (El Boukhari and al., 2023; Shukla and al., 2019). In addition to these direct physiological effects, recent evidence indicates that both protein hydrolysates and seaweed extracts can modify root exudation patterns and stimulate beneficial microbial communities in the rhizosphere, creating conditions that favor plant–microbe interactions (Costa and al., 2024; Roche and al., 2024).

Recent experimental studies on Phaseolus vulgaris have demonstrated that improving the efficiency of the legume–rhizobium symbiosis significantly enhances plant growth, chlorophyll content and productivity. For example, Athul and al. (2022) reported that inoculation with efficient native rhizobial strains substantially increased nodule number,

nodule biomass and grain yield under rainfed conditions. Kouki and al., (2021) showed that efficient rhizobial inoculation alleviated the negative effects of salinity while improving nodulation and biological nitrogen fixation. More recently, Ayala-García and al., 2022) demonstrated that modifications in Nod factor signaling broadened the symbiotic compatibility of Rhizobium tropici, whereas Shen and Feng, (2023) highlighted the central regulatory role of NIN in controlling nodule organogenesis and symbiotic development. These studies emphasize that optimizing the physiological and molecular mechanisms governing nodulation remains an effective strategy for improving common bean productivity.

Despite these advances, most published studies have focused on rhizobial inoculation, genetic regulation of nodulation or symbiotic signaling pathways, whereas comparatively little attention has been devoted to the influence of non-microbial biostimulants, particularly the combined application of protein hydrolysates and seaweed extracts, on the temporal dynamics of nodulation under field conditions. Furthermore, the physiological mechanisms linking improved root growth and plant metabolism with sustained nodule development remain poorly documented under semi-arid environments.

In our previous studies, we demonstrated that the same protein hydrolysate- and seaweed extract-based biostimulants program significantly improved root biomass (Tahiri and al., 2023), as well as vegetative growth, chlorophyll content and yield of green bean cultivated under semi-arid conditions (Tahiri and al., 2024). These findings suggested that the stimulation of plant physiological performance could also create favorable conditions for enhanced nodulation and structural development of the symbiotic organs.

Therefore, the objective of the present study was to evaluate the effects of a combined protein hydrolysate- and seaweed extract-based biostimulant program on the temporal dynamics of nodulation in green bean (Phaseolus vulgaris L.) cultivated under semi-arid field conditions in central Morocco. Particular emphasis was placed on the evolution of nodule number and nodule dry biomass throughout seven phenological stages to determine whether improvements in plant physiological performance were associated with enhanced structural development of the legume–rhizobium symbiosis.

2. Materials and Methods

2.1. Experimental site

The field experiment was conducted during the 2022 growing season on a commercial farm located in El Hajeb Province (33°41′ N, 5°22′ W), central Morocco. The region is characterized

by a semi-arid Mediterranean climate with hot, dry summers and cool winters. Annual rainfall ranges from approximately 350 to 450 mm, most of which occurs between November and April, whereas the mean annual temperature varies between 15 and 18 °C. These climatic conditions frequently expose crops to periods of water deficit during vegetative and reproductive growth, making the region particularly suitable for evaluating agronomic strategies designed to improve crop resilience under semi-arid environments.

The soil of the experimental field was slightly alkaline (pH ≈ 8.0) and presented moderate fertility. Before sowing, composite soil samples (0–30 cm depth) were collected and analyzed according to standard laboratory procedures. The principal physicochemical characteristics of the soil are presented in Table 1.

2.2.Plant material and crop management

Green bean (Phaseolus vulgaris L.) was cultivated according to the local commercial production practices. Sowing was carried out on 15 February 2022 using a planting density of approximately 62,500 plants ha^{-1}, with row spacing of 0.80 m and plant spacing of 0.40 m.

All agronomic operations, including irrigation, weed control, fertilization and phytosanitary management, were identical for both treatments throughout the cropping cycle. The only difference between treatments was the application of the formulated biostimulants.

2.3.Experimental design

The experiment consisted of two field treatments:

Control: plants cultivated according to the standard agricultural practices without biostimulant application.

Biostimulant treatment: plants receiving the combined application of a protein hydrolysate-based biostimulant and a seaweed extract-based biostimulant.

Each treatment occupied one commercial field plot managed under identical agronomic conditions.

At each phenological stage, ten plants were randomly sampled from the control plot and ten plants from the treated plot, giving a total of twenty sampled plants per stage. Sampling was repeated throughout the crop cycle from T1 to T7.

Because only one commercial plot was available for each treatment, the sampled plants represent subsamples within each field treatment rather than independent field replicates.

Consequently, the statistical analyses should be interpreted as exploratory comparisons describing treatment differences under commercial farming conditions rather than as evidence derived from replicated field experiments. Similar limitations have previously been discussed for exploratory biostimulant field studies.

2.4.Biostimulant formulations and application program

Two complementary non-microbial biostimulants developed from natural resources were evaluated.

The first formulation consisted of a protein hydrolysate-based biostimulant applied through fertigation at a dose of 5 L ha^{-1}.

The second formulation consisted of a seaweed extract-based biostimulant applied as a foliar spray at a concentration of 500 mL hL^{-1}.

Applications were performed according to the manufacturer's recommendations during specific phenological stages (Table 1).

Table 1: Application protocol of the biostimulants formulations used in the field experiment

Formulation	**Application method**	**Dose**	**Timing**	**Number of applications**
Protein-hydrolysate-based biostimulant	Fertigation	5 L ha^{-1}	T1, T3, and T4	3
Seaweed-based biostimulant	Foliar spray	500 mL hL^{-1}	T3, T4, and T6	3

2.5.Phenological stages

Plant development was monitored according to seven phenological stages representative of the crop cycle (Table 2):

Table 2 : Phenological stages (T1–T7) and sampling times of Phaseolus vulgaris L

Stage	**Phenological phase**	**Approximate timing**
T1	Emergence/seedling establishment	7 days after planting
T2	First trifoliate leaf	12 days after planting
T3	Third trifoliate leaf/vegetative development	30 days after planting
T4	Pre-flowering	45 days after planting
T5	Flowering	55 days after planting

T6	Pod formation (>2.5 cm)	65 days after planting
T7	Pod filling/maturation	75 days after planting

2.6.Nodulation measurements

At each developmental stage, ten plants were randomly excavated from each treatment.

Root systems were carefully removed with the surrounding soil and gently washed under running tap water to avoid mechanical damage to the nodules.

All visible nodules were manually detached and counted.

The number of nodules was expressed as nodules plant^{-1}.

For determination of nodule dry biomass, nodules from each sampled plant were oven-dried at 70 °C until constant weight and weighed using an analytical balance.

Dry biomass was expressed as mg plant^{-1}.

2.7.Statistical analysis

All data are presented as mean ± standard deviation (SD).

Homogeneity of variances was verified using Levene's test before statistical comparisons.

Differences between the control and biostimulant treatments at each phenological stage were evaluated using Student's independent t-test at $P < 0.05$.

The relative gain (%) induced by the biostimulant treatment was calculated as

$$\text{Relative change (\%)} = \frac{X_B - X_C}{X_C} \times 100$$

Because the study was conducted on one commercial plot per treatment, the statistical analyses describe differences between sampled plants within the experimental conditions and should therefore be interpreted with appropriate caution. Future studies based on replicated randomized block designs will be necessary to confirm the observed responses under broader agronomic conditions.

3. Results

3.1.Effects of biostimulants on nodule number

No nodules were observed during the first three phenological stages (T1–T3) in either treatment, indicating that nodulation had not yet been established during the early vegetative growth of Phaseolus vulgaris (Figure 1).

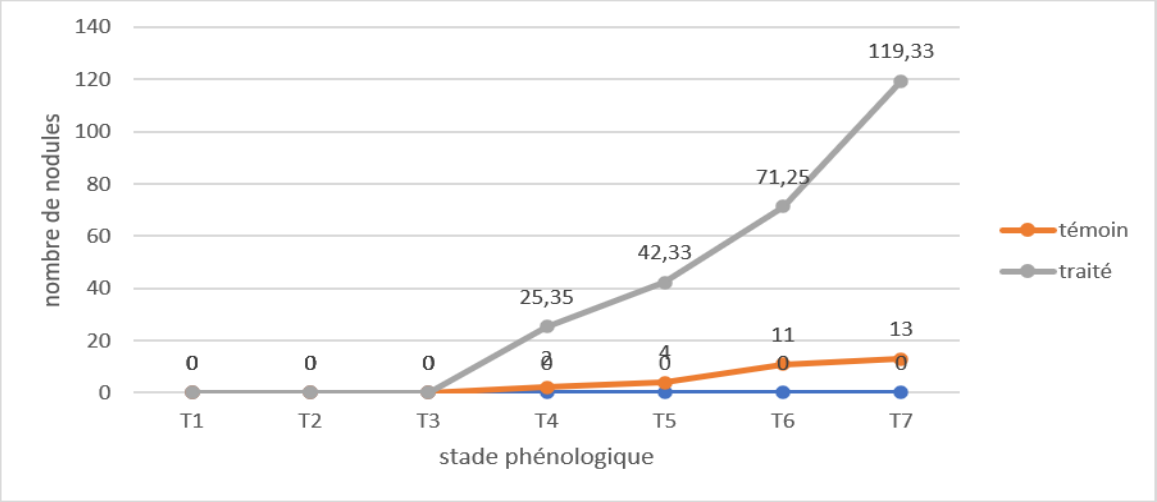


Figure 1: Temporal dynamics of root nodulation in control and biostimulant-treated plants across phenological stages

Nodule formation started at the pre-flowering stage (T4), with significant differences between treatments ($P < 0.05$). Control plants developed an average of 2.00 nodules plant^{-1}, whereas biostimulant-treated plants reached 25.35 nodules plant^{-1}, corresponding to a relative increase of 1167.5%.

At flowering (T5), nodulation continued to increase in both treatments; however, treated plants produced 42.33 nodules plant^{-1}, compared with 4.00 nodules plant^{-1} in the control, representing a relative gain of 958.3%.

The largest increase in absolute nodule number occurred during pod formation (T6), when treated plants developed 71.25 nodules plant^{-1}, whereas untreated plants produced only 11.00 nodules plant^{-1}, corresponding to an increase of 547.7%.

At physiological maturity (T7), nodulation reached its maximum values in both treatments. Treated plants produced 119.33 nodules plant^{-1}, while control plants reached only 13.00 nodules plant^{-1}, corresponding to an increase of 817.9%.

Overall, the combined application of protein hydrolysates and seaweed extracts consistently promoted nodulation throughout the reproductive period, resulting in significantly higher nodule numbers than those observed in untreated plants (Figure 1).

3.2.Effects of biostimulants on nodule dry biomass

The evolution of nodule dry biomass followed a pattern similar to that observed for nodule number (Figure 2).

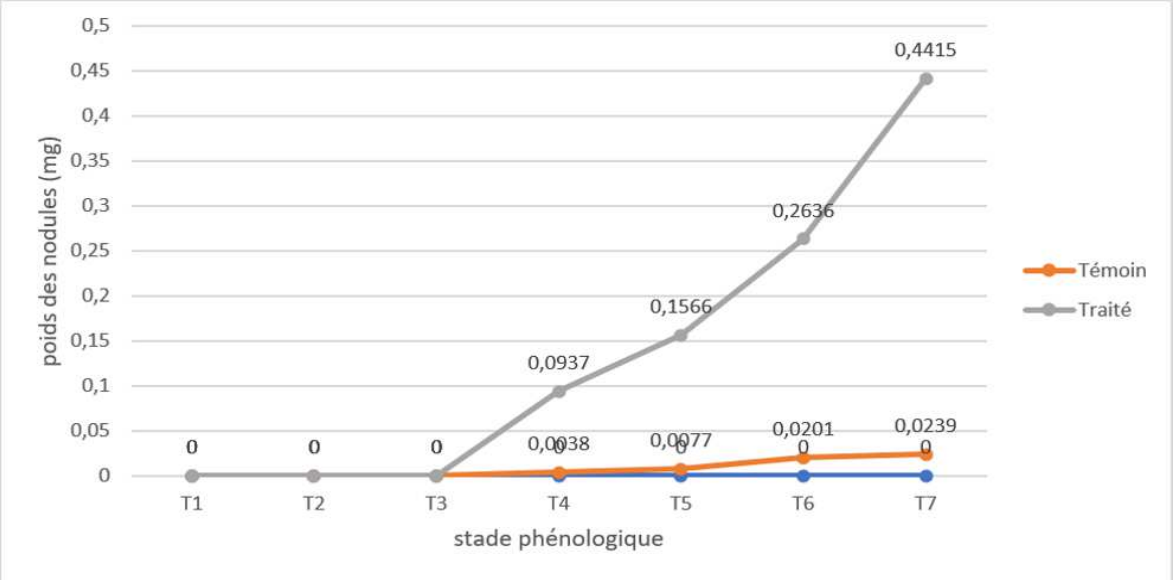


Figure 2: Changes in nodule dry biomass during crop development in response to the biostimulants treatment

No measurable nodule biomass was recorded during T1–T3 because nodules were absent in both treatments.

Beginning at T4, biostimulant-treated plants exhibited significantly greater nodule dry biomass than control plants ($P < 0.05$).

Nodule biomass increased progressively from T4 to T7 in both treatments; however, treated plants consistently accumulated larger amounts of nodule dry matter.

The greatest difference between treatments was recorded at physiological maturity (T7), indicating that nodules produced under biostimulant application continued to enlarge throughout the reproductive period rather than undergoing premature senescence.

Overall, the combined biostimulant program significantly promoted the structural development of nodules during the entire reproductive phase.

4. Discussion

4.1. Biostimulants enhanced nodulation dynamics

The progressive increase in nodule number observed from T4 to T7 demonstrates that the formulated biostimulants substantially enhanced the establishment and persistence of the Phaseolus vulgaris–rhizobium symbiosis under semi-arid field conditions. Nodulation was absent during the first three developmental stages but increased markedly once reproductive development began, suggesting that the physiological status of the host plant became progressively more favorable for symbiotic establishment.

At the molecular level, nodulation is initiated by the perception of rhizobial Nod factors, which activate the common symbiosis signaling pathway and induce the expression of NODULE INCEPTION (NIN), the master regulator of nodule organogenesis (Roy and al., 2020; Shen and Feng, 2023). Once nodules begin to develop, their number is regulated through the Autoregulation of Nodulation (AON) pathway involving CLE peptides and long-distance shoot–root signaling (Chaulagain and Frugoli, 2021). Although these molecular processes were not investigated in the present study, the markedly higher nodulation observed in treated plants suggests that the improved physiological condition of the host favored successful rhizobial infection and sustained nodule initiation.

The magnitude of the response was particularly remarkable. Compared with the untreated control, treated plants exhibited increases of 1167.5%, 958.3%, 547.7% and 817.9% at T4, T5, T6 and T7, respectively. Such responses considerably exceed the modest increases generally reported following conventional agronomic practices and highlight the potential of combined protein hydrolysate and seaweed extract applications to promote nodulation under semi-arid conditions.

Protein hydrolysates are known to stimulate root growth through the action of amino acids and bioactive peptides, whereas seaweed extracts enhance photosynthesis, antioxidant metabolism and nutrient acquisition (Jolayemi and al., 2022; Malécange and al., 2023b). These complementary effects probably increased the number of young lateral roots available for rhizobial infection while simultaneously improving the metabolic capacity of the host plant to sustain continuous nodule formation. In addition, recent studies have shown that non-microbial biostimulants can modify rhizosphere functioning by stimulating beneficial microbial communities and altering root exudation patterns, thereby indirectly facilitating plant–microbe interactions (Costa and al., 2024; Roche and al., 2024).

4.2.Biostimulants promoted structural development of nodules

The temporal evolution of nodulation observed in the present study demonstrates that the combined application of protein hydrolysate- and seaweed extract-based biostimulants markedly enhanced the establishment and persistence of the Phaseolus vulgaris–rhizobium symbiosis under semi-arid field conditions. No nodules were observed during the first three phenological stages (T1–T3), whereas nodulation started at the pre-flowering stage (T4) and increased progressively until physiological maturity (T7). This developmental pattern is consistent with the ontogeny of common bean nodulation, in which rhizobial infection occurs shortly after root emergence, but visible nodules develop only after the establishment of an active root system capable of supporting the high carbon and energy demands of symbiosis (Roy and al., 2020).

At the molecular level, nodulation is initiated by the perception of rhizobial Nod factors by LysM receptor-like kinases, activating the common symbiosis signaling pathway and inducing the expression of the transcription factor NODULE INCEPTION (NIN), which coordinates infection thread formation, cortical cell division and nodule organogenesis (Roy and al., 2020; Shen and Feng, 2023). Following nodule initiation, the Autoregulation of Nodulation (AON) pathway regulates nodule number through systemic signaling mediated by CLE peptides and shoot receptor kinases, thereby balancing the energetic cost of symbiosis with the nutritional requirements of the host plant (Chaulagain and Frugoli, 2021). Although these molecular processes were not investigated in the present study, the markedly greater nodulation observed in treated plants strongly suggests that the biostimulant program created physiological conditions more favorable for successful rhizobial infection and sustained nodule formation.

The magnitude of the response observed was particularly remarkable. Compared with untreated plants, biostimulant-treated plants exhibited increases in nodule number of 1167.5% at T4, 958.3% at T5, 547.7% at T6 and 817.9% at T7. These exceptionally high relative gains should, however, be interpreted in the context of the very low initial nodulation recorded in untreated plants during the early reproductive stages. Rather than indicating an unusual stimulation of the symbiotic machinery itself, these results suggest that the biostimulants accelerated the onset of nodulation and maintained the production of new nodules throughout reproductive development.

Recent experimental studies on Phaseolus vulgaris support the hypothesis that improving the physiological environment of the root system enhances nodulation. Athul and al. (2022)

demonstrated that inoculation with efficient native Rhizobium strains significantly increased nodule number, nodule biomass and grain yield under rainfed conditions compared with non-inoculated plants. Likewise, dos Santos Sousa and al. (2022), in a meta-analysis including numerous field experiments on common bean, concluded that rhizobial inoculation consistently improved nodulation and crop productivity across contrasting environments, although the magnitude of the response depended on environmental conditions, indigenous rhizobial populations and crop management. Similarly, co-inoculation strategies combining Rhizobium tropici with plant growth-promoting bacteria have been shown to further enhance nodulation and plant performance through complementary effects on root colonization and symbiotic efficiency (Hungria and Vargas, 2000). These studies collectively indicate that improving the conditions governing the legume–rhizobium interaction is an effective strategy for enhancing nodulation.

Unlike these microbial approaches, the present study evaluated non-microbial biostimulants. Consequently, the stimulation of nodulation observed here is unlikely to result from an increase in rhizobial population density or infectivity. Instead, it probably reflects indirect improvements in root architecture, nutrient acquisition and plant physiological performance. Protein hydrolysates provide amino acids and bioactive peptides known to stimulate root elongation and nutrient uptake, whereas seaweed extracts supply polysaccharides, betaines and hormone-like compounds that improve photosynthetic activity, antioxidant metabolism and tolerance to abiotic stress (Jolayemi and al., 2022; Malécange and al., 2023b). The complementary action of these formulations probably increased the production of young lateral roots available for rhizobial infection while simultaneously improving the carbon and nutritional status of the host plant.

Our interpretation is further supported by previous studies conducted under the same experimental conditions. Tahiri and al. (2023) demonstrated that the protein hydrolysate-based formulation significantly stimulated root biomass, whereas Tahiri and al. (2024) reported substantial improvements in chlorophyll content, vegetative growth and green bean productivity following the combined application of protein hydrolysates and seaweed extracts. Enhanced root development and increased photosynthetic capacity are expected to promote rhizobial colonization by increasing the number of infection sites and by supplying the carbohydrates required for sustained nodule initiation and development.

Recent evidence also indicates that non-microbial biostimulants influence the rhizosphere beyond their direct effects on plant metabolism. Protein hydrolysates and seaweed extracts can modify root exudation patterns, stimulate beneficial microbial communities and improve nutrient cycling, thereby creating a rhizosphere environment more favorable for plant–microbe interactions (Costa and al., 2024; Roche and al., 2024). Although the rhizosphere microbiome was not characterized in the present study, these indirect mechanisms provide a biologically plausible explanation for the substantial increase in nodulation observed under biostimulant application.

Nevertheless, nodule number alone cannot be considered direct evidence of enhanced biological nitrogen fixation. The efficiency of the symbiosis depends not only on the number of nodules but also on their structural organization, oxygen regulation, bacteroid differentiation and nitrogenase activity (Booth and al., 2021; Roy and al., 2020). Therefore, the present results should be interpreted as evidence of improved establishment and persistence of the symbiotic organs rather than direct confirmation of increased nitrogen fixation. Future studies integrating acetylene reduction assays, ^{15}N isotope techniques and transcriptomic analyses of symbiosis-related genes would provide a more comprehensive understanding of the mechanisms through which protein hydrolysates and seaweed extracts enhance nodulation under semi-arid field conditions.

5. Conclusion

The present study demonstrates that the combined application of protein hydrolysate- and seaweed extract-based biostimulants significantly improved the nodulation dynamics of green bean (Phaseolus vulgaris L.) cultivated under semi-arid field conditions. Compared with untreated plants, the biostimulant program markedly increased both nodule number and nodule dry biomass throughout the reproductive stages, indicating enhanced establishment and structural development of the legume–rhizobium symbiosis. These responses suggest that improvements in root growth, nutrient acquisition and plant physiological performance created favorable conditions for sustained nodule formation and development during the crop cycle

The originality of this study lies in demonstrating that a combined non-microbial biostimulant strategy, based on protein hydrolysates and seaweed extracts, can substantially enhance nodulation under field conditions without the direct application of microbial inoculants. Unlike rhizobial inoculation approaches that increase symbiotic efficiency through the introduction of beneficial microorganisms, the present biostimulant program appears to act indirectly by

improving the physiological status of the host plant and the rhizosphere environment, thereby facilitating the establishment of indigenous rhizobial symbiosis.

These findings complement our previous studies, which showed that the same biostimulant formulations improved root development, chlorophyll content, vegetative growth and yield (Tahiri and al., 2023, 2024). Collectively, these results indicate that protein hydrolysates and seaweed extracts represent promising components of sustainable crop management strategies capable of improving both plant performance and the development of symbiotic organs under water-limited environments. Their use could contribute to reducing dependence on synthetic nitrogen fertilizers while enhancing crop resilience in semi-arid agricultural systems.

Nevertheless, the present study focused on the structural development of nodules and did not directly assess the functional efficiency of biological nitrogen fixation. Consequently, further investigations should combine nitrogenase activity measurements, acetylene reduction assays, ^{15}N isotope techniques, and molecular analyses of symbiosis-related genes (e.g., NIN, ENOD, CLE, and CEP) together with rhizosphere microbiome characterization to determine how protein hydrolysates and seaweed extracts regulate the physiological and molecular mechanisms governing the legume–rhizobium symbiosis. Such multidisciplinary approaches will provide valuable information for optimizing biostimulant formulations and supporting the development of climate-resilient and environmentally sustainable legume production systems.

Funding

This research received no specific funding from public, commercial, or non-profit funding agencies.

Bibliographic References

Athul, P. P., Patra, R. K., Sethi, D., Panda, N., Mukhi, S. K., Padhan, K., ... & Pattanayak, S. K. (2022). Efficient native strains of rhizobia improved nodulation and productivity of French bean (Phaseolus vulgaris L.) under rainfed condition. *Frontiers in Plant Science*, *13*, 1048696.https://doi.org/10.3389/fpls.2022.1048696

Ayala-García, P., Jiménez-Guerrero, I., Jacott, C. N., López-Baena, F. J., Ollero, F. J., Del Cerro, P., & Pérez-Montaño, F. (2022). The Rhizobium tropici CIAT 899 NodD2 protein promotes symbiosis and extends rhizobial nodulation range by constitutive nodulation factor

synthesis. *Journal of Experimental Botany*, *73*(19), 6931-6941.https://doi.org/10.1093/jxb/erac325

Booth, N. J., Smith, P. M., Ramesh, S. A., & Day, D. A. (2021). Malate transport and metabolism in nitrogen-fixing legume nodules. *Molecules*, *26*(22), 6876.https://doi.org/https://doi.org/10.3390/molecules26226876

Chaulagain, D., & Frugoli, J. (2021). The regulation of nodule number in legumes is a balance of three signal transduction pathways. *International journal of molecular sciences*, *22*(3), 1117.https://doi.org/10.3390/ijms22031117

Costa, O. Y., Chang, J., Li, J., van Lith, W., & Kuramae, E. E. (2024). Unraveling the impact of protein hydrolysates on rhizosphere microbial communities: Source matters. *Applied Soil Ecology*, *196*, 105307. https://doi.org/10.1016/j.apsoil.2024.105307

Cramer, W., Guiot, J., Fader, M., Garrabou, J., Gattuso, J. P., Iglesias, A., ... & Xoplaki, E. (2018). Climate change and interconnected risks to sustainable development in the Mediterranean. *Nature climate change*, *8*(11), 972-980.https://doi.org/10.1038/s41558-018-0299-2

Dos Santos Sousa, W., Soratto, R. P., Peixoto, D. S., Campos, T. S., Da Silva, M. B., Souza, A. G. V., ... & Gitari, H. I. (2022). Effects of Rhizobium inoculum compared with mineral nitrogen fertilizer on nodulation and seed yield of common bean. A meta-analysis: Effects of Rhizobium inoculum compared with mineral nitrogen fertilizer on nodulation and seed yield of common bean. A meta-analysis. *Agronomy for Sustainable Development*, *42*(3), 52.https://doi.org/10.1007/s13593-022-00784-6

El Boukhari, M. E. M., Barakate, M., Drissi, B., Bouhia, Y., & Lyamlouli, K. (2023). Seaweed extract biostimulants differentially act in mitigating drought stress on faba bean (Vicia faba L.). *Journal of Plant Growth Regulation*, *42*(9), 5642-5652.https://doi.org/10.1007/s00344-023-10945-w

Graham, P. H. (1981). Some problems of nodulation and symbiotic nitrogen fixation in Phaseolus vulgaris L.: a review. *Field crops research*, *4*, 93-112.https://doi.org/10.1016/0378-4290(81)90060-5

Hungria, M., & Vargas, M. A. (2000). Environmental factors affecting N2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field crops research*, *65*(2-3), 151-164. https://doi.org/10.1016/S0378-4290(99)00084-2

Jolayemi, O. L., Malik, A. H., Ekblad, T., Fredlund, K., Olsson, M. E., & Johansson, E. (2022). Protein-based biostimulants to enhance plant growth—State-of-the-art and future direction with sugar beet as an example. *Agronomy*, *12*(12), 3211. https://doi.org/10.3390/agronomy12123211

Kouki, S., L'taief, B., Al-Qthanin, R. N., & Sifi, B. (2021). Impacts of Rhizobium strain Ar02 on the nodulation, growth, nitrogen (N2) fixation rate and ion accumulation in Phaseolus vulgaris L. under salt stress. *Legum. Res*, *44*, 1521-1528.

Lindström, K., & Mousavi, S. A. (2020). Effectiveness of nitrogen fixation in rhizobia. *Microbial biotechnology*, *13*(5), 1314-1335. https://doi.org/10.1111/1751-7915.13517

Lionello, P., & Scarascia, L. (2018). The relation between climate change in the Mediterranean region and global warming: P. Lionello, L. Scarascia. *Regional Environmental Change*, *18*(5), 1481-1493. https://doi.org/10.1007/s10113-018-1290-1

Malécange, M., Sergheraert, R., Teulat, B., Mounier, E., Lothier, J., & Sakr, S. (2023). Biostimulant properties of protein hydrolysates: Recent advances and future challenges. *International Journal of Molecular Sciences*, *24*(11), 9714.https://doi.org/10.3390/ijms24119714

Reinprecht, Y., Schram, L., Marsolais, F., Smith, T. H., Hill, B., & Pauls, K. P. (2020). Effects of nitrogen application on nitrogen fixation in common bean production. *Frontiers in plant science*, *11*, 1172. https://doi.org/10.3389/fpls.2020.01172

Roche, D., Rickson, J. R., & Pawlett, M. (2024). Moving towards a mechanistic understanding of biostimulant impacts on soil properties and processes: A semi-systematic review. *Frontiers in Agronomy*, *6*, 1271672. https://doi.org/10.3389/fagro.2024.1271672

Roy, S., Liu, W., Nandety, R. S., Crook, A., Mysore, K. S., Pislariu, C. I., ... & Udvardi, M. K. (2020). Celebrating 20 years of genetic discoveries in legume nodulation and symbiotic nitrogen fixation. *The Plant Cell*, *32*(1), 15-41.https://doi.org/10.1105/tpc.19.00279

Serraj, R., Sinclair, T. R., & Purcell, L. C. (1999). Symbiotic N2 fixation response to drought. *Journal of Experimental Botany*, *50*(331), 143-155.https://doi.org/https://doi.org/10.1093/jxb/50.331.143

Shen, L., & Feng, J. (2024). NIN—at the heart of NItrogen-fixing Nodule symbiosis. *Frontiers in Plant Science*, *14*, 1284720. https://doi.org/10.3389/fpls.2023.1284720

Shukla, P. S., Mantin, E. G., Adil, M., Bajpai, S., Critchley, A. T., & Prithiviraj, B. (2019). Ascophyllum nodosum-based biostimulants: Sustainable applications in agriculture for the stimulation of plant growth, stress tolerance, and disease management. *Frontiers in plant science*, *10*, 655. https://doi.org/10.3389/fpls.2019.00655

Tahiri, H., El yachioui, M., El Amraoui, K., El Oihabi, M., & Khadmaoui, A. (2024). Effect of biostimulants on growth and production parameters of green beans (Phaseolus vulgaris L.) cultivated under North African climate. *Journal of the Saudi Society of Agricultural Sciences*, *23*(5), 384-391. https://doi.org/10.1016/j.jssas.2024.03.007

Tahiri, H., El yachioui, M., & khadmaoui, A. (2023, June). Effect of biological biostimulator on green bean root biomass. In *E3S Web of Conferences* (Vol. 412, p. 01017). EDP Sciences.https://doi.org/10.1051/e3sconf/202341201017

Zahran, H. H. (1999). Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and molecular biology reviews*, *63*(4), 968-989.https://doi.org/10.1128/mmbr.63.4.968-989.1999