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Comparative Assessment of Biostimulants in Enhancing Nitrogen Assimilation of Organic Spinach

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Abstract. **In organic farming, matching nitrogen (N) availability to key developmental stages of plants with traditional compost or manure inputs can be challenging. Given its high solubility, limiting Chilean nitrate (CN; sodium nitrate) to 20% of the total N per crop cycle has been recommended because of potential environmental impacts on soil biology and nitrate (NO_3) leaching in organic farming. Although it effectively enhances N availability in the initial stages of growth, the impacts on N assimilation and its interactions with other organic amendments remain poorly understood. This study examined the effects of CN and its interactions with commercial biostimulants (iNvigorate®, B Sure®, Agrinos®, Manage™) on N assimilation in organic spinach production. We assessed their effects on yield, N utilization (total N, NO_3, ammonia, free amino acids), and mineral competition across four developmental stages in four spinach cultivars: Acadia, Corvair, Escalade, Shelby. The application of iNvigorate® led to increased total amino acid accumulation under CN, with the highest levels observed in 'Escalade' and 'Shelby'. Total N showed incremental increases, peaking at harvest, whereas free nitrates decreased progressively across all cultivars during development, regardless of CN application. Agrinos® application enhanced potassium accumulation in 'Acadia' at harvest when CN was used. Interestingly, sodium accumulation was less with CN across all biostimulants than the control, except for Manage™ during the developmental stages. CN application at sowing did not improve yields significantly, but Agrinos® affected biomass production positively, indicating a beneficial interaction with supplemental nitrates from CN. Based on N utilization, we identified spinach cultivars and biostimulants suitable for commercial organic production.**

Organic farming typically yields lower production than conventional methods because of the limited use of resources such as synthetic fertilizers and pesticides (Parađiković et al. 2019). There is a growing demand for new sustainable solutions and integrated management approaches that improve soil health and optimize nutrient cycling to meet consistent organic productivity and quality. Given the challenges of resource constraints in soil nutrition for organic farming, biostimulants have emerged as a viable and sustainable method for maintaining agricultural productivity, offering a nature-based solution. Biostimulants consist of various substances, beneficial microorganisms, and formulated compounds applied to plants to enhance nutrient availability and increase crop yield (du Jardin 2015). These may include natural elements such as fulvic acid, humic acids, seaweed extracts, and protein hydrolysates, along with growth-promoting rhizobacteria, fungi, and various polymicrobial inoculants, which serve as effective solutions for boosting sustainability and productivity in organic farming (Povero et al. 2016). Biostimulants provide several benefits for plants, including stimulating strong root systems, enhancing nutrient absorption, increasing photosynthetic activity, regulating flowering and fruit development, promoting larger fruit size, and improving overall crop yield (Calvo et al. 2014).

While collectively representing products with various modes of action, biostimulants can enhance organic production by improving nutrient uptake and assimilation, thereby narrowing the yield gap between conventional and organic systems (De Pascale et al. 2017). The diverse mechanisms of different biostimulants contribute to increased nutrient absorption and assimilation. Despite their widespread adoption across horticultural crops, only a few studies have assessed various biostimulants in spinach (*Spinacia oleracea*) under field conditions. Research on different biostimulants in spinach, including those derived from seaweed or protein hydrolysates, has shown enhanced bioactive properties and altered chemical composition, such as mineral content, amino acid profiles, and secondary metabolites such as ascorbic acid, phenolics, and flavonoids (Carillo et al. 2019; Papa et al. 2022; Pereira et al. 2019; Rouphael et al. 2018). However, their impact on nitrogen (N) metabolism and interactions with Chilean nitrate (CN; $NaNO_3$) or genotypes under field conditions has yet to be evaluated.

A field experiment was conducted at a certified organic farm in Texas, USA, to examine four spinach cultivars: Acadia, Corvair, Escalade, and Shelby. With its shallow root system and short life cycle, spinach faces challenges with early N uptake, which is crucial for its growth and development. Identifying cultivars suitable for low-input organic farming demonstrating improved N uptake and utilization remains a significant challenge. This study used CN as the initial N source. CN is applied in agricultural systems to fulfill early N requirements when traditional methods such as cover crops, crop rotation, and composting fail to provide adequate N during early development. CN is a natural source of water-soluble mineral N approved by the Organic Materials Review Institute for organic use. Unlike other organic slow-release N fertilizers, CN does not undergo soil volatilization or rapid microbial immobilization and is, therefore, readily available for early N needs. However, because of the potential leaching impacts on soil health, the National Organic Program (NOP) recommends limiting its application to 20% of the total N for each cropping phase. Our objectives were 1) to assess the efficacy of selected biostimulants—specifically, iNvigorate®, B Sure®, Agrinos®, and Manage™—on N assimilation, nutrient uptake, and biomass production in spinach cultivars under CN supply within the organic system; and 2) to explore the interactions among biostimulants, N sources, growth stages, and spinach cultivars. To our knowledge, this study is the first evaluation of multiple biostimulants on various commercial spinach cultivars. Our hypothesis posited that applying CN would enhance N uptake, assimilation, and metabolism in spinach grown under organic conditions. Furthermore, we anticipated that applying biostimulants would improve plant N distribution and increase biomass production.

Materials and Methods

Experimental site. The field experiment was carried out at a certified organic farm at Texas A&M AgriLife Research in Uvalde, TX, USA (lat. 29°12′57.6″N, long. 99°45′21.6″W). Preplanting soil analysis was conducted at the Soil, Water, and Forage Testing Laboratory within the Department of Soil and Crop Sciences in College Station, TX, USA. The soil texture of the experimental area was silty clay loam, calcareous, with a pH of 7.9 and an

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electrical conductivity of 290 μmho·cm^{-1}. The nitrate (NO_3)-N concentration in the soil was 19 mg·kg^{-1}, whereas the concentrations of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur, and sodium (Na) were 38, 828, 14,203, 318, 14, and 19 mg·kg^{-1}, respectively.

Plant material and treatments. Four distinct spinach cultivars—Acadia, Shelby, Escalade, and Corvair—were chosen for this study. Ten seeds were hand-sewn into the soil according to standard agronomic practices, maintaining a 3-inch spacing between seeds in a plot that was 30 inches long and 40 inches wide for each cultivar (Supplemental Fig. 1). The experiment involved two primary base N treatments: CN, derived from natural sodium nitrate ($NaNO_3$), and non-Chilean nitrate (NCN; without any additional N source). The subtreatments included biostimulants (iNvigorate®, B Sure®, and Agrinos®; Agrinos S.A. de C.V., Davis, CA, USA) and a polymicrobial inoculant (Manage™; Pathway BioLogic LLC, Plant City, FL, USA) under both CN and NCN conditions, administered through a drip irrigation system. The biostimulants (iNvigorate®, B Sure®, and Agrinos®) and the microbial inoculant (Manage™) were obtained from Agrinos® and Pathway®, respectively. iNvigorate® is made via a proprietary fermentation process of naturally occurring soil-borne microbes, B Sure® is a foliar nutrient solution derived from microbial fermentation to enhance key metabolic and photosynthetic pathways, and Agrinos® is a nutrient powder that supports crop productivity and a healthy soil microbiome (further details on the biostimulants and microbial inoculant can be found in Table 1). For the CN application, per the manufacturer's guidelines and NOP standards, we applied CN in the plots four times at 2.24 kg·ha^{-1} each time. In addition, three applications of Agrinos® (ranging from 0.56 to 4.50 kg·ha^{-1}), B Sure® (246.58 kg·ha^{-1}), and iNvigorate® (246.58 kg·ha^{-1}) were done at planting, 7 d postplanting, and 14 d postplanting. Manage™ was applied four times: 0.70 kg·ha^{-1} at planting and 0.28 kg·ha^{-1} at 14 d, 21 d, and 28 d postplanting). Control plots, which did not receive CN or biostimulant applications, were also included. The experimental design used a factorial randomized block design with two subfactors (biostimulants and spinach cultivar) (Supplemental Fig. 1). Three replications for each treatment were conducted in our study.

Extraction and analysis of amino acids. The amino acid analysis involved collecting fresh leaf samples at various developmental stages—early vegetative (DS1), vegetative (DS2), reproductive (DS3), and postreproductive (DS4)—and placing them in 2-mL centrifuge tubes. These tubes were promptly flash-frozen in liquid nitrogen (liquid N_2) and stored at −80 °C until needed. Amino acid extraction was conducted by homogenizing the samples in liquid N_2, following the protocol outlined by Joshi et al. (2019). Approximately 20 mg of lyophilized plant tissue was ground into a fine powder using 3-mm Demag stainless steel balls (Abbott Ball Company, CT, USA) and a Harbil model 5G-HD paint shaker to determine the amino acids. The homogenized tissue was suspended in 20 mM cold hydrogen chloride (10 μL·mg^{-1} of tissue), incubated on ice for 20 min, and centrifuged at 14,600 g_n for 20 min at 4 °C. The supernatant was filtered using 0.45-μm 96-well filters (Pall Life Sciences, USA). The filtrate was derivatized with minor modifications using the AccQ-Tag Ultra-Fluor™ derivatization kit (Waters Corporation, Milford, MA, USA). The derivatization was performed as follows: Five microliters of plant extract was mixed with 35 μL borate buffer, and the reaction was initiated by adding 10 μL AccQ-Tag Ultra-Fluor™ reagent (Waters Corporation). The reaction was allowed to proceed for 10 min at 55 °C.

Ultraperformance liquid chromatography (UPLC)–electrospray ionization (ESI)–tandem mass spectrometry analysis of the derivatized samples was conducted using a Waters Acquity H-Class UPLC system linked to a Waters Xevo TQ mass spectrometer fitted with an ESI probe. The Waters Acquity H-Class UPLC system included a binary solvent manager, an autosampler, a column heater, a Waters® ACQUITY UPLC® Fluorescence detector (Waters Corporation), and a Waters AccQ-Tag Ultra column (2.1 × 100 mm, 1.7-μm particles). The mobile phase was comprised of water with 0.1% formic acid (v/v) (A) and acetonitrile with 0.1% formic acid (v/v) (B). The column heater was set to 60 °C, and the mobile phase flow rate was maintained at 0.6 mL·min^{-1}. The nonlinear separation gradient was programmed as follows: 0−1.5 min (96% A), 3.0 min (95.0% A), 5.0 min (92% A), 5.10 min (72% A), and 6.10 min (5% A). A volume of 1 μL of the derivatized sample was injected for amino acid analysis. Using Waters IntelliStart™ software, multiple reaction monitoring (MRM) transitions for each amino acid, along with cone voltage and collision energy values, were optimized. The ESI source operated at 150 °C, with a desolvation temperature of 450 °C, a desolvation gas flow rate of 900 L·h^{-1}, and a capillary voltage of 3.2 kV. The cone voltage ranged from 27 to 39 V to detect all amino acids. Argon was used as the collision gas, with collision energies varying from 19 to 35 eV. MRM was performed in positive mode. Instrument monitoring and data acquisition were carried out using Waters MassLynx™ software, whereas data integration and quantification were executed using Waters TargetLynx™ software.

Analysis of nutrient components. The total N and total P in fresh leaf tissue samples were estimated using the Kjeldahl method (Easy Chem Plus; Chinchilla Scientific, Oak Brook, IL, USA), supplemented with a Kjeldahl-formulated catalyst (Pro-Pac-CT 37; Alfie Packers, Inc., Omaha, NE, USA) as outlined in (Ketterings 2017). Leaf tissue was homogenized, and 100 mg from each sample was placed in a clean Pyrex test tube with a screw cap for assessing NO_3-N and ammonium (NH_4)-N. Potassium chloride (20 mL) was added to each tube using a dispenser. The solution was vortexed for 30 min and then filtered through filter paper. The analysis of NO_3-N and NH_4-N was performed on the filtered samples following standard protocols as described by Ketterings (2017). Leaf samples were taken weekly (four times total) in triplicate to represent the various plant developmental stages (DS1–DS4) for analyzing N uptake and partitioning. Before nutritional component analysis, leaf samples were lyophilized. A 100-mg sample was ground using a homogenizer, digested with concentrated nitric acid, then treated with a 30% hydrogen peroxide solution, followed by a second digestion. The solution was cooled and filtered into scintillation vials using filter paper. The Ca, Mg, K, and Na concentrations were determined in the filtered solution using an atomic absorption spectrophotometer (model AAnalyst-400; Perkin Elmer, Waltham, MA, USA).

Biomass measurements. Fresh weights of the aboveground leaf biomass were recorded on the day of harvest in triplicate. The samples were placed in a hot-air oven set at 70 °C for 72 h to determine their dry weight.

Data analysis. The data collected in the study underwent statistical analysis using the JMP Pro 15 (SAS Institute, Cary, NC, USA) software package, following standard procedures. Analysis of variance (ANOVA) and multiple comparisons (Tukey's post hoc test) were used to assess the significant differences in the data at $P < 0.05$.

Results

Nitrogen assimilation. The total amino acid content increased significantly in response to the application of iNvigorate® at all developmental stages across all tested cultivars under both CN and NCN conditions, except for 'Escalade' at DS4, which showed no significant increase compared with the respective controls (Fig. 1). Among the cultivars, the greatest increase in total amino acid accumulation was observed in 'Escalade', displaying a 14-fold increase, followed by 'Shelby' (9.3-fold), 'Corvair' (7.9-fold), and 'Acadia' (7.2-fold) at DS3 (Fig. 1). In addition, a substantial increase was noted at DS1 with the CN treatment compared with the NCN treatment (Fig. 1). Multivariate ANOVA (MANOVA) analysis indicated a significant effect of developmental stage (developmental stage), N source (base N), treatment, and their interaction (developmental stage × base N × treatment) on total amino acid accumulation (Table 2).

The total N content exhibited a varied response among the tested cultivars across different developmental stages (Fig. 2). Under CN conditions, the application of Agrinos® reduced total N levels significantly for all cultivars, except for 'Acadia' at DS2, when no significant decrease was observed (Fig. 2). The greatest reduction in total N was recorded in 'Corvair' (34.2%), followed by 'Escalade' (28%) and 'Shelby' (24%) under the CN treatment (Fig. 2). However, variations in total N levels among treatments at other

Table 1. Details of biostimulants used in our study.

Biostimulant	Manufacturer	Ingredients	Purpose
iNvigorate®	Agrinos®	Soluble potash (from molasses) and microorganisms (1%) such as *Azotobacter vinelandii* and *Clostridium pasteurianum*	Enhanced root system development for efficient nutrient uptake, release of soil-bound nutrients
B Sure®	Agrinos®	Total N (0.5%), soluble potash (0.5%) from molasses, and shrimp protein hydrolysate	Root growth stimulation, increase in the activity of plant metabolism and photosynthesis
Agrinos®	Agrinos®	Total N (5%), of which 4% is slowly available N from shrimp meal	Improvement in plant nutrition, enhanced soil–microbial environment
Manage™	Pathway®, BioLogic	Microbial inoculant consisting of *Bacillus* sp., kelp (*Ascophyllum nodosum*), and humic acid	Enhancement of soil structure, root architecture, and nutrient cycling

N = nitrogen.

developmental stages remained statistically nonsignificant across the tested cultivars (Fig. 2).

Nitrate-N and NH_4-N showed distinct accumulation patterns across treatments and cultivars under CN and NCN conditions (Figs. 3 and 4). At DS1, NO_3-N levels decreased significantly under various treatments under the NCN condition. However, at DS2, NO_3-N concentrations increased significantly under different treatments under CN and NCN conditions (Fig. 3). The greatest NO_3-N accumulation occurred with the Agrinos® treatment, followed by B Sure® (Fig. 3). 'Shelby' demonstrated the most notable increase (5.8-fold), followed by 'Acadia' (4.8-fold), 'Corvair' (4-fold), and 'Escalade' (3.6-fold) with Agrinos® treatment under NCN conditions (Fig. 3). Under CN conditions, maximum NO_3-N accumulation was recorded in 'Shelby' (5.4-fold), followed by 'Escalade' (4.3-fold), 'Acadia' (3.5-fold), and 'Corvair' (3.2-fold) with the Agrinos® treatment (Fig. 3).

A similar trend was observed with the CN treatment, where Agrinos® resulted consistently in the greatest NO_3-N accumulation (Fig. 3). Ammonium-N displayed a pattern similar to NO_3-N, showing significant variation across developmental stages and treatments (Fig. 4). Statistical analysis revealed significant effects of developmental stage, the interaction of developmental stage and treatment, and the interaction of developmental stage, base N, and treatment on total N, NO_3-N, and NH_4-N, respectively (Tables 3–5).

Mineral nutrients. Our study observed significant effects on mineral nutrient accumulation with the addition of Agrinos® under CN conditions compared with NCN (Figs. 5–9). Among the tested cultivars, 'Acadia' showed a marked increase in K content at harvest with the Agrinos® treatment under CN conditions (Fig. 5). A 36.8% enhancement in K accumulation was noted compared with NCN (Fig. 5). Calcium accumulation varied among cultivars, with 'Corvair' and 'Escalade' exhibiting the most substantial increases under CN conditions (Fig. 6). At DS2, Ca levels rose by 92.6% in 'Corvair' and 71.2% in 'Escalade' compared with NCN (Fig. 6). The multivariate ANOVA indicated significant effects resulting from developmental stage, base N, treatment, developmental stage × treatment, base N × treatment, and developmental stage × base N × treatment for K (Supplemental Table 1) and developmental stage, base N, treatment, developmental stage × treatment, and developmental stage × base N × treatment for Ca (Supplemental Table 2). No notable differences in Mg (Fig. 7) and Na (Fig. 8) contents were observed across cultivars under either CN or NCN conditions. The multivariate ANOVA revealed a significant effect from developmental stage,

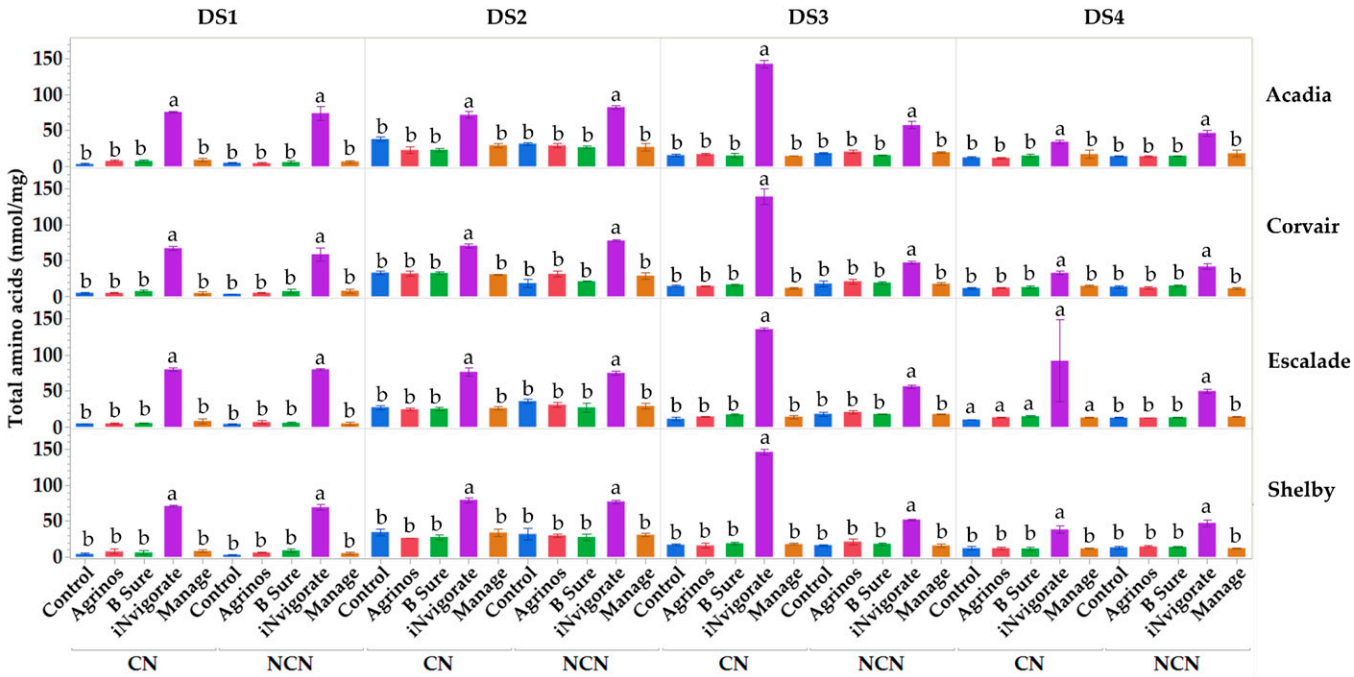

Fig. 1. Variations in total amino acids at different developmental stages (DS1–DS4) across cultivars under various biostimulant treatments under Chilean nitrate (CN) and non-Chilean nitrate (NCN). Bars with different letters indicate significant differences among treatments ($P < 0.05$) according to Tukey's test (values are mean ± standard error).

Table 2. Main effects and multivariate analysis of variance for total amino acids (measured in nanomoles per milligram).

Source	df	Sum of squares	F ratio	Prob. > F
Dev. stage	3	32,245.55	124.6598	<0.0001*
Base nitrogen	1	1,829.83	21.2221	<0.0001*
Dev. stage × base nitrogen	3	4,889.03	18.9008	<0.0001*
Treatments	4	249,684.19	723.9505	<0.0001*
Dev. stage × treatment	12	23,794.99	22.9976	<0.0001*
Base nitrogen × treatment	4	10,492.04	30.4213	<0.0001*
Dev. stage × base nitrogen × treatment	12	29,496.26	28.5078	<0.0001*
Cultivar	3	464.04	1.7939	0.1482
Dev. stage × cultivar	9	756.51	0.9749	0.4607
Base nitrogen × cultivar	3	70.08	0.2709	0.8464
Dev. stage × base nitrogen × cultivar	9	1,009.62	1.3011	0.2353
Treatment × cultivar	12	2,071.72	2.0023	0.0236*
Dev. stage × treatment × cultivar	36	2,988.98	0.9629	0.5337
Base nitrogen × treatment × cultivar	12	806.02	0.7790	0.6721
Dev. stage × base nitrogen × treatment × cultivar	36	2,573.69	0.8291	0.7476

Dev. = developmental; Prob. = probability. The asterisk (*) confirms that the MANOVA result is statistically significant.

cultivar, base N, treatment, developmental stage × treatment, base N × treatment, and developmental stage × base N × treatment for Mg (Supplemental Table 3), and for Na, from base N, treatment, and base N × treatment (Supplemental Table 4). Manage™ application resulted in significantly greater P levels under CN across various spinach cultivars (Fig. 9), with a consistent trend noted for 'Escalade' at different developmental stages (Fig. 9). The multivariate ANOVA indicated significant effects from developmental stage, cultivar, treatment, developmental stage × treatment, base N × treatment, and developmental stage × base N × treatment for P (Supplemental Table 5).

Biomass. Table 6 details the fresh and dry weights of the spinach cultivars under various treatments. The application of Agrinos® affected biomass accumulation significantly, particularly in 'Escalade' and 'Acadia'. Under CN conditions, the Agrinos® treatment led to a substantial increase in the fresh weight of 'Escalade', reflecting a 122.8% increase compared with the control (Table 6). Likewise, under NCN conditions, the Agrinos® treatment enhanced the fresh and dry weights of 'Acadia' significantly. The fresh weight exhibited a significant increase of 105.8%, whereas the dry weight showed an even more notable rise of 156% compared with their respective controls (Table 6). However, a restricted maximum likelihood ANOVA revealed significant effects resulting from treatment for fresh and dry weights (Supplemental Tables 6 and 7).

Discussion

Plant biostimulants present a promising method to enhance N use in organic systems by improving uptake, stimulating growth, and increasing productivity and quality. Nitrogen assimilation involves crucial biochemical and molecular processes that convert nitrates into amino acids, which are essential for various physiological functions in plants (Krapp 2015). Our study observed a significant increase in free amino acid accumulation during the later phases of plant development. The combination of iNvigorate® and CN led to the greatest total amino acid accumulation, particularly in 'Escalade' and 'Shelby'. Although other treatments had limited effects on amino acid accumulation, the results indicate improved NH_4 use for amino acid synthesis during the later growth stages. These findings underscore the substantial influence of developmental stage, base N levels, treatments, and their interactions on total amino acid accumulation, highlighting the intricate

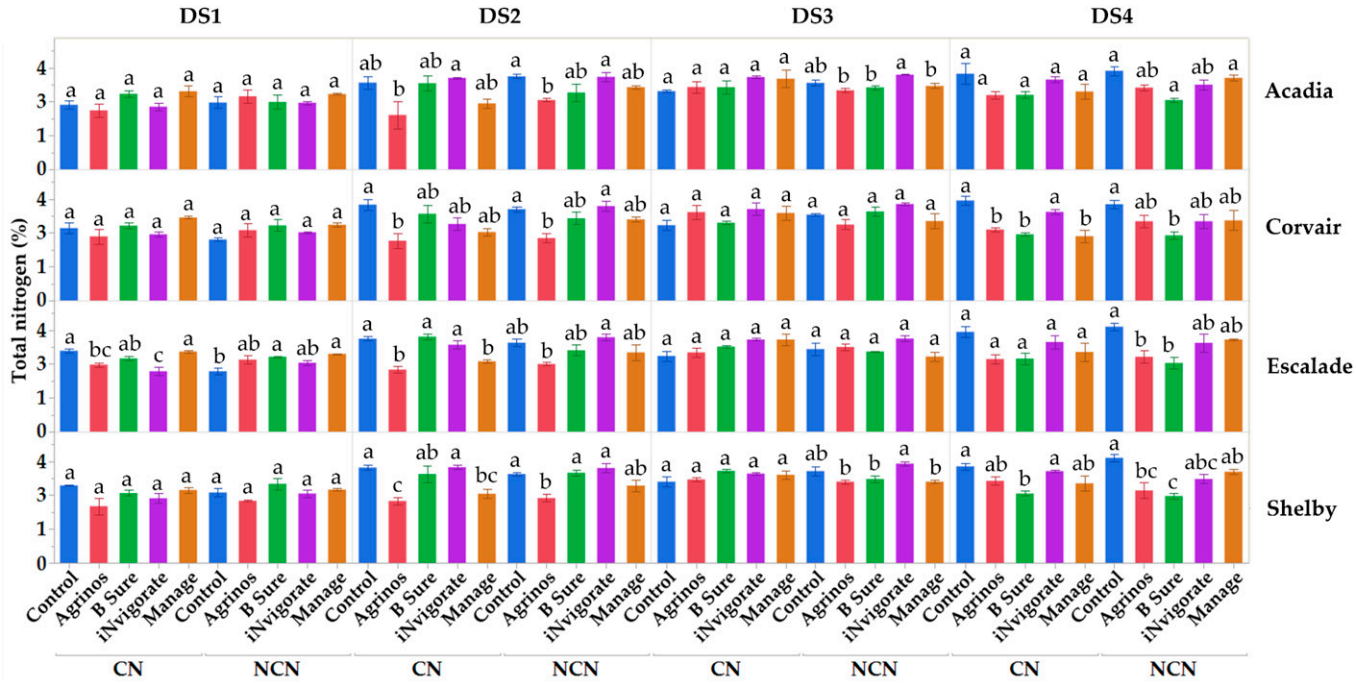

Fig. 2. Total nitrogen under various treatments across cultivars at different developmental stages (DS1–DS4) under Chilean nitrate (CN) and non-Chilean nitrate (NCN). Bars with different letters indicate significant differences among treatments ($P < 0.05$) according to Tukey's test (values are mean ± standard error).

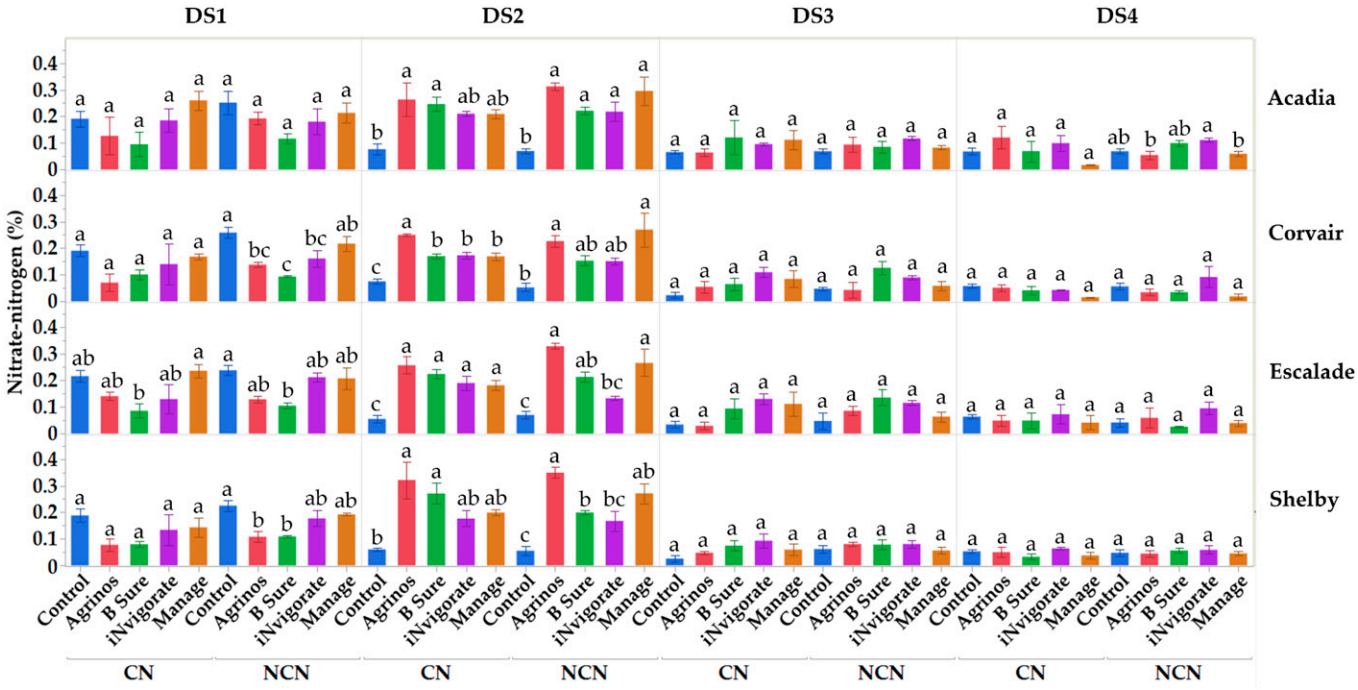

Fig. 3. Variations in nitrate nitrogen under various treatments across cultivars at different developmental stages (DS1–DS4) under Chilean nitrate (CN) and non-Chilean nitrate (NCN). Bars with different letters indicate significant differences among treatments ($P < 0.05$) according to Tukey's test (values are mean ± standard error).

interplay of factors that affect N assimilation in plant metabolism and growth.

Nitrate (NO_3^-) content influences the quality of fresh leafy vegetables significantly (Di Mola et al. 2019). Previous studies have shown that plants treated with biostimulants exhibit increased NO_3^- accumulation (Colla et al. 2018; Tsouvaltzis et al. 2014), often because of enhanced root system development, facilitating greater NO_3^- uptake from the soil and subsequent transport within the plant. The incremental changes in NO_3-N levels with Agrinos® application were independent of CN addition, particularly under NCN conditions. This effect was most pronounced in 'Shelby', followed by 'Escalade'. The rise in NO_3-N and NH_4-N levels at a specific developmental stage indicates an increased N assimilation response under the applied treatments, especially in cultivars such as 'Shelby' and 'Escalade'. These findings align

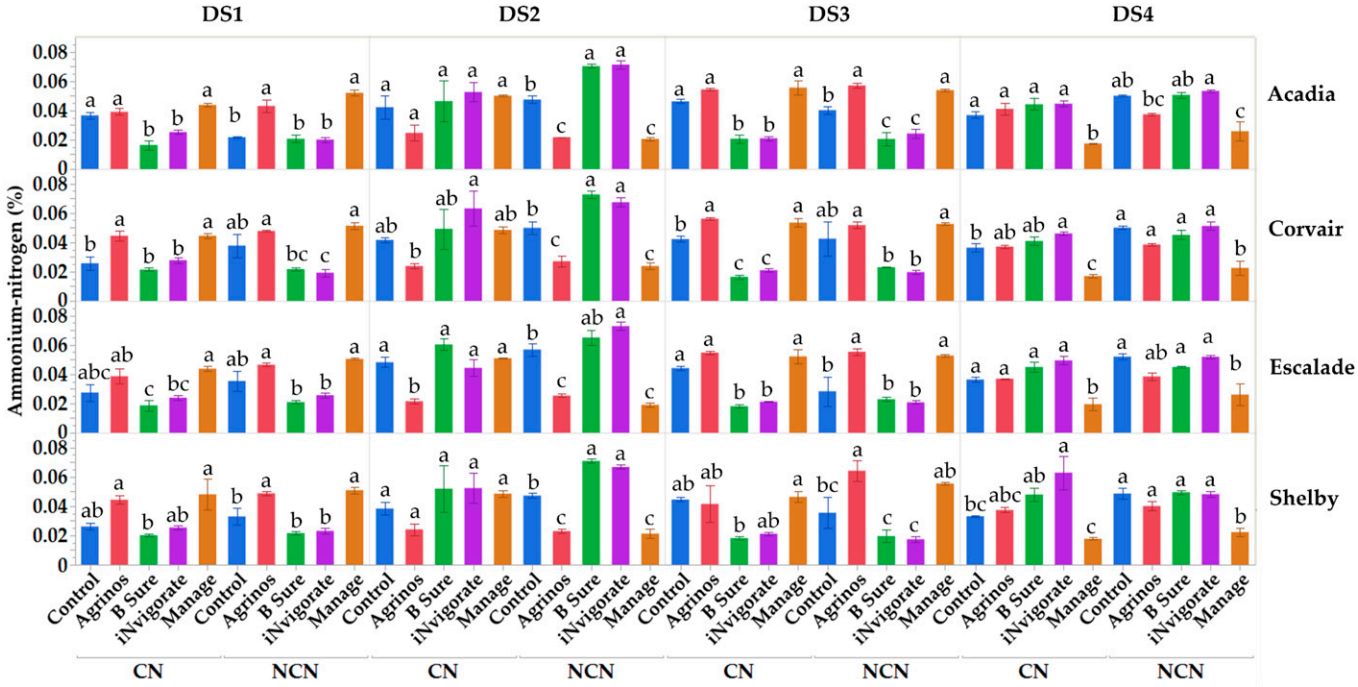

Fig. 4. Changes in ammonium nitrogen under various treatments across cultivars at different developmental stages (DS1–DS4) under Chilean nitrate (CN) and non-Chilean nitrate (NCN). Bars with different letters indicate significant differences among treatments ($P < 0.05$) according to Tukey's test (values are mean ± standard error).

Table 3. Main effects and multivariate analysis of variance for total nitrogen (as measured as a percentage).

Source	df	Sum of squares	F ratio	Prob. > F
Dev. stage	3	23.117279	77.9368	<0.0001*
Cultivar	3	0.485832	1.6379	0.1805
Dev. stage × cultivar	9	1.058263	1.1893	0.3010
Base nitrogen	1	0.305936	3.0943	0.0795
Dev. stage × base nitrogen	3	0.291003	0.9811	0.4019
Cultivar × base nitrogen	3	0.137468	0.4635	0.7080
Dev. stage × cultivar × base nitrogen	9	0.374719	0.4211	0.9235
Treatment	4	17.831032	45.0862	<0.0001*
Dev. stage × treatment	12	29.478418	24.8456	<0.0001*
Cultivar × treatment	12	0.692662	0.5838	0.8551
Dev. stage × cultivar × treatment	36	2.206652	0.6200	0.9586
Base nitrogen × treatment	4	0.825965	2.0885	0.0821
Dev. stage × base nitrogen × treatment	12	6.386053	5.3824	<0.0001*
Cultivar × base nitrogen × treatment	12	1.175792	0.9910	0.4572
Dev. stage × cultivar × base nitrogen × treatment	36	2.639797	0.7416	0.8614

Dev. = developmental; Prob. = probability. The asterisk (*) confirms that the MANOVA result is statistically significant.

with previous research demonstrating the role of biostimulants in promoting N assimilation and metabolism (Baglieri et al. 2014; Colla et al. 2015; Rouphael et al. 2017). The enhanced NO_3^- accumulation observed with the Agrinos® treatment suggests that this biostimulant may improve N utilization efficiency, potentially boosting overall plant performance. These insights deepen our understanding of how biostimulants affect plant N dynamics, providing valuable guidance for optimizing nutrient management strategies and enhancing crop productivity in agricultural systems.

In our investigation, we examined the impact of biostimulants on mineral nutrient levels, particularly when paired with CN, compared with control conditions. We found that applying biostimulants boosted mineral nutrient levels under CN conditions, demonstrating their potential to enhance nutrient uptake and improve plant health significantly. Under NCN conditions, we observed no significant differences between treatments at harvest, indicating a nuanced relationship between biostimulants and nutrient availability influenced by environmental factors.

Furthermore, our study examined the effects of a polymicrobial inoculant (Manage™) on P content. This finding is crucial because low P availability in the soil presents a significant challenge, particularly in organic farming systems. 'Escalade' had increased P levels consistently across various developmental stages under CN conditions, indicating a sustained response to the Manage™ application. The improved P uptake under CN suggests that Manage™ may enhance P availability and assimilation, which is vital for energy metabolism and root development. Microbial inoculants such as Manage™ can address this challenge by promoting nutrient solubilization and uptake, enhancing soil fertility and plant nutrient status. Our analysis further highlighted the complexity of nutrient dynamics, revealing significant effects arising from developmental stage, cultivar, treatment, and their interactions concerning total P. These results underscore the need for tailored nutrient management strategies that consider plant developmental stages, cultivar-specific requirements, and the influence of treatments such as biostimulants and microbial inoculants. These findings align with previous research indicating that microbial inoculants improve macro- and micronutrient uptake, consequently boosting plant vigor and productivity (Calvo et al. 2014). Our study contributes to existing research, emphasizing the potential of biostimulants and microbial inoculants as effective agents for addressing nutrient deficiencies, and optimizing nutrient utilization in organic spinach production systems.

The soluble peptides and essential amino acids in Agrinos® may act as signaling molecules, triggering physiological responses in plants that enhance nutrient uptake and accumulation (Colla et al. 2017; Ertani et al. 2013). These compounds could improve nutrient transport processes within plant cells, increasing the availability of K and Ca for metabolic functions and structural integrity. The observed increase in nutrient accumulation may also result from developing a robust root system in plants treated with Agrinos® under CN conditions. A well-developed root system can explore a larger soil volume, accessing more nutrients and water, which improves nutrient uptake and plant vigor (Lucini et al. 2018). Furthermore, applying Agrinos® under CN conditions could promote the upregulation of gene expression related to macronutrient transporters in cell membranes. This regulation might enhance the efficient uptake and translocation of essential nutrients such as K and Ca within the plant, leading to increased nutrient accumulation (Sestili et al. 2018). Overall, these mechanistic actions and physiological responses highlight the potential of Agrinos® and similar products in promoting nutrient acquisition and use in plants, especially under challenging conditions such as low N. Gaining insights into these mechanisms can help develop strategies for improving nutrient management and enhancing crop productivity in agricultural systems.

Table 4. Main effects and multivariate analysis of variance for nitrate nitrogen (as measured as a percentage).

Source	df	Sum of squares	F ratio	Prob. > F
Dev. stage	3	0.39761863	251.4642	<0.0001*
Cultivar	3	0.01586831	10.0355	<0.0001*
Dev. stage × cultivar	9	0.00665484	1.4029	0.1857
Base nitrogen	1	0.00449826	8.5344	0.0037*
Dev. stage × base nitrogen	3	0.00292224	1.8481	0.1383
Cultivar × base nitrogen	3	0.00005664	0.0358	0.9909
Dev. stage × cultivar × base nitrogen	9	0.00166233	0.3504	0.9571
Treatment	4	0.02705492	12.8327	<0.0001*
Dev. stage × treatment	12	0.22495309	35.5665	<0.0001*
Cultivar × treatment	12	0.00314897	0.4979	0.9155
Dev. stage × cultivar × treatment	36	0.01243351	0.6553	0.9380
Base nitrogen × treatment	4	0.00137138	0.6505	0.6269
Dev. stage × base nitrogen × treatment	12	0.01828831	2.8915	0.0008*
Cultivar × base nitrogen × treatment	12	0.00178575	0.2823	0.9918
Dev. stage × cultivar × base nitrogen × treatment	36	0.01436554	0.7571	0.8437

Dev. = developmental; Prob. = probability. The asterisk (*) confirms that the MANOVA result is statistically significant.

Table 5. Main effects and multivariate analysis of variance for ammonium nitrogen (as measured as a percentage).

Source	df	Sum of squares	F ratio	Prob. > F
Dev. stage	3	0.00233610	56.2302	<0.0001*
Cultivar	3	0.00000290	0.0697	0.9760
Dev. stage × cultivar	9	0.00011242	0.9020	0.5237
Base nitrogen	1	0.00021812	15.7505	<0.0001*
Dev. stage × base nitrogen	3	0.00007693	1.8518	0.1377
Cultivar × base nitrogen	3	0.00000317	0.0763	0.9728
Dev. stage × cultivar × base nitrogen	9	0.00009491	0.7615	0.6522
Treatment	4	0.00018785	3.3912	0.0098*
Dev. stage × treatment	12	0.01958854	117.8747	<0.0001*
Cultivar × treatment	12	0.00004330	0.2605	0.9943
Dev. stage × cultivar × treatment	36	0.00020815	0.4175	0.9989
Base nitrogen × treatment	4	0.00033652	6.0751	0.0001*
Dev. stage × base nitrogen × treatment	12	0.00220458	13.2661	<0.000*
Cultivar × base nitrogen × treatment	12	0.00024475	1.4728	0.1328
Dev. stage × cultivar × base nitrogen × treatment	36	0.00049036	0.9836	0.5000

Dev. = developmental; Prob. = probability. The asterisk (*) confirms that the MANOVA result is statistically significant.

In our study, Agrinos® promoted biomass production effectively under CN fertilization by enhancing N assimilation and plant growth processes. The positive effects on growth and biomass from the synergistic interaction between the biostimulants and the supplemental N provided through CN were evident, especially in 'Escalade'. Furthermore, under NCN conditions, a significant increase in the fresh and dry biomass of 'Acadia' from the Agrinos® treatment indicates that the biostimulant enhanced plant growth even with limited N supplementation, demonstrating its potential to improve productivity in low-input farming systems. No significant effects on fresh or dry biomass were recorded for certain biostimulants or microbial inoculants. Interestingly, the additional N applied through CN at planting alone did not increase yield, suggesting that this approach may not enhance productivity. The significant effects of treatment and cultivar on fresh weight accumulation indicate a genotype-dependent response to the applied treatments. For dry weight, treatment effects were significant, suggesting that biomass allocation is influenced primarily by external treatment application rather than cultivar-specific traits. Our findings highlight the potential of Agrinos® in promoting spinach growth across different N sources, with varying degrees of effectiveness depending on the cultivar. The differential responses among cultivars highlight the need for genotype-specific optimization of biostimulants treatment to maximize biomass yield under different fertilization regimes.

Despite extensive scientific literature documenting the benefits of biostimulant application on growth, productivity, quality, and tolerance to abiotic stresses, particularly nutrient deficiency, in various vegetable crops under conventional farming, information on these advantages in organic farming systems remains lacking (De Pascale et al. 2017). Microbial inoculants play a crucial role in organic production by decomposing organic residues and enhancing nutrient uptake and availability, mineralization, recycling, and detoxifying organic and inorganic substances (Aasfar et al. 2021; Chatterjee et al. 2017). Numerous studies on other horticultural crops have shown that biostimulant applications affect growth traits positively, including root and shoot biomass, nutrient uptake, and overall yield (El-Nakhel et al. 2022; Liatile et al. 2022; Majkowska-Gadomska et al. 2021; Melini et al. 2023; Raza et al. 2024). These studies emphasize that biostimulants can enhance plant productivity by stimulating

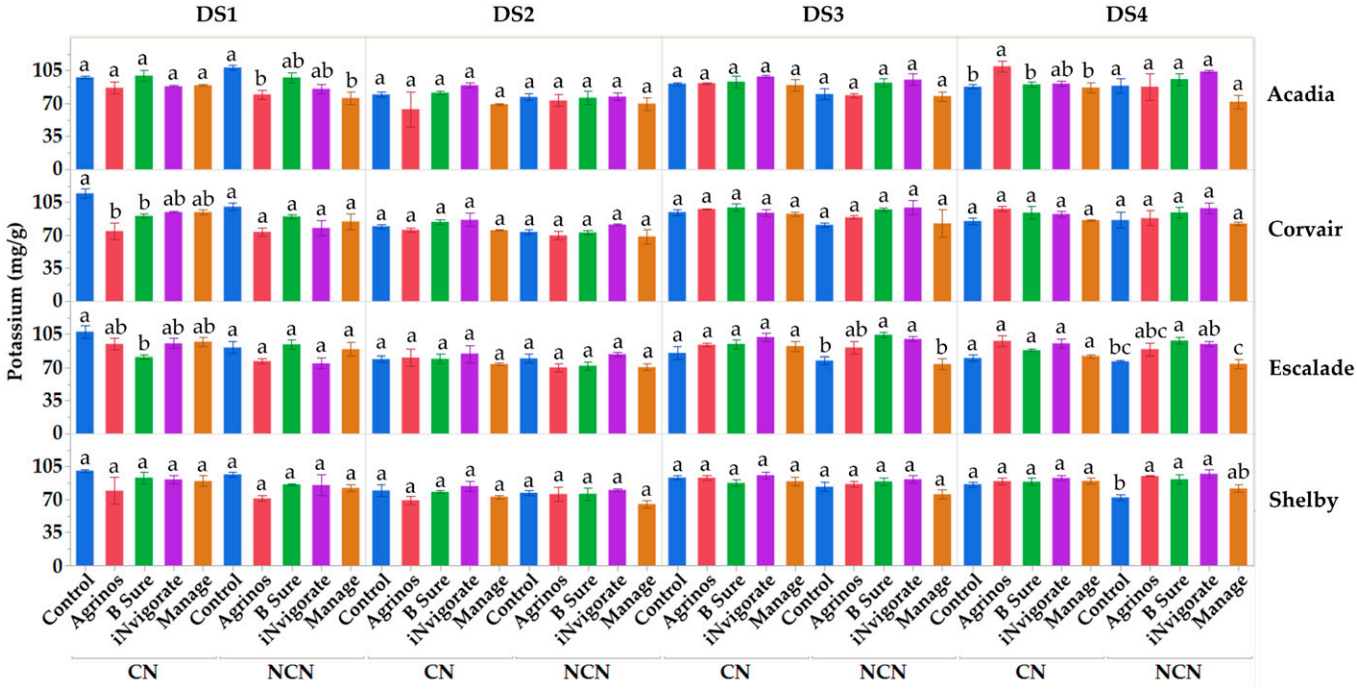

Fig. 5. Changes in potassium content under different treatments across cultivars at various developmental stages (DS1–DS4) under Chilean nitrate (CN) and non-Chilean nitrate (NCN). Bars with different letters indicate significant differences among treatments ($P < 0.05$) according to Tukey's test (values are mean ± standard error).

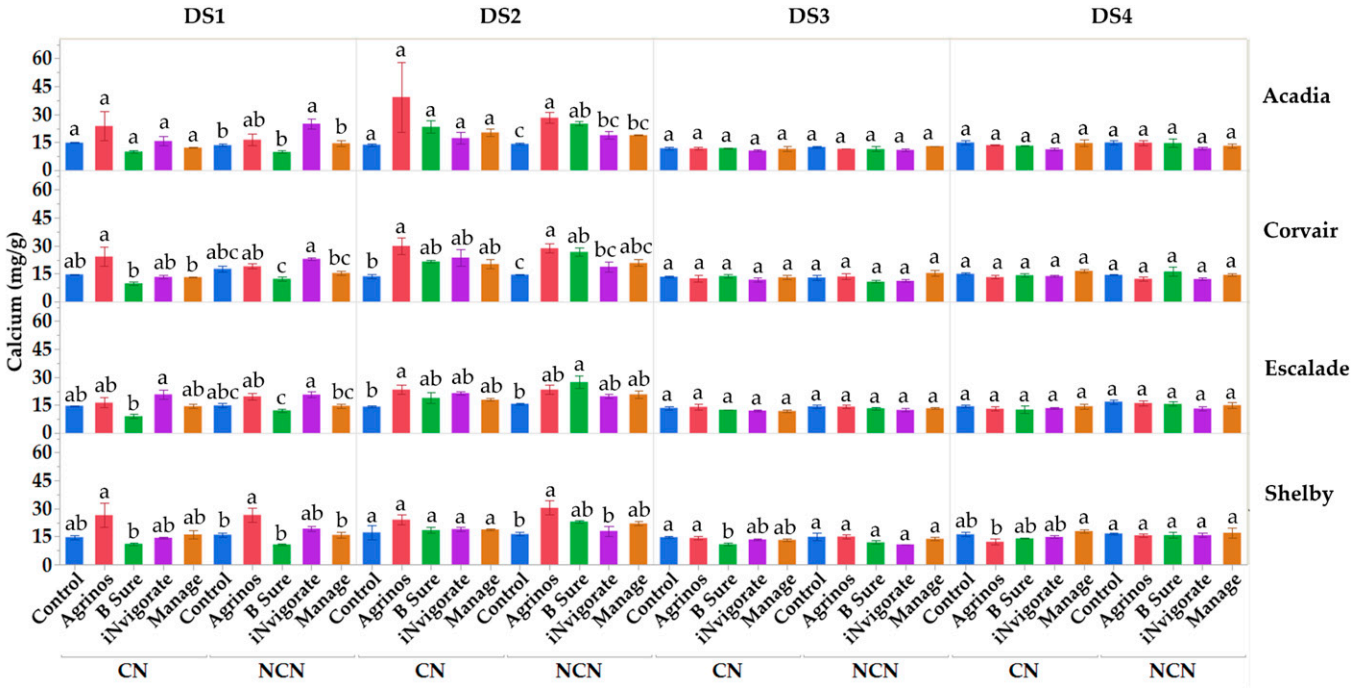

Fig. 6. Variations in calcium content under different treatments across cultivars at various developmental stages (DS1–DS4) under Chilean nitrate (CN) and non-Chilean nitrate (NCN). Bars with different letters indicate significant differences among treatments ($P < 0.05$) according to Tukey's test (values are mean ± standard).

primary metabolism through different signaling molecules (Ertani et al. 2015; Rouphael et al. 2017) or by improving soil health by influencing microflora and promoting plant growth (Nardi et al. 2009). However, there is limited information regarding the effects of such microbial inoculants on leafy vegetables such as spinach. Despite their significant impacts on plant growth and productivity, species of *Azotobacter* or *Clostridium* (Aasfar et al. 2021), components of iNvigorate®, have rarely been assessed in vegetable crops within organic systems. As a by-product of the shrimp processing industry, shrimp meal has excellent potential to supplement N in organic production, aligning with our findings for the Agrinos® product.

It is important to note that different species or cultivars within the same species may respond differently to biostimulant applications. Although many studies

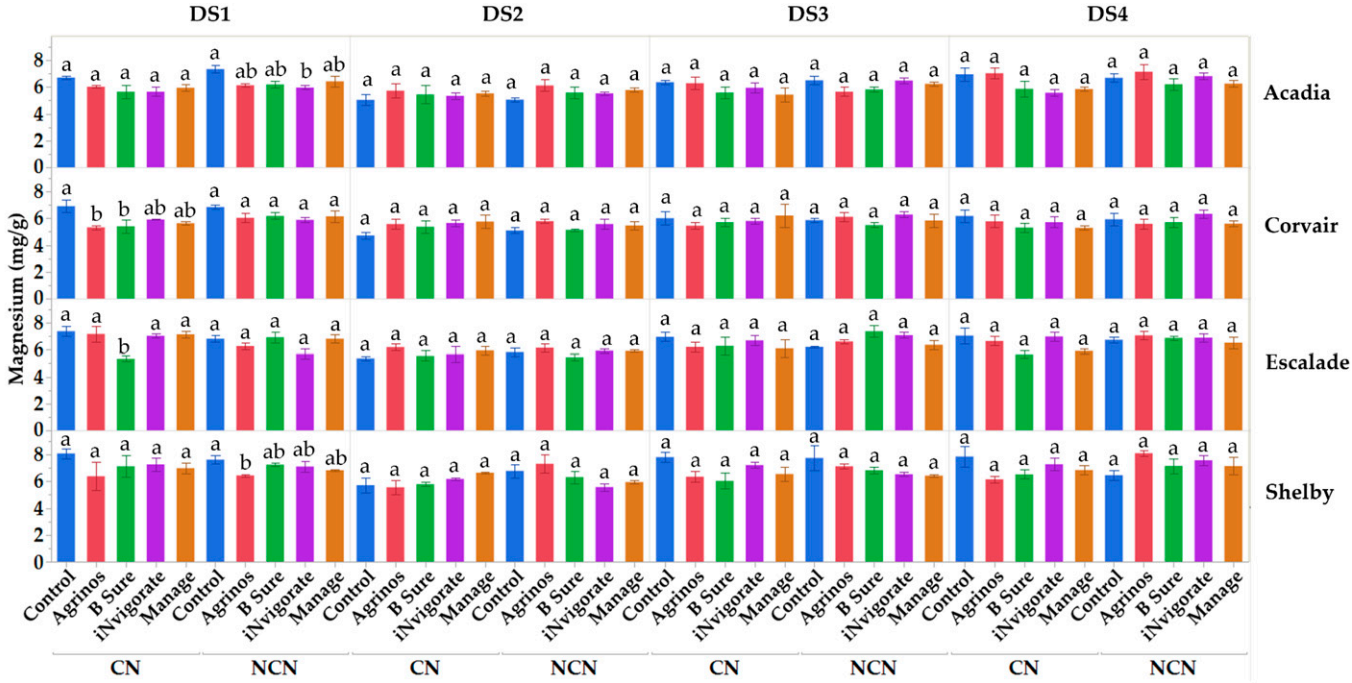

Fig. 7. Changes in magnesium content under various treatments across cultivars at different developmental stages (DS1–DS4) under Chilean nitrate (CN) and non-Chilean nitrate (NCN). Bars with different letters indicate significant differences among treatments ($P < 0.05$) according to Tukey's test (values are mean ± standard error).

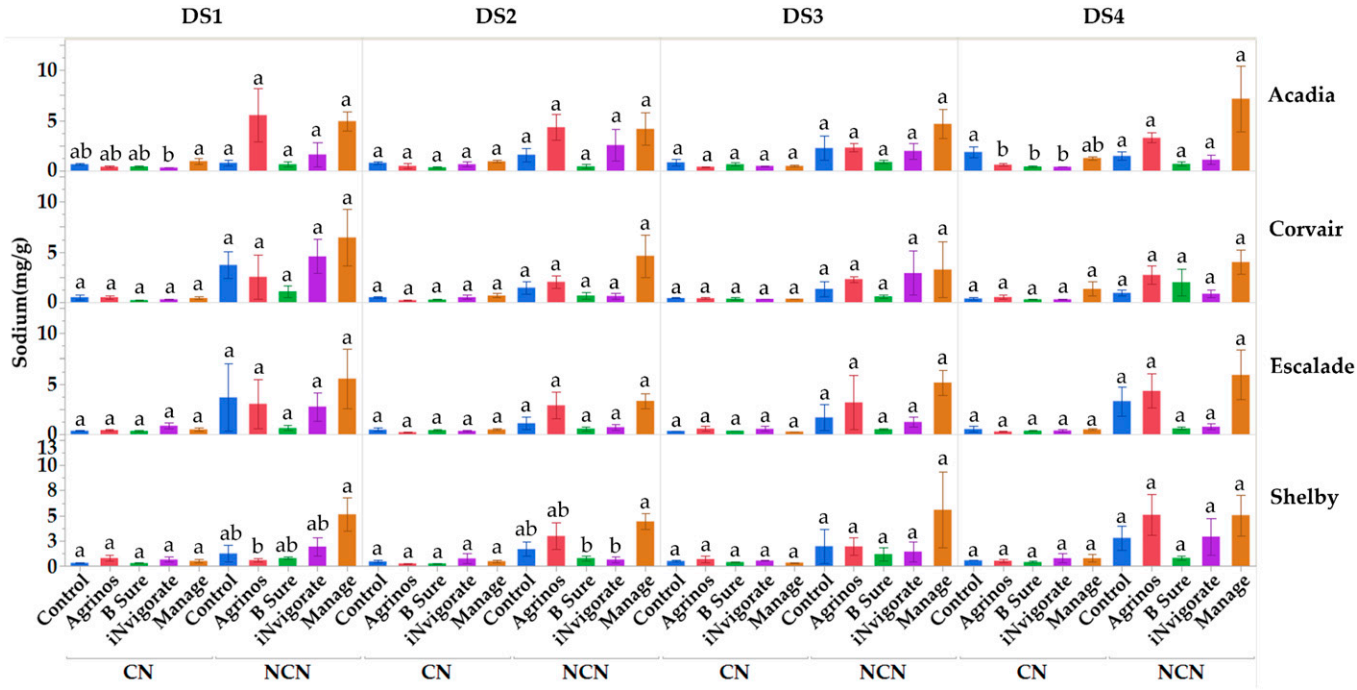

Fig. 8. Sodium content under different treatments across cultivars at various developmental stages (DS1–DS4) under Chilean nitrate (CN) and non-Chilean nitrate (NCN). Bars with different letters indicate significant differences among treatments ($P < 0.05$) according to Tukey's test (values are mean ± standard).

report positive effects, others also indicate cases in which biostimulants do not provide significant benefits (Canellas and Olivares 2014; Kirn et al. 2010; Qin and Leskovar 2020). Thus, the effectiveness of biostimulants can vary depending on specific conditions, crop species, and the types of biostimulants used. Overall, although there is substantial evidence supporting the positive impact of biostimulants on plant growth and productivity, factors such as plant species, environmental conditions, and the exact formulations of biostimulants must be considered when assessing their effectiveness in horticultural systems. The changes observed in N metabolism and biomass in our study suggest that components of the biostimulants may trigger signals necessary for N uptake and its efficient use in spinach. However, because of the diverse composition of biostimulants, these responses are expected to vary among spinach genotypes and production environments, requiring separate evaluations and functional validations.

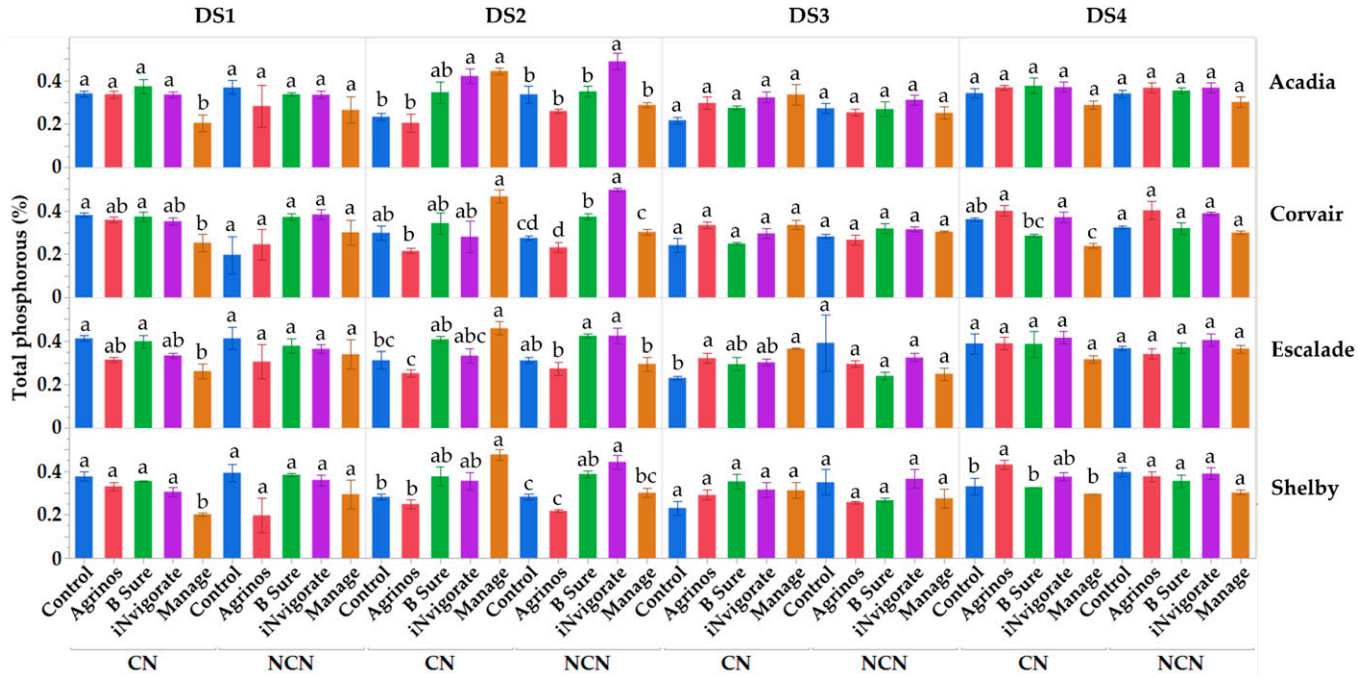

Fig. 9. Changes in total phosphorous under various treatments across cultivars at different developmental stages (DS1–DS4) under Chilean nitrate (CN) and non-Chilean nitrate (NCN). Bars with different letters indicate significant differences among treatments ($P < 0.05$) according to Tukey's test (values are mean ± standard).

Table 6. Fresh weight and dry weight of spinach cultivars across different treatments under two base nitrogen conditions: Chilean nitrate and non-Chilean nitrate.

	Cultivar			
Parameter/treatment	Acadia	Shelby	Escalade	Corvair
Chilean nitrate				
Fresh wt. (g)				
Control	28.7 ± 3.5 a	38.3 ± 2.4 a	29.9 ± 5.0 a	44.0 ± 7.1 a
Agrinos®	48.5 ± 10.3 a	57.8 ± 5.9 a	67.0 ± 12.1 a	66.0 ± 8.1 a
B Sure®	44.6 ± 7.7 a	53.9 ± 7.4 a	48.8 ± 7.2 a	57.3 ± 6.8 a
iNvigorate®	48.5 ± 12.2 a	53.2 ± 9.8 a	46.3 ± 4.6 a	49.4 ± 4.8 a
Manage™	39.1 ± 8.2 a	38.3 ± 3.5 a	51.6 ± 10.4 a	46.6 ± 5.7 a
Dry wt. (g)				
Control	3.6 ± 0.3 a	4.2 ± 0.4 a	3.5 ± 0.5 b	5.1 ± 0.9 a
Agrinos®	5.6 ± 1.3 a	6.4 ± 0.6 a	7.8 ± 1.0 a	7.6 ± 1.2 a
B Sure®	5.4 ± 1.0 a	6.9 ± 0.3 a	6.1 ± 0.3 ab	6.5 ± 1.0 a
iNvigorate®	5.6 ± 1.5 a	6.7 ± 1.3 a	5.4 ± 0.7 ab	5.6 ± 0.5 a
Manage™	5.5 ± 1.5 a	4.7 ± 0.4 a	6.4 ± 1.2 ab	5.6 ± 0.5 a
Non-Chilean nitrate				
Fresh wt. (g)				
Control	29.3 ± 2.3 b	35.4 ± 11.2 a	27.1 ± 3.9 a	57.9 ± 10.1 a
Agrinos®	60.3 ± 1.5 a	43.5 ± 4.6 a	53.6 ± 8.4 a	62.3 ± 7.3 a
B Sure®	48.2 ± 3.2 ab	56.3 ± 10.6 a	50.3 ± 6.4 a	54.7 ± 2.1 a
iNvigorate®	41.6 ± 8.8 ab	51.8 ± 3.3 a	52.8 ± 4.3 a	64.7 ± 6.5 a
Manage™	43.4 ± 7.8 ab	39.1 ± 1.8 a	50.7 ± 5.9 a	44.1 ± 5.1 a
Dry wt. (g)				
Control	3.4 ± 0.5 b	4.0 ± 1.0 a	3.1 ± 0.3 a	6.2 ± 1.4 a
Agrinos®	8.2 ± 0.3 a	5.5 ± 0.5 a	6.0 ± 0.8 a	6.8 ± 0.9 a
B Sure®	6.1 ± 0.3 ab	8.0 ± 2.5 a	6.2 ± 1.0 a	6.7 ± 0.6 a
iNvigorate®	4.6 ± 0.9 b	6.1 ± 0.8 a	6.4 ± 0.8 a	7.1 ± 0.7 a
Manage™	5.3 ± 0.9 ab	4.4 ± 0.2 a	6.9 ± 1.1 a	4.9 ± 0.6 a

Groups with by different letters are significantly different according to Tukey's test ($P < 0.05$).

Conclusion

This study assessed the effectiveness of specific biostimulants in enhancing N uptake and assimilation across four cultivars of spinach, particularly when used in conjunction with CN. The findings indicated that the application of iNvigorate® led to a significantly greater accumulation of total amino acids, suggesting improved N uptake and utilization. In addition, incorporating Agrinos® resulted in increased fresh and dry biomass production, promoting plant growth. In contrast, the microbial inoculant (Manage™) did not affect N assimilation or biomass production significantly. This highlights the specificity of various biostimulants and their differing impacts on N metabolism. 'Escalade' and 'Acadia' emerged as viable candidates for commercial spinach production under organic conditions because of their ability to use and assimilate N effectively. These findings reveal the varied responses of biostimulants among spinach cultivars, providing valuable insights for growers aiming to optimize biostimulant-based N supplementation strategies to enhance plant growth and biomass production. The positive outcomes observed in our study underscore the potential of biostimulants such as Agrinos® to increase the yield of spinach and other leafy vegetables.

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