Cyanobacteria-based nutrimental strategy to enhance yield and quality of spinach

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Abstract

The intricate interplay of plant-soil-microbial formulations was explored using cyanobacterium-based mixes (*Anabaena laxa* C11, *Nostoc carneum* BF2 and *Anabaena laxa* RPAN8) as seed coating, followed by soil drench at selected growth stages in spinach (variety *Pusa All green*). A field experiment under shade net conditions was conducted and data recorded 2, 3, 5 WAS (Weeks after sowing) and harvest stage. Cyanobacterial treatments led to significantly higher pigment content (0.58-1.29-fold), along with substantial enhancement in the enzyme activities of nitrate reductase (0.33 - 0.49-fold), glutamine synthetase (0.35 - 0.53-fold) and phosphoenol pyruvate carboxylase activity (0.17 -0.38-fold) in leaves. RPAN8 treatment led to a notable increase in spinach yield, while BF2 treatment stimulated ascorbic acid (0.37-fold), antioxidants (0.36-fold) and β-carotene (0.41-fold) in fruits, over control. Overall, C11 treatment consistently exhibited significantly superior results in terms of growth, soil microbiological, yield and quality attributes. Principal Component analyses illustrated a strong positive correlation of fruit ascorbic acid with soil chlorophyll and available iron. Path coefficient analysis revealed positive direct effects of available nitrogen with PEPCo, nitrate reductase, chlorophyll *a*, carotenoids, leaf area and antioxidants at different sampling intervals. Such organic formulations can be a valuable resource-efficient strategy for improving spinach yield and quality.

Keywords Amended mixes · Ascorbic acid · Leaves · Nitrogen · PEPCo · Pigments

Introduction

Spinach (*Spinacia oleracea*) is a highly popular nutritious and versatile leafy vegetable crop that is widely consumed worldwide due to its health benefits, vitality, and flavor. In developing countries, including India, phytonutrients such as β-carotene are of immense significance, 95% of β-carotene comes from fruits and vegetables and, among these, leafy greens comprise a substantial portion of about 52% of its total consumption (Gopalan 1992; Anjana et al. 2007). Among them, spinach boasts of an impressive array of properties, from being antimicrobial to anticarcinogenic and antioxidant rich (Vázquez et al. 2013). In addition, spinach contains important constituents, vitamins such as ascorbic acid, folic acid, riboflavin and minerals, including iron, calcium, (Prakash and Pal 1991), making it an excellent nutraceutical and culinary option (Miller et al. 1995).

The art of nurturing seedlings is a cornerstone in successful vegetable crop establishment. Furthermore, it is crucial to comply with all relevant regulations like providing optimal growing conditions; hence, ecological factors need to be prioritized as well to produce high-value vegetable seedlings. In modern agriculture, farmers have become reliant on synthetic fertilizers, pesticides, intensive ploughing, and intensive irrigation. While these approaches have been

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essential in keeping up with food demands of developing nations, they have raised some environmental and health concerns as well. The use of organic fertilizers, particularly biofertilizers or microbial consortia to enhance plant productivity are sustainable options to reduce the pressure on the environment (Cakmakci et al. 2009; Dineshkumar et al. 2019; Kholssi et al. 2021). Leveraging the beneficial effects of interactions between plants and microbes can lead to greater nutrient availability and their mobilization, which can lead to better growth of plants. This can be a boon for developing environmentally sustainable solutions for tackling the global challenge of dwindling crop yields.

Among photosynthetic prokaryotes, cyanobacteria with their multifaceted abilities, including beneficial effects on plant and soil health can help to promote eco-friendly farming practices for sustaining soil fertility and plant nutrition (Gupta et al. 2013; Renuka et al. 2018). When integrated effectively into agriculture, cyanobacteria hold significant potential for substantially augmenting crop yields (Higa and Wididana 1991). Incorporation of various cyanobacterial species into the soil illustrated their impact on growth, health and productivity across diverse crops (Garlapati et al. 2019; Kholssi et al. 2021; Prasanna et al. 2021). These positive effects are conventionally attributed to the provisioning of nutrients essential for the growth of plants and improvement in soil tilth, infiltration of water and nutrients and storage capacity of soil (Singh et al. 2016; Carillo et al. 2020). Several researchers also have highlighted the role of cyanobacteria in nutrient cycling and use efficiency, emphasizing their ability to enhance mineral content in soils and promote plant growth (Rana et al. 2012; Prasanna et al. 2018; Shivay et al. 2022).

Although there is evidence supporting the efficacy of cyanobacteria in enhancing yields in various vegetable and fruit crops (Geries and Elsadany 2021), studies investigating their impact on the commercial and qualitative aspects of spinach remain limited. The application of aqueous extract of *Anabaena sphaerica* on spinach resulted in enhanced plant dry weight, improved nutrient absorption and elevated protein content in the leaves (Mahmoud et al. 2019). Salamah et al. (2019) used *Nostoc* sp. SO-A31 to bring about significant enhancement in the growth of water spinach. Such interventions led to improvement in biomass yield, leaf numbers and vigorous stem and root development, even under nitrogen deficit conditions.

Besides nutritional inputs, vegetables also need to be protected from excessive sunlight and other environmental stressors, for which shading nets are commonly used in tropical and subtropical regions (Fallik et al. 2008). Shade net cultivation also can help growers to overcome the challenges of unfavorable natural conditions by enhancing the thermal climate, leading to improved crop protection against excessive radiation, insects, and environmental fluctuations (Kittas et al. 2008) and produce high-quality vegetables more efficiently and profitably.

With this background, the primary objective of the present investigation was aimed towards evaluating the efficacy of combining cyanobacteria-based mixes as seed coating along with drench application in stimulating the growth, yield and quality aspects of *Pusa All Green* variety of spinach at different stages of maturity, when grown under shade net conditions. Moreover, the novelty of this research lies in developing a nutrient-enriching strategy as an environmentally friendly organic option for boosting spinach cultivation.

Materials and methods

Cultivation of cyanobacterial strains for drench application and their maintenance

Anabaena laxa C11, *Nostoc carneum* BF2 and *Anabaena laxa* RPAN8 belong to the Division of Microbiology, IARI, New Delhi, India, and are maintained routinely by culturing in a nitrogen-free BG11 medium in Haffkine flasks (Stanier et al. 1971) at 27±1 °C under 16 h light and 8 h darkness. The cultures (thereafter referred to as C11, BF2 and RPAN8) were grown in stationary mode, and subjected to white light with an intensity of 50-55 μmol photons m^{-2} s^{-1}. These cultures are deposited in the National Agriculturally Important Microbial Culture Collection (NAIMCC), ICAR-National Bureau of Agriculturally Important Microorganisms (NBAIM), India.

These cyanobacterial cultures serve as the basis for developing seed coating and soil drenching formulations. The cultures were harvested, meticulously rinsed with distilled water to eliminate any residual media components, and then blended with a carrier mixture containing vermiculite and paddy straw as its constituents (1:1 mix of Vermiculite:paddy straw compost, having a C/N ratio of 16.22:1) The humus content of paddy straw compost is 13.8%, and pH is 7.34 to which the dry powdered biomass of cyanobacterial cultures was added. The amended mix contained 2-4 μg chlorophyll g^{-1} carrier. Chlorophyll was used as an index of biomass, as the CFU of filamentous cyanobacteria is often erratic. The treatments for the spinach variety (*Pusa All green*) included - T1-Control, T2-*A. laxa* C11, T3-*N. carneum* BF2 and T4-*A. laxa* RPAN8, as both seed coating and drench formulations. The drench formulations of C11, BF2 and RPAN8 were prepared by blending 300 g of carrier mixture and those amended with cyanobacteria in 3 L of tap water. After thorough blending, the mixture was allowed to incubate in darkness for 48 h, with periodic agitation. Subsequently, after filtration through a muslin cloth, as formerly optimized (Prasanna et al. 2018;

Bhardwaj et al. 2024), these drench formulations were stored at 4°C for further analyses and use.

Analyses of drench

The drench formulations were also analyzed in relation to the content of total, released and reducing sugars, proteins, indole acetic acid (IAA), total phenolic and glomalin content. The total and released sugar content were expressed as $\mu g\ mL^{-1}$, after being assayed in drench formulations using the method of Dubois et al. (1956). The reducing sugars assay was carried out using 3,5-dinitrosalicylic acid (DNSA) reagent by the method of Miller (1959) and the values expressed as $\mu g\ mL^{-1}$. In the drench formulations, the total soluble proteins were quantified spectrophotometrically (Herbert et al. 1971) with bovine serum albumin as standard. The IAA in the formulations was quantitatively determined by the Salkowski method through spectrophotometric measurement at 530 nm (Gordon and Weber 1951). Total phenols in the drench formulations was quantified, following Singleton et al. (1999) using Folin–Ciocalteu reagent and spectrophotometric analysis, where the development of blue color at 660 nm was measured. The total phenolic content was expressed in μg caffeic acid equivalent (CAE) per mL of formulation mix. Glomalin-related soil proteins in the drench formulations were extracted and quantified, as per the standard procedure (Wright and Upadhyaya 1996).

Experimental set-up

The performance of the carrier-based mixes of selected cyanobacterial strains was assessed in a field trial involving spinach (*Spinacea oleracea*; variety *Pusa All Green*) grown in 12 beds, each accommodating 1000 plants per bed, within a 4 m^2 area. The experiment was set up in the shade nets covered polyhouse belonging to the Centre for Protected Cultivation and Technology (CPCT), ICAR-Indian Agricultural Research Institute (ICAR-IARI), New Delhi, situated at 28.4° N latitude, 77.2° E longitude, having an altitude of 228.6 m above mean sea level, in relation to the Arabian Sea. Prior to sowing, spinach seeds were initially coated with cyanobacterial carrier mix, involving a mechanized process known as seed-coating, carried out 24 h before sowing. The air-dried seeds were sown at the rate of 50 g per bed. At the time of sowing, the sandy loam soil had 166.67 kg ha^{-1} available N, available Fe and Zn as 1.13 and 0.95 mg kg^{-1}, with dehydrogenase activity of 84.64 μg TPF g^{-1} soil d^{-1} and 84.77 μg g^{-1} soil microbial biomass carbon.

The experimentation involved four treatments, arranged in a randomized block design (RBD), each with four replicates: T1 Control; T2 C11; T3 BF2; T4 RPAN8. The recommended chemical fertilizer doses for spinach crop were 50 kg ha^{-1} nitrogen, applied as two split doses of urea, with only one dose being given to treatments (T2-T4). At the time of sowing, single super phosphate (SSP), to provide 25 kg ha^{-1} phosphate and muriate of potash (MOP) applied to provide 50 kg ha^{-1} potassium were given as single basal doses. Irrigation was through drip system on alternate days, while fertigation was done twice a week, at 3-day intervals. The experimental area comprised a total of 12 beds, each containing 1000 seeds/saplings per bed, with an inter-row spacing of 1 m between each bed; the total row length was 4 m and the width was 1 m. Soil drenching using the formulations was initiated at 2 WAS (Weeks after sowing) and continued until 5 WAS, at 10-day intervals; application rate was 1000 mL bed^{-1}, approximately 1 mL plant^{-1}. Plant as well as rhizospheric soil samples were collected at 2, 3, 5 WAS and at the harvest stage. Analyses for various parameters, including soil microbiological attributes (soil chlorophyll, soil microbial biomass carbon, soil dehydrogenase), nutrient levels (soil organic carbon, available nitrogen, available phosphorus, available iron and zinc), leaf pigment composition, enzymatic assays (glutamine synthetase, nitrate reductase and phosphoenol pyruvate carboxylase activity), leaf nitrogen and calcium analyses. Plant yield as well as quality traits were recorded at harvest stage (60 days after sowing). All the plant and soil samples were analyzed immediately or stored at 4°C in sterile polybags for subsequent analysis.

Soil microbiological and nutrient analysis

Soil chlorophyll content was quantified utilizing soil cores collected from a depth of 0 -15 cm. These cores were treated with a mixture of acetone and DMSO (1:1) added at the rate of 4 mL per g of soil. After mixing, the samples were kept under dark at normal room temperature for 48 h. Periodic shaking at 24-h intervals ensured complete chlorophyll extraction. Spectrophotometric readings were taken at 663, 645, 630, and 775 nm. The chlorophyll content in the soil samples was as quantified, represented the abundance of photosynthetic biomass (Nayak et al. 2004). The soil chlorophyll content was calculated using the SCOR/UNESCO formula and expressed in mg g^{-1} soil.

$$\text{Soil chlorophyll}\ (\text{mg g}^{-1}\ \text{soil}) = (11.64 \times OD_{663})$$
$$- (2.16 \times OD_{645}) + (0.10 \times OD_{630})$$

To assess the carbon content as microbial biomass (SBMC), soil samples equivalent to 3.5 g in dry weight were added to glass vials. In one batch of vials, chloroform was added, followed by sealing the lids tightly and vigorous shaking. The other batch of vials has no chloroform added. Thereafter, the vials were incubated in dark for a day before being flushed with N_2 under a fume hood at 40-50°C to remove chloroform completely. Upon complete removal of

chloroform, both fumigated and un-fumigated samples were supplemented with 0.5 M K_2SO_4. These vials were shaken for 30 min and the resultant extracts were filtered through Whatman No.42 filter paper, the optical density at 280 nm was measured (Nunan et al. 1998), and microbial biomass carbon calculated using the following formula:

$$SMBC \left(\mu g\ g^{-1}\ dry\ soil\right) = (ECF - ECNF)/\ kEC$$

where *ECF* and *ECNF* represent the values of extractable carbon in both the fumigated (*ECF*) and non-fumigated soil samples (*ECNF*) respectively, with *kEC* having a value of 0.45.

Dehydrogenase activity was determined by incubating 6 g soil with a 3% triphenyl tetrachloride solution and measuring the absorbance at 485 nm (Casida et al. 1964). The values were expressed as μgtriphenyl formazan (TPF) g^{-1} soil d^{-1}.

For macro and micronutrient analysis, rhizospheric soil samples were collected at 0 -15 cm depth using a soil auger and subsequently subjected to airdrying, grinding and sieving. Soil organic carbon content (SOC) was determined (Walkley and Black 1934) and expressed as a percentage. Available nitrogen (Subbiah and Asija 1956) and phosphorus content (Olsen 1954) in soil were also quantified. Available iron and available zinc were evaluated using a wet digestion method with a di-acid mixture and the concentrations measured at 248.4 nm and 213.7 nm, using an Atomic Absorption Spectrophotometer (Prasad 2006). The nitrogen content of spinach leaves was assessed at 2, 3, 5 WAS and harvest stage, through a process of digestion with H_2SO_4 and subsequent distillation; the quantity of acid consumed in the titration was measured, and the nitrogen content in the spinach leaves expressed as percent (%).

Estimation of pigment content in leaves

Spinach leaf samples were collected for photosynthetic pigment estimation, as per the standard method (Lichtenthaler 1987). For the pigment extraction process, 100 mg of fresh spinach leaves (without the roots) were weighed; an aliquot of 10 mL dimethyl sulphoxide (DMSO) was added and kept overnight in dark at room temperature. The volume was then made up to 10 mL using DMSO and optical density recorded at 480, 510, 645 and 663 nm. The formulae of Arnon (1949) for chlorophyll *a*, chlorophyll *b*, carotenoids and total pigments were used and expressed as mg g^{-1} fresh weight (FW):

Chlorophyll *a* (mg g^{-1} FW) = $(12.7 \times A_{663}) - (2.69 \times A_{645}) \times V/(1000 \times W)$

Chlorophyll *b* (mg g^{-1} FW) = $(22.9 \times A_{645}) - (4.68 \times A_{663}) \times V/(1000 \times W)$

Carotenoids (mg g^{-1} FW) = $(7.6 \times A_{480}) - (1.49 \times A_{510}) \times V/(1000 \times W)$

Total chlorophyll + Carotenoids (mg g^{-1} FW) = (Chlorophyll *a*)

+ (Chlorophyll *b*) + (Carotenoids)

where, *V* represents the total volume of DMSO added and *W* denotes the weight of spinach leaf sample in g.

Plant enzymatic assays

The activity of glutamine synthetase (GS) in leaf tissues was determined by measuring the absorbance at 540 nm denoted as μmoles γ-glutamyl hydroxamate produced per g fresh weight of leaves per min, following the method of Shapiro and Stadtman (1970). Nitrate reductase activity (NR) was determined as per the method of Snell and Snell (1961) and expressed as μmoles nitrite per g fresh weight. Phosphoenol pyruvate carboxylase activity (PEPCo) was determined following the protocol of Wu and Wedding (1985) and expressed as micromoles per g protein weight per min.

Yield characteristics

At harvest stage (120 days after sowing), spinach plants were randomly selected from each experimental plot, in triplicates, to record the biometric and yield parameters, including plant height (cm), root length (cm), number of branches, number of leaves, leaf area (m^2 $plant^{-1}$), yield (kg m^{-2}) and leaf weight (g $plant^{-1}$). Plant height was measured using a ruler scale at harvest stage for each individual plant. The height was assessed from the plant's base to the tip of the main branch in 15 randomly tagged plants. Root length was the distance from the root's starting point at the base of the plant to its endpoint, and the mean data for 15 plants were documented. The number of branches was measured from the base of the stem where the branches originate. The individual number of branches was counted and their total number recorded at harvest stage. Healthy plants with an adequate number of leaves were chosen. Any deceased or impaired leaves were eliminated. The number of leaves was then subsequently ascertained by tallying them from the uppermost part of the spinach plant to the lowest. The leaf area of three plants was measured using an automated leaf area meter, and the mean leaf area was computed and documented at harvest stage as m^2 per plant. The spinach plants were harvested at maturity phase and the weight of fresh leaves from each plant was measured in g. Subsequently, the relative yield was computed and expressed in kg m^{-2}.

Quality traits estimation in leaves

Total soluble solids (TSS) of spinach leaves were determined by a handheld refractometer (ERMA; Japan) and expressed in °Brix, based on the method of Ranganna

(1986). The firmness of spinach leaves (often denoted as film burst) was assessed by the optimized protocol of Sun et al. (2015), using a texture analyzer (model: TA.Xtplus, Stable Micro System, UK) equipped with a spherical probe. This assesses the firmness of spinach leaves through the application of a regulated force and measures the resultant resistance encountered which is then analyzed to obtain a firmness value (g m^{-2}), which denotes the maximum force exerted during the puncture. Titrable acidity values were computed using the method given by Ranganna (1986). The extracts of the macerated spinach leaf samples in distilled water (10 g in 100 mL distilled water) were filtered through Whatman no. 1 filter paper and titrated with 0.1 N NaOH, using phenolphthalein as the indicator. Titrable acidity (%) can be expressed as:

$$\text{Titrable acidity (\%)} = \text{Titre value} \times 0.1N \text{ NaOH} \times \text{Volume made up (mL)}$$
$$\times \text{ Equivalent weight}$$
$$\times 100/[\text{Aliquot taken (mL)}$$
$$\times \text{ Weight of the spinach sample (g)} \times 1000]$$

Ascorbic acid (vitamin C) was measured by the visual titrimetric technique as outlined in AOAC (2000).

Protein content of spinach leaves was measured with bovine serum albumin (BSA) as standard (Herbert et al. 1971). The assessment of antioxidant activity in fresh spinach leaves was conducted using the CUPRAC (Cupric Reducing Antioxidant Capacity) method, following the procedure outlined by (Apak et al. 2004). Absorbance of the samples was measured at 460 nm and expressed as micromoles of Trolox equivalents per 100 g (Trolox equivalent antioxidant capacity-TEAC):

$$\text{Antioxidant activity } (\mu mol \text{ TEAC } (100g)^{-1}) = (\text{Absorbance} \times 4.1 \times \text{ volume made up})$$
$$/(1.67 \times 10^4 \times \text{ aliquot taken } \times \text{ weight of spinach sample})$$

The total phenolic content in the raw spinach leaves were determined using the Folin-Ciocalteau reagent method as described by Singleton et al. (1999). The total phenolic content was expressed in mg of gallic acid equivalents (GAE) per 100 g of extract:

$$\text{Total phenols } (GAE \text{ mg } (100 \text{ g})^{-1}) = (\text{Absorbance} \times \text{ volume made up} \times 100)$$
$$/(0.02 \times \text{ weight of spinach sample taken } \times \text{ aliquot } \times 1000)$$

The β-carotene, carotenoids and vitamin A were also determined in spinach leaves using the formula given below.

$$\text{Beta carotene } (\mu g \text{ } (100 \text{ g})^{-1}) = (\text{Absorbance} \times 13.9 \times 10^4 \times 100)$$
$$/(\text{weight of the leaf sample taken} \times 560 \times 1000)$$
$$\text{Vitamin A (IU)} = \beta - \text{carotene } (\mu \text{ g } (100 \text{ g})^{-1})$$
$$/0.6 \text{Carotenoids } (\text{mg } (100 \text{ g})^{-1}) = (3.85 \times OD \times V \text{ (mL)} \times 10^4)$$
$$/ \text{ leaf sample weight (g)} \times 1000$$

The calcium content of spinach leaves was determined at harvest stage using a Flame photometer equipped with an LPG flame system, Jaisbro microprocessor and silicon photodiode

detector, following the wet ashing digestion (di-acid digestion) method outlined by Raghuramulu et al. (1983). The values were expressed as mg calcium (100 g)$^{-1}$ dry weight.

Statistical analysis

The experiment used a randomized block design (RBD), comprising four treatments, including control. The data obtained in triplicate, at four-different time intervals (2, 3, 5 WAS and harvest stage) were analyzed for the various soil, plant, quality and yield parameters at a probability level of 0.05 %, through ANOVA using WASP 2.0 (Web Agri Stat Package, Indian Council of Agricultural Research, India) statistical tools, with critical differences computed. Pearson's Correlation coefficient analysis using GRAPE (GRAphics Programming Environment) software tool (v1.0.0) (Gopinath et al. 2020) was utilized. Path coefficient analysis was performed using Windostat version 8.5 software (Singh and Chaudhary 1977).

Results

Analyses of the drench formulations

The total sugar values were highest in T2 (C11) and T4 (RPAN8) and lowest in Control. As compared to control (uninoculated), the released sugars were 2- fold higher in T4 (RPAN8) over control; T2 (C11) had the highest reducing sugar content which was 2-fold higher over control. In terms of protein content, both T3 (BF2) and T4 (RPAN8) showed high values (43.39 and 36.73 μg mL^{-1}). IAA values were 2-fold higher in T2 (C11; 39.86 μg mL^{-1}) amended drench formulations compared to control. In terms of total phenol content, it was observed that T4 (RPAN8) showed higher values (36.43 μg CAE mL^{-1}), as compared to control (T1). In terms of Glomalin content, T4 drench formulation (RPAN8) exhibited 18.72% higher values, as compared to control (Table 1).

Growth attributes, as influenced by cyanobacterial treatments

Total pigment content of the leaves was greater in cyanobacterium-treated plants and highest with C11 (0.95 mg g^{-1} FW) at 2 WAS, however, at later stages RPAN8 and BF2 were most promising respectively, as compared to control (Fig. 1a).

The components of total pigments such as chlorophyll values were also higher in leaves of seeds treated with cyanobacteria +drench, as compared to the control. Chlorophyll *a* was higher with C11 treatment at all stages of crop

growth (Fig. S1a) i.e., 2, 3, 5 WAS and harvest stage (72%, 67.74%, 1.02-fold and 38.29% in C11 treated plants respectively). Similarly, treatment with cyanobacteria resulted in the enhancement of chlorophyll *b* content of the leaves at all stages, which was higher in plants treated with C11 (0.50-fold increase at 2 WAS, 0.048-fold increase at 3 WAS, 1.09-fold increase at 5 WAS and 0.82-fold increase at harvest stage respectively), in comparison with control (Fig. S1b). carotenoid content showed no significant differences in the cyanobacterium treated plants as compared to the uninoculated control, particularly at harvest stage (Fig. S1c). At 2 and 5 WAS, carotenoid content was significantly higher in plants treated with C11 (62.5% and 80.76% increase over control) respectively. However, changes in carotenoids content were significantly higher at 3 WAS in cyanobacteria-treated plants, while BF2 and RPAN8 significantly enhanced the content in leaves (0.22 mg g^{-1} FW and 0.21 mg g^{-1} FW).

Activity of plant enzymes

A gradual reduction in glutamine synthetase activity in leaves was recorded with increasing age of the plants, and all three cyanobacterial treatments brought about a positive influence. Values ranged from 4.88 to 7.23 μmol γ-glutamyl hydroxamate g^{-1} FW min^{-1} at 2WAS, with highest in C11 (7.23 μmol γ-glutamyl hydroxamate g^{-1} FW min^{-1}), followed by BF2 (6.05 μmol γ-glutamyl hydroxamate g^{-1} FW min^{-1}) and RPAN8 (6.20 μmol γ-glutamyl hydroxamate g^{-1} FW min^{-1}) respectively (Fig. 1b). In terms of cyanobacterial treatments, RPAN8 treatment recorded significantly higher values of 5.40 μmol γ-glutamyl hydroxamate g^{-1} FW min^{-1} respectively. At 5 WAS, the values ranged from 2.79 to 3.77 μmol γ-glutamyl hydroxamate g^{-1} FW min^{-1} and among the treatments, the top performers were C11 and BF2, respectively (3.77 and 3.73 μmol γ-glutamyl hydroxamate g^{-1} FW min^{-1}). The cyanobacterial treatments, in particular, C11 considerably enhanced the glutamine synthetase activity even at harvest stage, by 53.02% compared with Control, followed by 37.2% enhancement by RPAN8, respectively.

The activity of nitrate reductase (NR) in leaf samples ranged from 183.09 to 256.80 μmol NO$_2$ g^{-1}FW at 2 WAS, 157.49 to 210.27 μmol NO$_2$ g^{-1}FW at 3 WAS, 132.34 to 180.31 μmol NO$_2$ g^{-1}FW at 5 WAS and 114.64 to 171.53 μmol NO$_2$ g^{-1}FW at harvest stage respectively (Fig. 1c), while among treatments (Fig. 1c), the highest values were observed in C11 (256.80, 194.64, 179.37 and 161.81 μmol NO$_2$ g^{-1}FW) and RPAN8 (250.35, 210.27, 180.31 and 171.53 μmol NO$_2$ g^{-1}FW) at all 4 stages respectively. Overall, C11 and RPAN8 were the top performers, at all stages of growth.

Phosphoenol pyruvate carboxylase (PEPCO) activity determined in leaves at 2, 3, 5 WAS and harvest stages were enhanced by all cyanobacterial treatments, up to harvest stage (Fig. 1d). BF2 was the top ranked, followed by C11 and RPAN8 at all stages, except at 2WAS, in which RPAN8 exhibited a greater increment.

Soil microbiological analyses

The soil microbial biomass carbon in treatments receiving cyanobacterial treatment (seed coating and drench) ranged from 94.47 to 132.47; μg g^{-1} soil, with highest in C11 (132.47 μg g^{-1} soil), followed by BF2 (117.29 μg g^{-1} soil), as given in Table 2. SMBC content at 3 WAS significantly increased in cyanobacterial treatments, as compared to control, with highest values recorded in C11 and RPAN8 203.81 and 223.63 μg g^{-1} soil, followed by BF2 (137.52 μg g^{-1} soil).A similar trend was also observed at 5 WAS, wherein T2 (C11) and T4 (RPAN8) showed highest total SMBC content (316.35 and 320.37 μg g^{-1} soil), followed by T3 (BF2; 238.60 μg g^{-1} soil) respectively. SMBC content showed a progressive increase in RPAN8 treatment throughout the period of the experiment. Dehydrogenase activity in soil enhanced with cyanobacterial treatments at all 4 stages. Among the cyanobacterial treatments, dehydrogenase activity in T2 (C11) was almost 40.72% higher at 2WAS, with 46.09% enhancement at 3 WAS, 35.80% at 5 WAS and 30.33% enhancement at harvest stage, respectively, compared to the control (Table 2). Chlorophyll content was highest in T4 (RPAN8) treatment at both 2 WAS (0.48 mg g^{-1}) and harvest stage (0.67 mg g^{-1}) respectively (Table 2). Among the cyanobacterial treatments evaluated

Table 1 Biochemical analyses of cyanobacterial drench formulations

Treatments	Total sugars (μg mL^{-1})	Released sugars (μg mL^{-1})	Reducing sugars (μg mL^{-1})	Proteins (μg mL^{-1})	IAA (μg mL^{-1})	Total Phenols (μg CAE mL^{-1})	Glomalins (μg g^{-1} mix)
T1Control	52.28± 2.20b	29.38± 1.65c	29.43 ± 0.65d	19.58±2.03b	17.30± 1.83d	15.40 ± 1.04c	16.88 ± 0.34c
T2 C11	73.41 ± 1.98a	46.49 ± 2.28b	65.79 ± 2.54a	33.77± 1.74b	39.86± 3.65a	22.84± 0.65b	18.85 ± 0.08b
T3 BF2	53.22 ± 3.01b	43.04 ± 1.10b	37.72 ± 1.87c	43.39 ± 2.63a	23.24± 1.57c	24.57± 1.61b	18.34 ± 0.36b
T4 RPAN8	70.57 ± 1.84a	56.26 ± 2.04a	56.03 ± 3.08b	36.73 ± 2.31a	33.72± 2.93b	36.43± 1.43a	20.04 ± 0.11a
CD(P≤0.05)	7.56	5.85	7.26	4.90	7.06	0.43	0.833

T2 C11 (*Anabaena laxa*); T3 BF2 (*Nostoc carneum*); T4 RPAN8 (*Anabaena laxa*); CAE – Caffeic acid equivalent; the values presented are the mean of three replicates; CD, Critical difference at 5% significance level.

Fig. 1 Influence of cyanobacterial treatments on photosynthetic pigments and enzymatic activities of spinach crop at different crop growth stages in terms of **(a)** Total pigments, **(b)** Glutamine Synthetase activity, **(c)** Nitrate reductase activity, **(d)** PEPCO. Error bars represent standard deviation of mean of three replicates. Abbreviations for treatments and attributes: T1: Control; T2:C11 (*Anabaena laxa*), T3: BF2 (*Nostoc carneum*), T4: RPAN8 (*Anabaena laxa*); WAS: weeks after sowing
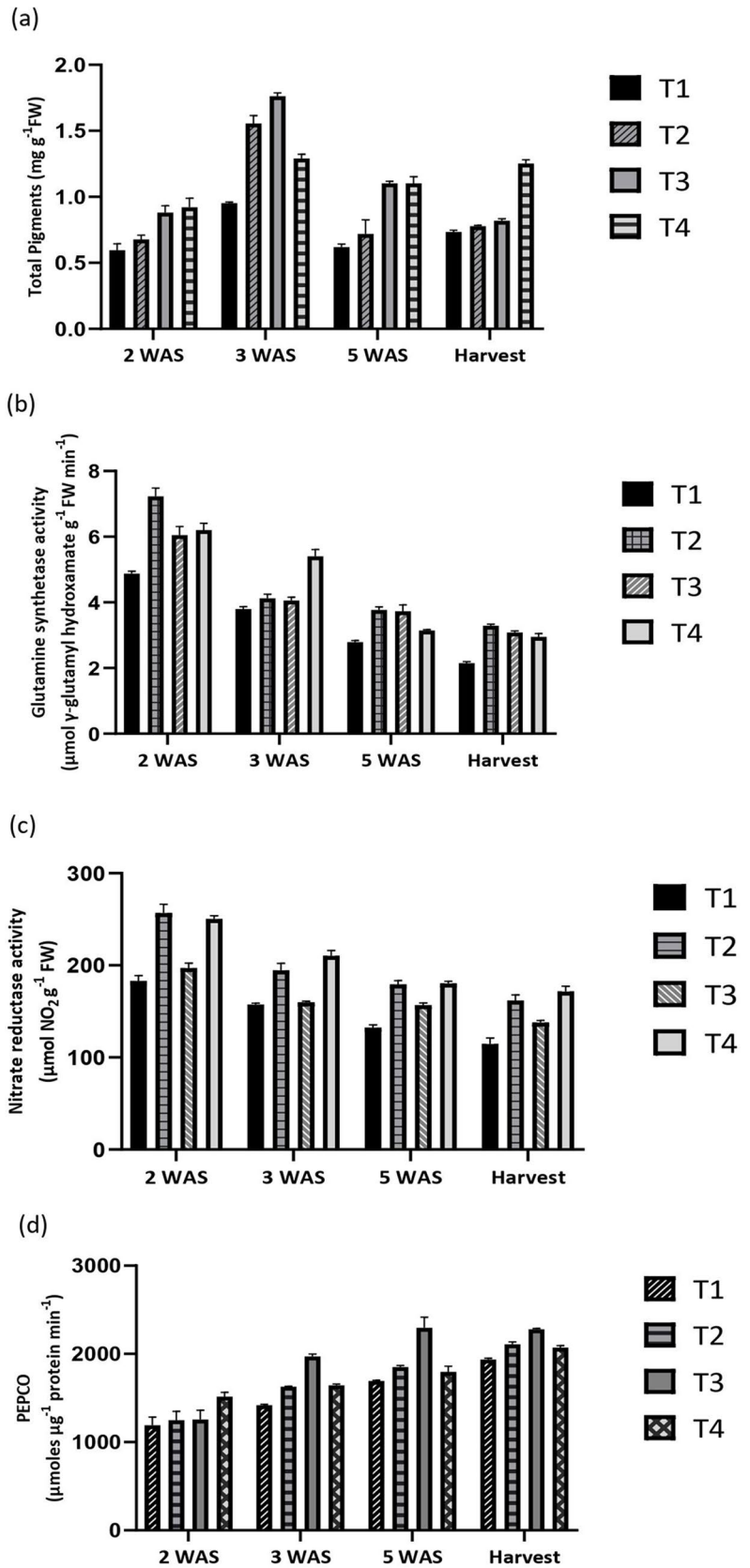
at 3 WAS, the highest chlorophyll values of 0.56 mg g^{-1} was observed in T2 (C11), followed by 0.51 mg g^{-1} in RPAN8. The soil chlorophyll content in all the treatments at 5 WAS varied from 0.24 to 0.63 mg g^{-1}, with all the cyanobacterial treatments (C11, BF2 and RPAN8) exhibiting the highest values (0.63, 0.53 and 0.52 mg g^{-1}).

Analysis of soil and plant nutrient attributes

The organic carbon content in soil samples ranged from 0.25 to 0.38 %, with all the cyanobacterial amendments (C11, BF2 and RPAN8) showing significantly higher values (0.38, 0.37 and 0.35%) than the control. At 2, 5 WAS and harvest stage, T2 (C11) treatment showed an increase of 52 %, 70.83% and 37.93% increase in terms of organic carbon content, in contrast to a 32%, 45.8% and 27.58 % increment caused by RPAN8 treatment (Fig. 2a). All the cyanobacterial treatments exhibited significant differences in available soil nitrogen content; soil under C11, BF2 and RPAN8 treatments experienced significantly higher available soil nitrogen content at both 2 and 5 WAS compared to the control (Fig. 2b). However, no significant differences in available nitrogen in soil was recorded at harvest stage, although C11 showed higher values. Among the treatments at 3 WAS, T4 (RPAN8) showed an 8.72% increase as compared to control (Fig. 2b). The available phosphorus content in soil at 3 WAS ranged from 6.82.2 – 11.95.2 mg kg^{-1}; among the treatments, T2 (C11) showed higher total phosphorus content of 11.19 mg kg^{-1} compared to T1 (control). At 5 WAS, available phosphorus content was significantly higher in all the cyanobacterial treatments (C11, BF2 and RPAN8 -11.31, 11.27 and 11.37 mg kg^{-1}) compared to the control (7.10.53 mg kg^{-1}) respectively. Among the cyanobacterial treatments, the highest available phosphorus content of 12.12.07 mg kg^{-1} was observed in RPAN8 (Fig. 2c) at harvest stage. However, at 2WAS, no significant differences were observed among the cyanobacterial treatments.

The effect of cyanobacterial treatments (Table 3), in terms of available soil iron revealed that in all treatments (C11, BF2, RPAN8), excluding control, the highest values were recorded at 3 WAS, at 5 WAS and at harvest stage. However, no significant differences were recorded at 2 WAS. The available zinc content in soil showed significantly higher values, with a 1.22-fold and 1.20- fold increments in T4 (RPAN8) and T3 (BF2) respectively. However, all cyanobacterial treatments (C11, BF2, RPAN8), showed the highest increase of 74.33 %, 79.64% and 76.10 % at 3 WAS, (56.37 %, 64.42 % and 52.34 % increase) at 5 WAS and (34.65%, 46.59% and 36.93% increments) at harvest stage as compared to the control, respectively (Table 3). Interestingly, nitrogen content in leaves at 2 WAS exhibited significantly higher values in all the cyanobacterial treatments (C11, BF2 and RPAN8 - 3.67, 3.90 and 3.80 %), compared to

Table 2 Evaluation of soil microbiological parameters, as influenced by cyanobacterial treatments in spinach crop

Treatments	Soil microbial biomass carbon (SMBC; μg g^{-1} soil)				Soil Dehydrogenase activity (μg TPF g^{-1} soil day^{-1})				Soil Chlorophyll (mg g^{-1} soil)			
	2 WAS	3 WAS	5 WAS	Harvest	2 WAS	3 WAS	5 WAS	Harvest	2 WAS	3 WAS	5 WAS	Harvest
T1Control	94.47 ± 7.28c	108.72 ± 3.92c	105.91 ± 8.16c	121.31± 9.40c	78.74 ± 1.56d	79.70 ± 0.06c	87.11± 0.53d	90.88 ± 0.84c	0.18 ± 0.002d	0.21 ± 0.006d	0.24 ± 0.007b	0.31 ± 0.003c
T2 C11	132.47 ± 4.97a	203.81 ± 10.75a	316.35 ± 11.53a	366.63 ± 6.40ab	110.81 ± 1.72a	116.44 ± 2.38a	118.30 ± 0.17a	118.45 ± 0.69a	0.43 ± 0.004b	0.56 ± 0.023a	0.63 ± 0.012a	0.57 ± 0.001b
T3BF2	117.29 ± 5.35b	137.52 ± 4.98b	238.60 ± 12.38b	358.47 ± 9.82b	78.76 ± 4.14c	93.80 ± 0.75b	97.41 ± 0.62c	106.25 ± 0.63b	0.40 ± 0.006c	0.46 ± 0.027c	0.53 ± 0.075a	0.54 ± 0.031b
T4 RPAN8	118.47 ± 7.45ab	223.63 ± 3.57a	320.37 ± 4.60a	393.16 ± 14.33a	93.37 ± 2.37b	96.08 ± 0.60b	102.17 ± 0.55b	107.72 ± 1.84b	0.48 ± 0.013a	0.51 ± 0.035b	0.52 ± 0.026a	0.67 ± 0.020a
CD(P≤0.05)	16.647	22.944	37.712	26.919	4.013	4.143	1.265	4.245	0.027	0.045	0.131	0.073

WAS- weeks after sowing; T2 C11 *Anabaena laxa*; T3 BF2 *Nostoc carneum*; T4 RPAN8 *Anabaena laxa*; TPF- Tri phenyl formazan; ± denotes standard error; CD represents critical difference among treatment means (P≤0.05) based on Duncan's multiple range test (DMRT)

Fig. 2 Modulation of soil macronutrient levels as influenced by cyanobacterial treatments at different stages of growth **(a)** soil organic carbon, **(b)** available soil nitrogen, **(c)** available soil phosphorus. Error bars represent standard deviation of mean of three replicates. Abbreviations for treatments and attributes: T1: Control; T2:C11 (*Anabaena laxa*), T3: BF2 (*Nostoc carneum*), T4: RPAN8 (*Anabaena laxa*); WAS: weeks after sowing
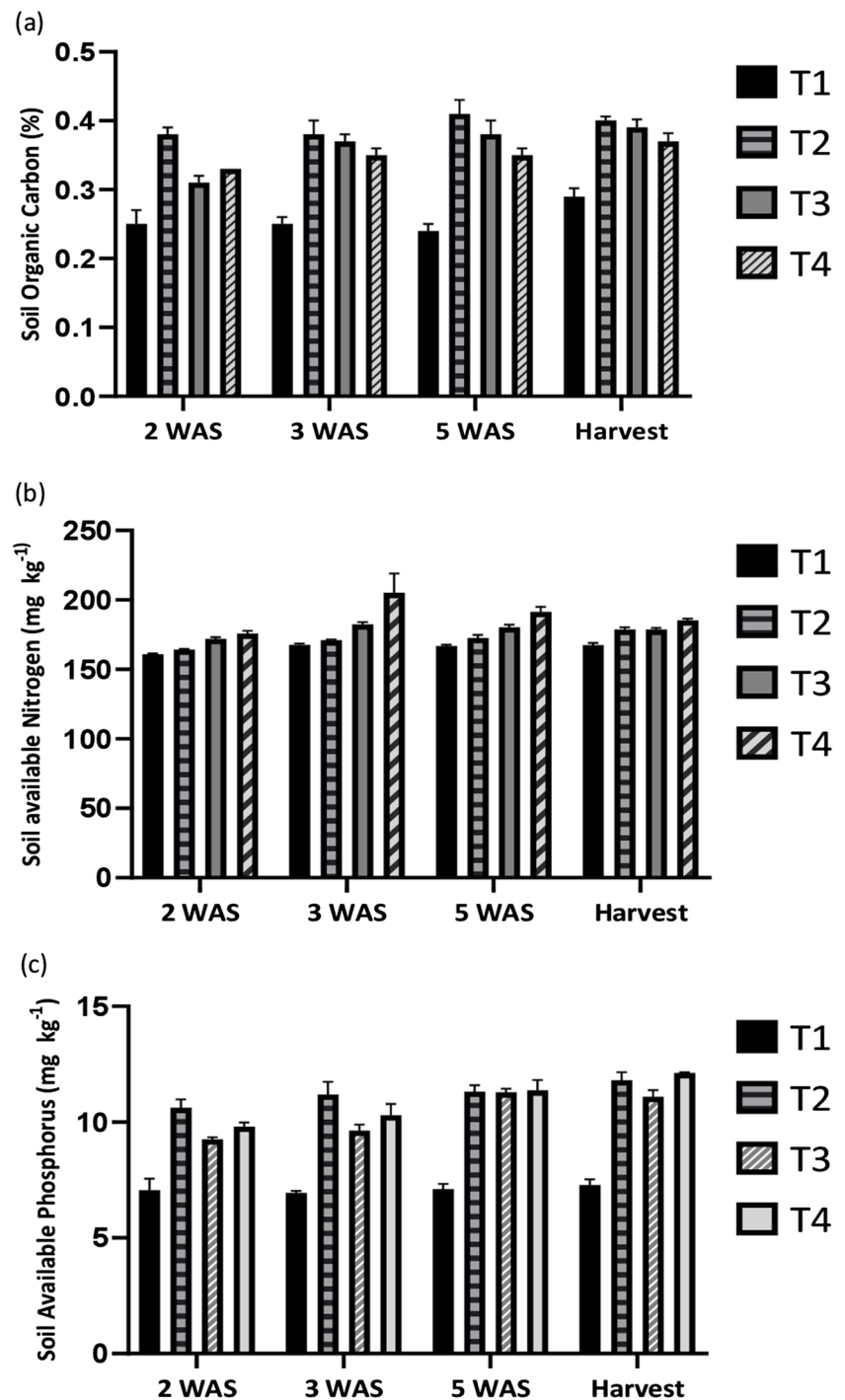
to control (3.40%) respectively (Table 3), with highest values recorded at harvest stage. C11 (4.18%) recorded significantly higher leaf nitrogen content at 3WAS, but at 5 WAS, RPAN8 recorded statistically at par values (Table 3). Comparison across cyanobacterial treatments at harvest stage showed significant differences (4.58 and 4.52%), with T2 (C11) and T4 (RPAN8) being the top performers. Analyses of leaf calcium content in cyanobacterial treatments illustrated that RPAN8

brought about an increase of 6.33%, while C11 and BF2 exhibited a 5.73 and 5.37% increment over control (Table 3).

Yield attributes

Cyanobacterial treatment resulted in a 54.35 % increase of leaf area, over the uninoculated control estimated at harvest stage (Table 4). Comparatively, the treatments C11 (35.92%

Table 3 Assessment of soil micronutrient levels and plant nutrient attributes, as influenced by cyanobacterial treatments

Treatments	Available soil Iron (mg kg⁻¹ soil)				Available soil Zinc (mg kg⁻¹ soil)				Leaf Nitrogen (%)				Calcium content in leaves (mg (100 g)⁻¹)
	2 WAS	3 WAS	5 WAS	Harvest	2 WAS	3 WAS	5 WAS	Harvest	2 WAS	3 WAS	5 WAS	Harvest	
T1 Control	0.84 ± 0.15	0.93 ± 0.11b	0.84 ± 0.15	0.93 ± 0.11b	0.84 ± 0.15	0.93 ± 0.11b	0.84 ± 0.15	0.93 ± 0.11b	3.40 ± 0.08b	3.68 ± 0.16c	3.93 ± 0.08c	4.10 ± 0.03b	91.00 ± 0.06b
T2 C11	1.43 ± 0.24	1.62 ± 0.13a	1.43 ± 0.24	1.62 ± 0.13a	1.43 ± 0.24	1.62 ± 0.13a	1.43 ± 0.24	1.62 ± 0.13a	3.67 ± 0.15a	4.18 ± 0.07a	4.41 ± 0.12a	4.58 ± 0.06a	96.22 ± 0.26a
T3 BF2	1.33 ± 0.09	2.05 ± 0.15a	1.33 ± 0.09	2.05 ± 0.15a	1.33 ± 0.09	2.05 ± 0.15a	1.33 ± 0.09	2.05 ± 0.15a	3.90 ± 0.09a	3.99 ± 0.11b	4.15 ± 0.09bc	4.25 ± 0.12b	95.89 ± 0.41a
T4 RPAN8	1.48 ± 0.15	1.64 ± 0.20a	1.48 ± 0.15	1.64 ± 0.20a	1.48 ± 0.15	1.64 ± 0.20a	1.48 ± 0.15	1.64 ± 0.20a	3.80 ± 0.04a	4.08 ± 0.16b	4.28 ± 0.08ab	4.52 ± 0.09a	96.78 ± 0.07a
CD (P≤0.05)	NS	0.596	NS	0.596	NS	0.596	NS	0.596	0.295	0.165	0.245	0.261	0.968

WAS- weeks after sowing; T2 C11 *Anabaena laxa*; T3 BF2 *Nostoc carneum*; T4 RPAN8 *Anabaena laxa*; ± denotes standard error. NS indicates the treatments are non-significant; CD represents critical difference among treatment means (P≤0.05) based on Duncan's multiple range test (DMRT)

increase) and RPAN8 (11.65% increase) showed better yield over control (Fig. 3). In terms of individual leaf weight, RPAN8 showed a 1.24-fold increase, followed by C11 exhibiting a 0.82-fold enhancement, respectively (Table 4). All the treatments with cyanobacteria significantly improved plant height, when compared with T1 (control) (Table 4). The root length of plants treated with C11 (14.96 cm) was higher than the control, and the number of branches were more in RPAN8 (12) by 1.19% (Table 4). The number of leaves was highest in T4 (RPAN8; 14), followed by T2 and T3, which were statistically at par (Table 4).

Quality of the produce

The leaves of *Pusa All Green* analyzed for ascorbic acid content at harvest stage showed higher values for all cyanobacterial respectively (Fig. 3). Total soluble solids also exhibited a similar trend with C11, BF2 and RPAN8 recording an increase of 14.45, 13.98 and 16.31%, over control, respectively (Table 5). Phenol content in the leaves showed a wide range and the highest values of 391.17 mg GAE (100 g)⁻¹ was recorded in RPAN8 treatment, which reflected 86.29% enhancement respectively (Table 5). In general, the titrable acidity content was not significantly modified by the cyanobacterial treatments (Table 5). As compared to control (uninoculated), the anti-oxidant activity in plants was significantly higher (1.51 µmol Trolox (100 g)⁻¹) by the inoculation of either C11 or BF2 (Table 5). Analyses of protein content in leaves illustrated that cyanobacterial treatment (RPAN8) enhanced the content to the greatest extent, by 86.27%, followed by C11, which also brought about a significant increase in protein content in leaves (7.87 mg g⁻¹ leaves) (Table 5). BF2 increased the content of β-carotene (Fig. 3), Vitamin A and carotenoids (Table 5) and brought about significant enhancement over the control (T1). The textural analysis in terms of firmness of spinach leaves (also referred to as film burst) revealed that C11 treatment (seed coating + soil drench) showed a 30.18% increase over Control (Table 5). This was followed by BF2, which brought about a significant increase of 17.15%.

Phenotypical correlation of plant, soil, yield and quality traits at different stages of growth

To investigate the associations among plant, soil, yield and quality traits, Pearson's correlation coefficients were computed. A strong positive and significant association was observed between the soil nitrogen with plant/soil parameters and the values in ascending order are as follows: glutamine synthetase (GS) activity (r=0.85**) at 2 WAS (Fig. S2a) < available soil phosphorus (P) at 5 WAS (r=0.86**; Fig. S2c) < glutamine synthetase

Table 4 Yield attributes of Spinach crop, as influenced by the application of cyanobacterial treatments at harvest stage

Treatments	Plant height (cm)	Root length (cm)	No. of branches	No. of leaves	Leaf area (m^2 $plant^{-1}$)	Leaf Weight (g $plant^{-1}$)
T1Control	38.15 ± 0.68b	9.43 ± 0.24b	7 ± 0.60c	9 ± 0.66c	44.67 ± 1.45c	20.60 ± 0.58b
T2C11	39.93 ± 0.64a	14.96 ± 0.36a	9 ± 0.71b	12 ± 0.73b	56.47 ± 1.95b	37.57 ± 1.01a
T3BF2	39.89 ± 0.92a	10.49 ± 0.48b	7 ± 0.50b	10 ± 0.51b	46.50 ± 1.16c	21.13 ± 1.32ab
T4 RPAN8	40.14 ± 0.86a	10.09 ± 0.54b	12 ± 0.49a	14 ± 0.96a	68.95 ± 2.18a	46.20 ± 2.14a
CD (P≤0.05)	1.760	1.395	1.973	1.500	5.217	0.584

T2 C11 *Anabaena laxa*; T3 BF2 *Nostoc carneum*; T4 RPAN8 *Anabaena laxa*; ± denotes standard error. CD represents critical difference among treatment means (P≤0.05) based on Duncan's multiple range test (DMRT)

Fig. 3 Influence of cyanobacterial treatments on yield and quality traits of spinach at harvest stage. Error bars represent standard deviation of mean of three replicates. Abbreviations for treatments and attributes: T1: Control; T2:C11 (*Anabaena laxa*), T3: BF2 (*Nostoc carneum*), T4: RPAN8 (*Anabaena laxa*)
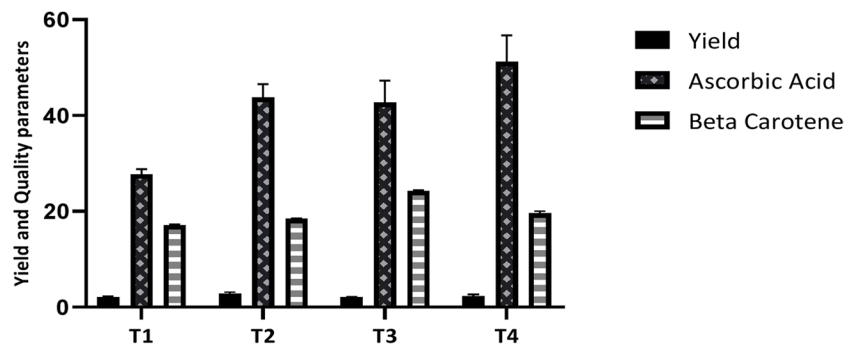
Table 5 Effect of cyanobacterial treatments on quality attributes of Spinach crop at harvest stage

Treatments	Antioxidant activity (μmol Trolox (100 g)$^{-1}$ leaves)	Total Phenols (mg GAE (100 g)$^{-1}$ leaves)	Titrable Acidity (%)	Leaf Proteins (mg g^{-1} leaves)	Total Soluble Solids (°Brix)	Vitamin A (IU)	Carotenoids (mg (100 g)$^{-1}$ leaves)	Spinach Firmness (g m^{-2})
T1 Control	1.09 ± 0.0042c	209.97 ± 4.35d	1.06 ± 0.20	4.30 ± 0.24c	4.29 ± 1.21b	28.55 ± 0.19d	66.43 ± 0.04d	245.23 ± 39.19c
T2 C11	1.51 ± 0.0084a	327.37 ± 6.53b	1.29 ± 0.12	7.87 ± 0.63b	4.91 ± 1.29a	30.73 ± 0.16c	71.49 ± 0.04c	319.26 ± 56.35a
T3 BF2	1.51 ± 0.0004a	246.40 ± 4.75c	1.17 ± 0.23	5.81 ± 0.37ab	4.89 ± 1.25a	40.42 ± 0.33a	94.04 ± 0.08a	287.30 ± 60.47ab
T4 RPAN8	1.18 ± 0.0004b	391.17 ± 3.24a	1.06 ± 0.20	8.01 ± 0.42a	4.99 ± 1.28a	32.66 ± 0.72b	75.98 ± 0.17b	263.26 ± 95.15bc
CD(P≤0.05)	0.017	19.327	NS	0.682	0.510	1.539	0.358	44.69

T2 C11 -*Anabaena laxa*; T3 BF2- *Nostoc carneum*; T4 RPAN8- *Anabaena laxa*; GAE – Gallic acid equivalent; IU- International unit; ± denotes standard error. CD represents critical difference among treatment means (P≤0.05) based on Duncan's multiple range test (DMRT)

activity (GS) & organic carbon (OC)/ soil chlorophyll (SC; r=0.87**) at 5, 2 WAS, respectively. The nitrogen content in leaves showed a significant positive correlation with soil parameters such as soil chlorophyll (SC; r=0.934**) at 3 WAS (Fig. S2b; Fig. S5) and available soil phosphorus (P; r=0.865**) at 3 WAS respectively (Fig. S4a). The available iron content in soil exhibited positive correlation with chlorophyll *a* (r=0.936**) at harvest stage respectively (Fig. S2d) and with soil parameters like soil microbial biomass carbon (SMBC; r=0.980**) at harvest stage, soil chlorophyll (r=0.942** at harvest stage) and Available soil phosphorus (P;

r=0.953**) at harvest stage, respectively. The phosphorus content in soil exhibited significant positive association with chlorophyll *a* (0.946**) at harvest stage, GS (r=0.910**) at harvest stage, SMBC (r=0.980**) at harvest stage, soil dehydrogenase (r=0.904**) at 3 WAS (Table S.3) and soil chlorophyll (r=0.966; 0.954**) at 3 WAS and harvest stage respectively. A positive and significant association was observed between the OC with GS (r=0.950;0.909/0.971**) at 2, 5 WAS (Fig. S6) and harvest stage respectively (Fig. S3d) and with soil parameters like SMBC (r=0.901**) at harvest stage, soil dehydrogenase (r=0.919**) at 2 WAS (Fig. S4) and soil

chlorophyll(r=0.924**) at 3 WAS respectively (Fig. S5). The chlorophyll content in soil exhibited significant positive correlation with phosphoenol pyruvate carboxylase activity (PEPCO; r = 0.904**) at 2 WAS (Table S.1) and SMBC (r = 0.952**) at harvest stage respectively. A positive and significant association was observed between dehydrogenase activity in soil with traits such as chlorophyll *a* (r= 0.941;0.903**) at 2 WAS and harvest stage, carotenoids (r= 0.911*) at 2 WAS, total pigments (r= 0.930;0.895**) at 2, 3 WAS (Table S.1), and GS (r= 0.913;0.934**) at 2 WAS and harvest stage and with SMBC (r=0.893**) at 2 WAS respectively. SMBC exhibited significant positive correlation with plant traits like chlorophyll *a* (r=0.923**) at harvest stage (Fig. S7), nitrate reductase (r=0.902;0.945/0.983**) at 2, 3, 5 WAS (Table S5); and GS (r=0.914**) at harvest stage respectively. Nitrate reductase activity showed a significantly positive correlation with chlorophyll *a*, chlorophyll *b*, carotenoids and total pigments content (r=0.910**; 0.821*; 0.713*; 0.899**) at 2 WAS and (r=0.772*; 0.845**; 0.822*;0 .816*) at harvest stage respectively (Table S.7).

Among yield variables recorded at harvest stage (Fig. 4a; Table S9), the leaf calcium content showed a strong significant positive correlation with parameters and the values were in ascending order are as follows: organic carbon (r=0.827**) <Chlorophyll *a* (*CHLA;* r=0.841**) < Soil Microbial Biomass Carbon (r=0.877**) < available soil phosphorus (r=0.881**) < available iron (r=0.887**) < soil chlorophyll (r=0.898**) at harvest stage (Fig. S8). There were higher correlations observed among individual plant weight/nitrate reductase activity and individual plant weight/Leaf nitrogen (r=0.933**) and (r=0.827**) at harvest stage respectively. However, there were also significant higher correlations observed between vitamin A with phosphoenol pyruvate carboxylase activity (r=0.912**) respectively (Fig. 4b; Fig. S9; Table S11). The leaf protein content showed a strong significant positive correlation with the values in ascending order: chlorophyll *a*, glutamine synthetase, organic carbon (r=0.84**) < soil available iron (r=0.90**) <available phosphorus (r=0.94**) < SMBC/SC (r=0.95**). Most interestingly, the ascorbic acid trait depicted a strong positive correlation with soil chlorophyll (r=0.890**), SMBC (r=0.850**) and soil available iron (r=0.851**).

Path coefficient analysis

Significant effects were recorded for path coefficient analyses of available soil nitrogen with other parameters at 2, 3, 5 WAS and harvest stage. Positive direct effects of PEPCO (2.66), carotenoids (2.19), chlorophyll *a* (1.40), soil dehydrogenase activity (1.32) and Glutamine synthetase activity

(1.27) besides organic carbon (0.13) and Soil Microbial Biomass Carbon (0.01) on available soil nitrogen at 2 WAS were recorded (Fig. 5). The residual effect was 0.001, which clearly depicted that all the parameters at 2 WAS were majorly involved in enhancing the soil available nitrogen, brought about by cyanobacterial treatments (Fig. S3a; Table S2). The positive direct effect of nitrate reductase activity (10.64) and chlorophyll *b* (10.23) on available soil nitrogen was found to be very high at 3 WAS are observed (Table S4). The residual effect was 0.002, which shows that the increase in soil available nitrogen is due to the other attributes at 3 WAS investigated, as influenced by cyanobacterial treatments (Fig. S3b). On the other hand, chlorophyll *a* (7.17) in the leaves exerted a major direct effect on available soil nitrogen values at 5 WAS in inoculated treatments (Fig. S3c). Furthermore, the direct positive effect of available soil phosphorus in the soil (5.28) carotenoids (3.39) and PEPCO (3.15) was found to be stronger in the inoculation treatments (Table S.6). At harvest stage, carotenoids (3.27), soil available iron (2.83) and chlorophyll *a* (2.65) had positive direct effects on available soil nitrogen at harvest stage followed by glutamine synthetase activity (1.78), available soil Zn (0.98), soil dehydrogenase activity (0.53) and soil chlorophyll (0.48). The residual value for the path analysis model was 0.001 at harvest stage was also observed (Fig. S3d; Table S8). Except for total soluble solids (8.02) and leaf firmness/ film burst (9.26); the assessed traits had positive direct effects on available soil nitrogen through quality traits, including antioxidant activity (25.82), phenols (24.96), leaf proteins (21.73), beta carotene (14.89) and titrable acidity (10.74). The residual value for the path analysis model was 0.003 at harvest stage was observed for quality traits with soil available nitrogen (Fig. 5; Table S12) whereas with yield traits; Leaf area (3.99) and Individual plant weight (1.42) had the greatest positive direct effect on soil available nitrogen (Fig. S3e; Table S10).

Discussion

Cyanobacteria are a valuable bioresource which play a significant role in the sustainability of various sectors, particularly agriculture and industry (Rana et al. 2012; Gupta et al. 2013; Grzesik and Romanovska-Duda 2015; Kholssi et al. 2021; Shivay et al. 2022). They are valuable as biofertilizers or nutrient-mobilizing inoculants, which have been successfully utilized in various crops, including rice, wheat, maize, tomatoes, and other vegetables (Kumar et al. 2013; Prasanna et al. 2014, 2021). Several cyanobacteria are known to enhance yields in various vegetable and fruit species as documented in several studies (Geries and Elsadany 2021; Kokila et al. 2023; Bhardwaj et al. 2024), however, little research

Fig. 4 Phenotypical Correlation network, depicting the relationships among plant, soil, yield and quality traits (**a**) yield traits with plant and soil parameters at harvest stage; (**b**) quality traits with plant and soil at harvest stage; P: available soil phosphorus; OC: soil organic carbon; SC: soil chlorophyll; DHY: soil dehydrogenase activity; SMBC: soil microbial biomass carbon; PEPC: Phosphoenol pyruvate carboxylase activity; GS: glutamine synthetase activity; NR: nitrate reductase activity; TP: total pigments; CAR: carotenoids; CHLB: chlorophyll *b*; LN: leaf nitrogen; SN: available soil nitrogen; LCA: leaf calcium; LA: leaf area; NOL: number of leaves; NOB: number of branches; PW: plant weight; IPW: individual plant weight; RL: root length; PH: plant height; CHLA: chlorophyll *a*; ZN: soil available zinc; FE: soil available iron; VITA: vitamin A; LP: leaf proteins; PHE: phenols; AOX: antioxidants; CAR: carotene; BCAR: beta carotene; FB: film burst; TSS: total soluble solids; TA: titrable acidity; AA: ascorbic acid
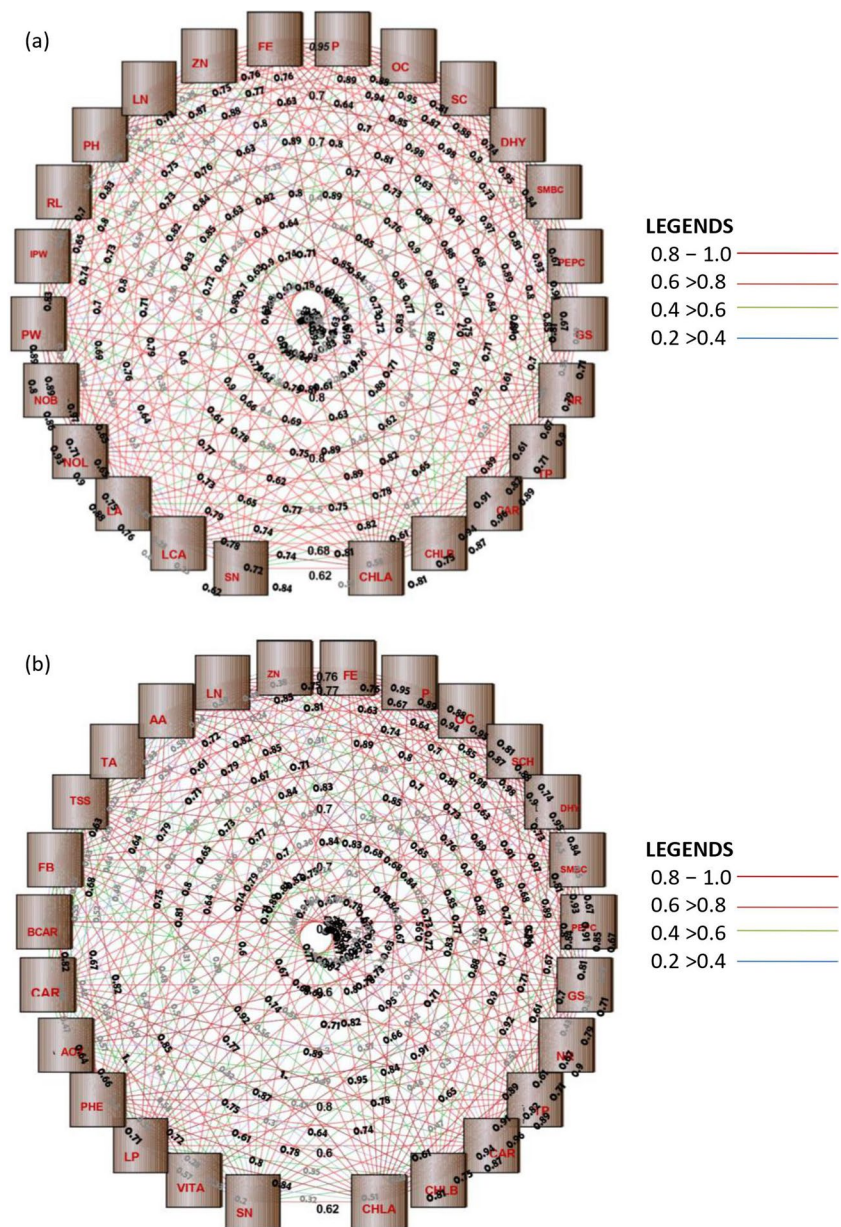
is documented on cyanobacteria as an input to enhance the commercial and qualitative attributes of spinach as a crop. In the current study, the use of carrier-based cyanobacterial formulations, as seed coating, specifically those amended with *A. laxa* C11, *N. carneum* BF2 or *A. laxa* RPAN8, along with their use as soil drench, in the cultivation of *Pusa All Green* variety of spinach in shaded net house was investigated. A range of growth-related, enzymatic, soil biological, yield, and quality parameters as indicators were evaluated the role of cyanobacteria as an organic intervention towards the production of a healthy spinach crop.

Dehydrogenase activity, microbial biomass carbon, and soil chlorophyll content serve as important soil microbiological indicators to gauge nutrient mobilization and soil quality

indices as they correlate positively with the abundance of microbial numbers (Filip 2002; Hanaka et al. 2019) Soil microbial biomass plays a fundamental role in regulating the transformation processes of elements, besides governing the accumulation and decomposition of organic matter, and serving as an early indicator of perturbations due to changes in agronomic practices or contamination through xenobiotics or heavy metals. The soil microbial biomass carbon in cyanobacteria-inoculated treatments was enhanced by 2-3 fold over control, underscoring the significant impact of cyanobacterial inoculation on soil quality. Several previous studies showed an increase in microbial biomass carbon by 15-30%, highlighting the remarkable ability of cyanobacteria to substantially contribute towards soil carbon accretion (Prasanna et al. 2014,

Fig. 5 Network path diagram depicting the association of soil available nitrogen with quality traits at harvest stage; PEPCO: Phosphoenol pyruvate carboxylase activity
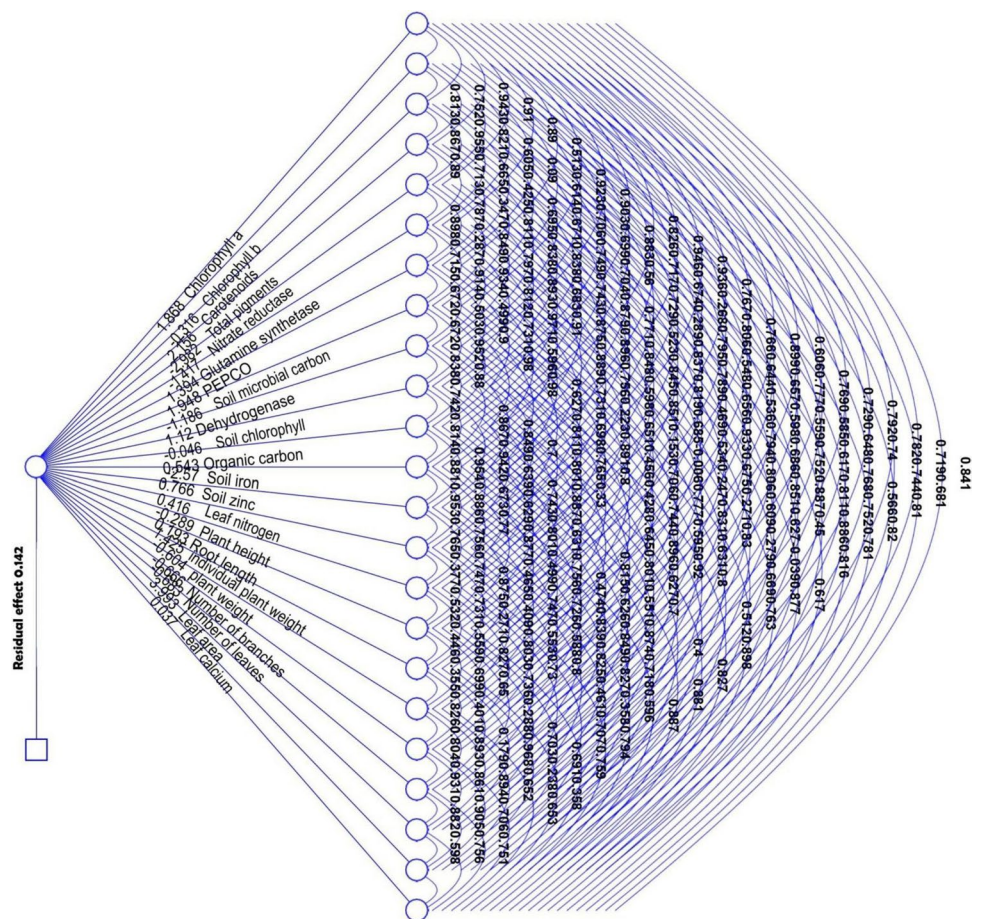
2021; Kokila et al. 2023). Photosynthetic prokaryotes, such as cyanobacteria have the capability to capture and fix atmospheric CO_2 leading to an anticipated increase in soil carbon with cyanobacterial treatment (Renuka et al. 2017), This may also lead to proliferation of soil microbial populations (Mager 2010), as recorded in cyanobacterial soil crusts of Kalahari sand, which contained large fractions of soil organic pools with carbohydrates as their major constituent. In addition, the proliferation of microalgae and cyanobacteria is known to be a source of organic matter for the growth of higher plants (Renuka et al. 2018). As an indirect impact, the decaying biomass of microalgae and cyanobacteria in agricultural fields can be decomposed or utilized by various other organisms and grazers, thereby augment the organic carbon levels in the soil. The significant role of cyanobacteria in paddy fields, besides nitrogen fixation was emphasized by Mandal et al. (1999) as they can trigger enzymatic processes in soil and plants, and promote fertility and plant health, as also observed in this study. The overall changes in all the microbiological and plant parameters recorded in the present investigation depict a beneficial relationship between cyanobacterial treatment and the tested spinach genotype, duly supported by previous studies in which soil microbiological activity, such as that of

dehydrogenase or those related to nutrient mobilization were greatly enhanced upon the addition of cyanobacterial biomass and their exopolysaccharides (De Caire et al. 2000; Rana et al. 2012; Prasanna et al. 2018). This has often been attributed to the beneficial impact of cyanobacterial inoculation on plant growth and development in terms of increased availability of nitrogen in the soil, microbial biomass carbon accretion, particularly photosynthetic biomass, measured as soil chlorophyll. Bergi and Trivedi (2020) discussed the role of cyanobacteria as biofertilizers in increasing phosphorus content in saline soils, while in our study, significant improvement in nitrogen content in spinach leaves was recorded. This aligns with prior research on the use of cyanobacterial biofertilizers, which as diazotrophs increase nitrogen availability, followed by its mobilization, enhancing plant nitrogen concentration, and promoting plant growth during the growth stage, as reported by Rodgers et al. (1979).

Similarly, IAA-producing cyanobacterial strains caused significant enhancements in both soil and plant chlorophyll content in wheat seedlings (Sood et al. 2011); earlier also reported by PGPR by Cakmakci et al. (2009). Bharti et al. (2021), amended the potting medium with a cyanobacterial strain and found significant increase in nitrogen availability

in the soil, resulting in improved flower traits and productivity in Chrysanthemum. Therefore, coating seeds with cyanobacterial formulations or its use as a drench can be instrumental in enhancing nutrient availability in the soil surrounding spinach plants, leading to improved growth and overall productivity of the crop. Chlorophyll molecules play a pivotal role in the light-dependent photosynthetic reactions by capturing light energy and transferring this absorbed energy to the photo system reaction centers in plants and algae (Ghotbi et al. 2019). Spinach, being valued for its culinary and nutritional attributes of its leaves, our effort was to enrich this trait and we observed a positive response to cyanobacterial inoculation. A significant increase in The levels of chlorophyll *a, b*, carotenoids, and total pigments was recorded in the treated plants as compared to the control, as earlier recorded by Grzesik and Romanovska (2015), in willows treated with *Microcystis aeruginosa* and *Anabaena* sp. An increased chlorophyll content can be attributed due to the presence of microelements (iron zinc and magnesium) being more available, as earlier also shown in rice and wheat plants receiving cyanobacterial inoculation (Rana et al. 2012; Shivay et al. 2022).

Cyanobacteria have garnered attention for their potential to enhance soil macro- and micronutrient availability and aid in the efficient mobilization of minerals by plants (Singh et al. 2016; Renuka et al. 2018). Furthermore, the cyanobacterial strains utilized in our study enhanced soil micronutrient parameters, including available iron and available zinc in soil, as also recorded earlier (Rana et al. 2012; Shivay et al. 2022). Manjunath et al. (2016) observed that soil available iron levels at the mid-crop stage recorded a threefold increase compared to the control when An–Az biofilm formulation was used in okra crop. Renuka et al. (2017) evaluated the promise of unicellular and filamentous micro algal-cyanobacterial consortial inoculation on soil composition and grain quality in a wheat crop. A significant increase in micronutrient availability in the soil, particularly zinc and iron, soil organic carbon content, led to improved crop yield, and enhanced grain quality.

Earlier economic analyses of spinach production undertaken illustrated that cultivating spinach under green shade net conditions showed promise, resulting in a substantial increase in yields and productivity (Meena et al. 2014). The cyanobacterial treatments resulted in significant changes in plant height, root length, increase in yield, individual leaf weight, and expansion in leaf area compared to the control (T1) plants. These findings align with previous observations (Salamah et al. 2019), in which the use of *Nostoc* sp. SO-A31 as a nitrogen source resulted in robust vegetative growth and increased biomass production in spinach. Similarly, the use of *Chlorella vulgaris* and *Spirulina platensis* in a pot experiment with maize, led to increase in the number of leaves from 6 in the control plants to 8 leaves in the treated plants

(Dineshkumar et al. 2019). In a study conducted by Kumar et al. (2013), the application of cyanobacterial strains, *A. laxa* and *Calothrix elenkinii* as seed treatment of spice crops led to significant improvement in the plant biometrical parameters and dry weight. Kholsi et al. (2021) examined the combined use of cyanobacteria, especially *Calothrix* sp. and *Anabaena cylindrica*, and plant-growth-promoting rhizobacteria (PGPR) and recorded more than 36% increase in plant height in wheat. Similar improvement in plant biometric parameters, including leaf area, and yield were recorded in the present investigation.

Significant enrichment to the quality attributes of the *Pusa All green* spinach cultivar using three different cyanobacterial treatments was observed. Specifically, an increase in phenol content, leaf proteins, along with boost in beta carotene content, and enhancement in leaf firmness. The most promising treatment RPAN8 was also found to elevate antioxidant activity significantly. Previous studies (Mala et al. 2017; Ertani et al. 2019) have demonstrated the effectiveness of cyanobacterial-based biostimulants, as soil inoculation or foliar application against oxidative stress. This is known to help in minimizing the damage caused by stress originated free radicals, by acting as scavengers, preventing the production of reactive oxygen species. Singh et al. (2011) documented that introducing *Oscillatoria acuta* and *Plectonema boryanum* through soil inoculation resulted in a notable enhancement of total phenolic content in rice. Mahmoud et al. (2019) demonstrated that the spinach leaves, when applied with an aqueous extract from *Anabaena sphaerica* increased the plant protein content.

Cyanobacterial inoculation can improve the micronutrient density of the produce, as shown in in rice, wheat and maize grains/seeds (Rana et al. 2012; Nishanth et al. 2021; Shivay et al. 2022). Kokila et al. (2023) recorded enhanced fruit quality in tomato, with higher titrable acidity, ascorbic acid, carotenoids and lycopene content in the tomato fruits. This is known to be mediated through siderophores or other chelating molecules and their respective transporters. The accumulation of phenolic acids, including caffeic, chlorogenic, ferulic, gallic, and gentisic acids, as well as increased levels of proteins and chlorophylls was shown in rice plants inoculated with various cyanobacterial strains (Singh et al. 2011). These findings support the beneficial effects of cyanobacteria as biofertilizers and effective biostimulatory agents for promoting plant growth and quality of produce, as also shown in spinach, in this investigation.

Phenotypic correlation analyses aligned with the biochemical and microbiological data recorded in spinach crop and brought to light the significant role played by cyanobacteria-based seed coating and drenching in fostering positive connections among a range of plant, soil, yield and quality factors. These results underscore the valuable contributions of diazotrophy, IAA and macro- and micronutrient mobilizing potential of cyanobacteria in stimulating the robust growth of

spinach crop. The correlation analyses spotlighted the strong associations existing between soil available nitrogen, yield and quality traits with cyanobacterial inoculants at various stages of growth. Utilization of these cyanobacterial formulations, specifically *A. laxa*, led to a substantial impact on a multitude of traits linked to plant, soil, yield and quality parameters.

Path coefficient analysis has conventionally been applied in subjects such as plant breeding, varietal selection and yield analyses. However, it has been relatively overlooked in the realm of microbiology, particularly, plant-microbe interactions. By using this analysis, the findings of this investigation were analysed through an innovative perspective, which provided insights regarding the substantial impact of cyanobacterial treatments in enhancing the plant growth, soil characteristics, yield as well as quality attributes in spinach crop. Path coefficient analyses was instrumental in elucidating the vital role of examined parameters, particularly in the context of improving plant health, as evidenced by the important role of soil available nitrogen with yield and quality traits at 2, 3, 5 WAS and harvest stage.

This study is noteworthy in that it resulted in a nutrimental intervention, through seed coating + drench using cyanobacterial formulations to reduce chemical inputs for a crop, particularly urea (N fertilizer; only one basal dose of urea, as against two in control, given in cyanobacterial treatments). This is often a costly input, which is environment-polluting and in excess, often a wasteful expenditure, due to its volatilization. Such a research mediation in spinach cultivation with better quality of produce, which is mostly consumed fresh, showcases microbial inputs as a more efficient and cost-effective approach. Further research is focused on evaluation across diverse agro-climatic conditions and varieties, to assess the viability and efficacy, thereby environmental proofing the technology and its widespread use in agriculture.

Declarations

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