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Article

Changes in Nitrate Concentration, Nitrate Reductase Activity, and Accumulation of Nutrients in Spinach (*Spinacia Oleracea* L.) in Response to *Streptomyces* Sp. Inoculation

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Abstract: A replicated outdoor pot experiment was conducted in order to investigate the effect of different phytohormone and siderophore producing, and P-solubilizing bacterial species on spinach nutrient uptake, nitrate concentration and nitrate reductase activity. The mentioned parameters were determined in spinach leaves (*Spinacia oleracea* L.), non-inoculated and inoculated with four plant growth–promoting actinobacteria (*Streptomyces griseus* (S1), *Streptomyces albogriseolus* (S2), *Streptomyces aurantiacus* (S3) and *Streptomyces kanamyceticus* (S4) under the influence of two sources of nitrogen fertilizers including potassium nitrate and urea (100 and 200 ppm). Inoculation with the strains increased spinach shoot fresh weight by 16%–43% over the control. Bacterial inoculation gave leaf chlorophyll increases of 15%–40%. Inoculation increased plant height by 2.2%–24.6% in spinach. A close reverse relationship between nitrate concentration and enzyme activity (r^2= 0.87) was demonstrated. The measured parameter responses were variable and dependent on the inoculant strain, with highest enzyme activity and lowest nitrate concentration exhibited in S2 (*S. albogriseolus*) inoculation. The source and application dose of nitrogen fertilizer had varied impact on measured parameter. The maximum phosphorous and iron concentration were measured by soil inoculation with S4 and applying 200 ppm nitrate potassium nitrogen fertilizer. Application of 200 ppm KNO_3 nitrogen fertilizer with different *Streptomyces* strains showed the capability of S2 in decreasing nitrate content while protein content increased. In particular, the strains S2 and S4 have great potential in being formulated and used as biofertilizers.

Keywords: *Streptomyces*; vegetable production; biofertilizer

1. Introduction

Nitrate is a common nutrient for all plants, and plays an important role in nutrition, growth and development and is particularly in vegetable production such as arugula and spinach [1,2,3]. In our bodies, nitrate can be converted into toxic substances which reduce the transport of oxygen, especially in infants. Considerable amounts of nitrate from nitrogen fertilizers accumulate in vegetables, especially leafy vegetables such as lettuce or spinach which contain the highest concentration of nitrate [4,5]. Vegetables, fruit, and processed meats are the source of nitrate that humans are exposed to; nitrate is ingested typically with vegetable consumption [5,6]. The toxic effects of nitrate accumulation are associated with the occurrence of methemoglobinemia, and some types of cancer which have been possibly attributed to creating metabolites such as nitrites, N-nitrosamines [7] and various other nitrogen compounds. However, some research suggest nitrate intake from vegetables could be associated with cardiovascular disease through the effects of nitric oxide [7; 8].

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The Scientific Committee on Food determined in 2002 that the acceptable nitrate daily intake ranges from 0 to 3.7 mg/kg body weight/da, which is equivalent to the intake of 222 mg nitrate/day for an adult weighing 60 kg [9]. Therefore, determination of nitrate content of vegetables is important. The accumulation of nitrate in plants happens due to the imbalance between ion absorption and assimilation, therefore the excess amounts are stored in vacuoles [10].

It has been revealed that a close relationship exists between the amounts of nitrate in plants and applied nitrogen fertilizer [9; 11; 12; 10). Spinach (Spinacia oleracea L.) is a nutritious vegetable as a good source of macro and micro nutrients and vitamins. However, spinach is a nitrate accumulator vegetable. Spinach has an inefficient regeneration system for nitrate-N and is one of the highest nitrate-accumulating vegetables [13]. Out of the two forms of available nitrogen (nitrate and ammonium), nitrate is of greater importance and is the main form of nitrogen taken up by plants. There is a reverse relationship between nitrate content and NRA (Nitrate reductase activity) [11]. Thus, accumulation or assimilation of nitrate in cells depends upon activity of Nitrate Reductase (NR).

The negative impacts of chemical fertilizers on the environment have resulted in a search for new ways to increase productivity in agriculture while mitigating the eradication of natural resources. Actinomycetes comprise 10 to 50% of soil microflora [14] and produce a wide range of effective compounds, including Nitrate reductase [15].

Streptomyces have been shown to effectively colonize the rhizoplane and rhizosphere. Due to their endophytic ability, they are capable of controlling gene expression and impacting the production of siderophores, phytohormones and some other traits [16; 17; 18; 19).

There are different solutions to reduce the amount of nitrate in plants. Perhaps one of these strategies can be the use of bacteria that assimilate nitrate in the soil or induce the activity of nitrate reductase to reduce nitrate in plants. Some studies have revealed nitrate reduction by *Streptomyces* genus [15; 20]. Fischer et al revealed the presence of three operons encoding respiratory nitrate reductases (NARs) in *Streptomyces griseorubens* and Feng et al provided the draft genome of the species[21, 37].

The absence of information on nitrogen fertilizer dose to improve yield and quality of spinach and the role of PGPB on increasing quality of plants caused to design the present study. Thus, the first aim of this study was to isolate *Streptomyces* from the rhizosphere of some different plants and trees at different locations of San Fernando Valley, Los Angeles, and to evaluate some PGP traits of the isolates. The second goal was to investigate the isolates' abilities to colonize spinach roots, as spinach is a highly consumed vegetable with high nutritional values. The nitrate content of spinach as a response to the applied dose of nitrogen fertilizer was evaluated and the impact of the best PGPS strains were considered as potential strains on nitrate accumulation reduction, nitrate reductase activity and some properties of plant and nutrients uptake in spinach leaves.**2. Materials and Methods**

Streptomyces strains

Four *Streptomyces* strains have been used in this study. *Streptomyces griseus*, *Streptomyces albogriseolus*, *Streptomyces aurantiacus* and *Streptomyces kanamyceticus* which were isolated from rhizosphere soil of tomatoes, tomatoes, lettuce, sunflowers collected from San Fernando Valley, Los Angeles, California. The root colonization ability, putative molecular identification, and functional traits of these strains was described in our previous work (Saraylou et al 2021).

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Pot experiment

The impacts of Streptomyces griseus (S1), Streptomyces albogriseolus (S2), Streptomyces aurantiacus (S3) and Streptomyces kanamyceticus (S4) on nitrate concentration, nitrate reductase activity, protein, chlorophyll and some nutrients uptake in spinach leaves were evaluated in pot experiment. The experiment was carried out in completely randomized design including two factors: bacterial inoculation (Streptomyces griseus (S1), Streptomyces albogriseolus (S2), Streptomyces aurantiacus (S3) and Streptomyces kanamyceticus (S4)) and nitrogen fertilizer from potassium nitrate and urea (100 and 200 ppm) with three replicates. Each pot was filled with 3 kg of soil and peat (1:1) and the soil was taken from the surface layer of the farms of Pierce College, Los Angeles. Ca. (Table 1). Six inoculated seeds were sown with the suspension of each strain (1*10 6 CFU ml-1) in each pot, at the depth of 1 cm from the soil surface, which was thinned to 3 plants after 2 weeks. The moisture of soil was maintained at 75 percent of field capacity with water. The grown plants were harvested 40 days after planting.

Elemental analysis of plant tissue

The concentration of iron (Fe) in dried and milled leaves was determined after wet digestion by nitric acid [23] via atomic absorption. The digested leave samples were analyzed using a spectrophotometer at 470 nm for the quantification of phosphorus [24]. For potassium determination, a flame photometer was used. For total nitrogen analysis, one mg of the dried and ground plants leaves was used to measure by a Kjeldahl apparatus (Labconco).

Nitrate content and Nitrate reductase activity measurement

Nitrate content in spinach leaves was determined based on the salicylic acid method [25]; 100 mg of fresh leaves was moisture with 1 mL deionized water and kept at 45°C for 60 minutes. The specimen was centrifuged at 600 rpm for 15 minutes, thereafter 200 µl of supernatant was mixed with 800 µl salicylic acid (%5) and kept at 24 °C for 20 minutes. The procedure was followed by addition of 19 ml 2N NaOH to the solution and adjustment of the pH to 12. The absorbance of the mixture was measured at 410 nm via spectrophotometer (Laxco, Alpha1502. USA). To measure nitrate reductase activity, fresh leaves were suspended in phosphate buffer (100 ml, pH 7.5) containing propranolol (4%) and potassium nitrate, which was kept in the dark at 30°C for 30 minutes. Then, 1 ml sulphanilic acid in chloric acid 2N and 1 ml N-(1-Naphthyl) ethylene diamine was added. Nitrate reductase activity was measured at 540 nm using spectrophotometry (Laxco, Alpha1502. USA). The amount of nitrite produced in nmol per FW (g) tissue per hour, was calculated by the following formula: NR activity (µg/g FW·h) = Nitrite content (µg) × sample dilution multiple/sample FW (g) × time (h) (Marek and Dominika, 2012).

Chlorophyll Measurement

Chlorophyll was extracted from 100 mg fresh leaves, cut into small pieces and ground for 5 min in 10 mL of 85% acetone with a mortar and pestle. The homogenized filtrate was transferred into a 15 mL Falcon tube and adjusted to a set volume with 85% acetone. The absorbance of the extract was measured at both 663 and 644 nm by spectrophotometry (Laxco, Alpha1502. USA). The concentrations of chlorophyll a and b, in mg per gram of FW sample, were calculated using the following formulae:

Milligrams chlorophyll a/g FW = 1.07 (OD663)−0.094(OD644)

Milligrams chlorophyll b/g FW = 1.77 (OD644)−0.280(OD663)

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Total chlorophyll = chlorophyll a + chlorophyll b

Statistical Analysis

Analyses were done using SAS software, version 9.4. The analysis of the pot experiment was carried out on factorial based on a completely randomized design. The Tukey grouping of means was performed at a significance level of α= 0.05. Data for root colonization were log-transformed.

3. Results

Plant analysis

The interaction between fertilizers and Streptomyces strains was a significant factor ($p<0.01$) in determining nitrate content of spinach leaves. Results showed that by increasing the rate of applied nitrogen fertilizer, the nitrate content in the leaves increased. However, this increase in control plants was far greater than the bacteria-treated plants. Furthermore, the bacteria-treated plants showed an increase in NRA. The plants treated with potassium nitrate (200 ppm), tended to have the maximum nitrate content in general (Figure 1).

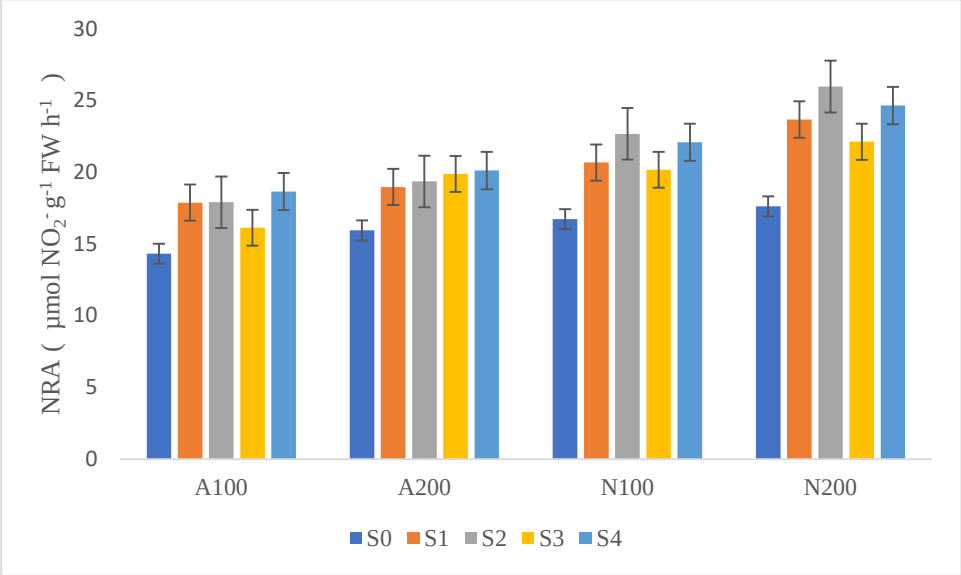

Figure 1. The results for protein levels in spinach leaves are shown for each potassium nitrate and Streptomyces strain combination. SO was the negative control.

Among the bacterial treatments, plants treated with S2 showed the lowest nitrate content, among the 200 mg kg-1 potassium nitrate-treated plants, with a mean of 4257.7 mg kg-1, as shown in Table 1. Meanwhile, the minimum nitrate content was exhibited by plants treated with S2 (3667 mg kg-1) and S4 (3683.3 mg kg-1), among those treated with 100 mg kg-1 Urea. The maximum amount of nitrate (8772 mg kg-1) was observed in treatment S0N200 using 200 mg kg-1 potassium nitrate and without bacterial inoculation, and was followed by S3N200 (7123.3 mg kg-1). The results of Tukey's test are summarized in Table 1.

Table 1. The results of Tukey's test for Chlorophyll content, nitrate content, nitrate reductase activity, iron, phosphorus, potassium, % protein, nitrogen, potassium, fresh weight, and dry weight are given.

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Treatment	S0	S1	S2	S3	S4	Mean
Chlorophyll mg/100g						
A100	93.30l	111.47h	113.43g	104.47j	126.67e	69.46D
A200	104.33j	129.73d	135.60b	120.50f	146.40a	127.31A
N100	90.80k	107.83i	114.07g	107.23i	114.43g	106.99C
N200	101.00m	125.07bc	131.90c	121.10f	137.03b	123.22B
Mean	97.35E	118.5C	123.75B	113.32D	131.1A	
Nitrate (mg/kg)						
A100	4524.7j	3667.0m	3734.0m	3975.7l	3683.3m	3916.9D
A200	5999.7f	4999.3h	4257.7k	5670.3g	4810.3i	5126.6C
N100	6871.0c	5563.3g	5001.3h	6493.7d	4836.3i	5753B
N200	8772.3a	6966.7c	5927.3f	7123.3b	6137.3e	6985.38A
Mean	6541.9A	5299C	4730E	5815.6B	4866.8D	
Nitrate reductase (µmol NO_2^- g^{-1} FW h^{-1})						
A100	14.30m	17.853j	17.907j	16.123l	18.657i	16.95D
A200	15.940l	18.99i	19.87h	19.87g	20.07g	18.94C
N100	16.73k	20.623f	22.657d	20.15g	22.60d	20.55B
N200	17.647j	23.67c	25.97a	22.60e	24.647b	23.50A
Mean	16.14E	20.52C	21.85A	19.68D	21.74B	
Fe (mg/kg)						
A100	93.67k	107.33gh	109.33fg	105.33h	110.67ef	105.26C
A200	98.00j	114.00cd	118.33ab	108.67fg	117.33b	111.26B
N100	94.67k	105.33h	107.33gh	103.00i	109.33fg	103.93D
N200	99.33j	114.67c	117.00b	112.33de	120.33a	112.73A
Mean	96.41E	110.33C	112.99B	107.33D	114.41A	
P (g/kg)						

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A100	4.90k	5.66hi	6.03fg	5.63i	5.80hi	5.6C
A200	5.36j	6.23ef	6.53bc	6.30de	6.73ab	6.23B
N100	4.83k	5.80hi	5.86gh	5.70hi	6.13ef	5.66C
N200	5.33j	6.63bc	6.66abc	6.46cd	6.86a	7.98A
Mean	5.09D	6.08C	6.27B	6.06C	53.14A	

Protein (%)

A100	1.32q	2.11o	2.87k	2.61m	2.22n	2.22D
A200	2.05p	2.95j	3.78c	3.58e	3.45f	3.16B
N100	2.20n	3.17i	3.33g	3.20i	3.28h	3.03C
N200	2.81l	3.88b	4.43a	3.72d	4.40a	3.84A
Mean	2.09E	3.02D	3.6B	3.27C	3.33A	

N (g kg^{-1} DW)

A100	18.2i	19.200hi	20.16fghi	19.7ghi	22.13efg	19.87D
A200	21.27efgh	23.76cde	25.20bcd	25.23abcd	25.80abc	24.24B
N100	17.9i	21.2efgh	21.73efgh	20.23fghi	22.76def	20.76C
N200	21.9efgh	25.36abcd	28.100a	23.23cde	27.833ab	25.28A
Mean	19.81E	22.38C	23.79B	22.09D	24.63A	

K (g kg^{-1} DW)

A100	5.76l	6.53j	5.93l	5.83l	6.86i	6.18D
A200	6.23k	7.20h	7.43g	6.86i	8.133f	7.71C
N100	7.13h	8.7e	8.90e	8.167f	9.93c	8.5B
N200	8.33f	9.23d	10.60b	8.73e	11.133a	9.6A
Mean	6.86E	7.9C	8.21B	7.39D	9.01A	

FW (g)

A100	6.157m	7.20i	8.72de	6.74k	8.59ef	7.48D
A200	6.907jk	8.50f	9.50c	8.057g	9.70c	8.53B
N100	6.437l	7.43h	8.463f	7.10ij	8.82d	7.64C

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N200	7.15i	8.943d	9.93b	8.76de	10.38a	9.03A
Mean	6.65E	8.01C	9.15A	7.64D	9.37B	
			DW (g)			
A100	0.73n	0.91ij	0.91ij	0.77m	0.92ij	0.84D
A200	0.89jk	1.18e	1.26c	0.97gh	1.340b	1.12B
N100	0.77m	0.89jk	0.94hi	0.82l	0.98g	0.88C
N200	0.87k	1.22d	1.19de	1.03f	1.420a	1.146A
Mean	0.81E	1.05C	1.07B	0.89D	1.16A	

Leaf nitrate reductase activity increased with the rate of applied fertilizer and with soil bacterial inoculation (Table 1). The S2 inoculated soil which contained 200 mg kg-1 potassium nitrate was shown to have the greatest leaf nitrate reductase activity (25.97 µmol NO2- g-1 FW h-1) and showed a 47.3% increase over the control. Next, treatment S4 plus 200 mg kg-1 potassium nitrate (24.64 µmol NO2- g-1 FW h-1) had a 39.6% increase in NRA compared to the control. In general, the rate and type of nitrogen fertilizer had a significant impact on this enzyme activity.

Among the bacterial treatments, S3A100 with 16.12 µmol NO2- g-1 FW h-1 had the least NRA after the S0A100 treatment. As shown in Table 2, there was a correlation between NRA and applied nitrogen fertilizers in the leaves (r^2=0.67). This demonstrated that, with increasing nitrate application, NRA increased. In treatments with high NRA, the amount of nitrate was lower. For instance, in S1N200 treatment nitrate decreased by 20.7%, followed by a 34.6% increase in NRA in the leaves.

Table 2. The results of the correlation analysis between factors in shown in the table.

	Chlorophyll	Protein	N	P	K	Fe	DW	FW	Nitrate	Nitrate reductase
Chlorophyll	1	0.70**	0.83**	0.91**	0.45**	0.94**	0.69**	0.85**	0.18-	0.61**
Protein		1	0.82**	0.87**	0.78**	0.79**	0.52**	0.83**	0.28*	0.88**
N			1	0.85**	0.65**	0.81**	0.51**	0.82**	0.17	0.88**
P				1	0.59**	0.95**	0.64**	0.9**	-0.04	0.77**
K					1	0.10	0.41**	0.65**	0.4**	0.91**
Fe						1	0.67**	0.88**	-0.19	0.71**
DW							1	0.65**	0.04	0.46**
FW								1	-0.01	0.76**
Nitrate									1	0.75**
Nitrate reductase										1

There is evidence that co-applying *Streptomyces* with fertilizers has increased chlorophyll content in the leaves. This enhancement was greater in the urea treatments. The

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200ppm urea-treated (A200) inoculated soils with S4 led to the greatest chlorophyll content in the leaves, followed by the 200ppm-urea plus S2 treatment, being 40% and 30% greater than the S0A200 control, and 62.2% and 49.3% higher than S0A100, respectively. Results also indicated that chlorophyll content in 200ppm urea treatment samples were greater than in the plants grown in potassium nitrate treated soil (Figure 2). Overall, chlorophyll pigments increased with increases in both sources of fertilizers.

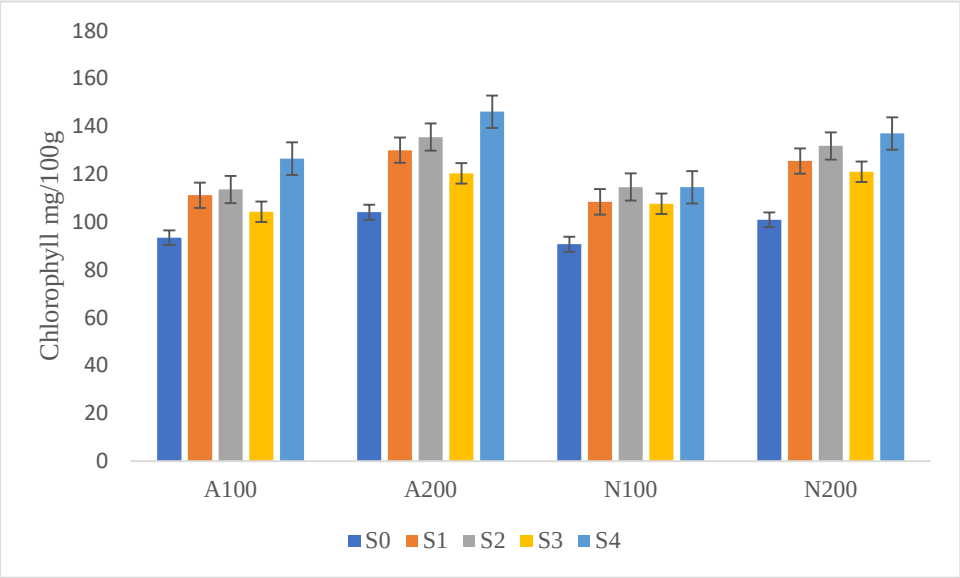

Figure 2. The results for chlorophyll levels in spinach leaves are given for each potassium nitrate and *Streptomyces* strain combination. SO was the negative control.

Protein content in spinach leaves varied among the different treatments, as shown in Figure 3. The impact of soil bacterial inoculation on leaf protein content was significant ($p<0.01$). The highest protein content was observed in the 200mg kg-1 nitrate potassium (N200) treatments with S2 and S4; these had 4.43% and 4.4% protein in the leaves which increased the protein content by 57% over the N200-control.

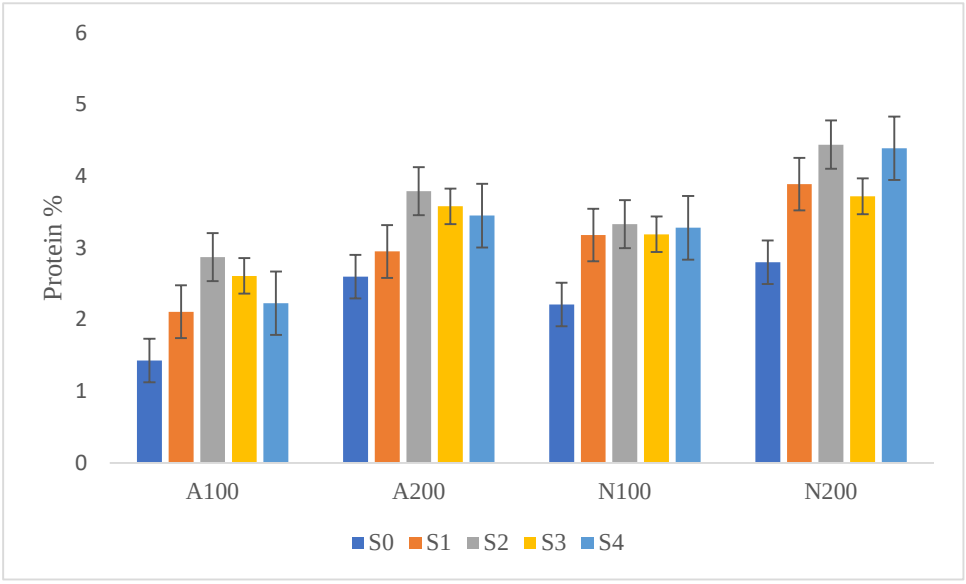

Figure 3. The results for protein levels in spinach leaves are shown for each potassium nitrate and *Streptomyces* strain combination. SO was the negative control.

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By increasing the rate of applied nitrogen fertilizers from both sources, the protein content of the leaves increased. However, the least protein content was observed in 100ppm urea-control. The highest increase of protein over the control was associated with the urea fertilizers, and S2 was the strain that had increased protein content by 84.2% over the control in 200 ppm urea treatment (Table 1).

Nutrient analysis showed bacterial treatments significantly increased nutrients compared to the non-inoculated treatments. S4 strain treatments significantly increased nitrogen, potassium and iron. 200ppm potassium nitrate treatments plus S2 and S4 were significantly high in phosphorous concentration in the leaves. N and K contents were significantly different in potassium nitrate fertilizer treatments. The most effective strains in N and K improvement were S1 with 29.87 and 7.1 (g kg-1), as well as S4 with 22.23 and 7 (g kg-1), respectively.

S4 was the best strain in increasing shoots' wet and dry weight among the strains. Also, the best fertilizer for increasing the yield parameters was 200 ppm potassium nitrate. Furthermore, similar positive trends with nitrate and chlorophyll in response to increasing nitrogen concentrations, were observed (Figure 4).

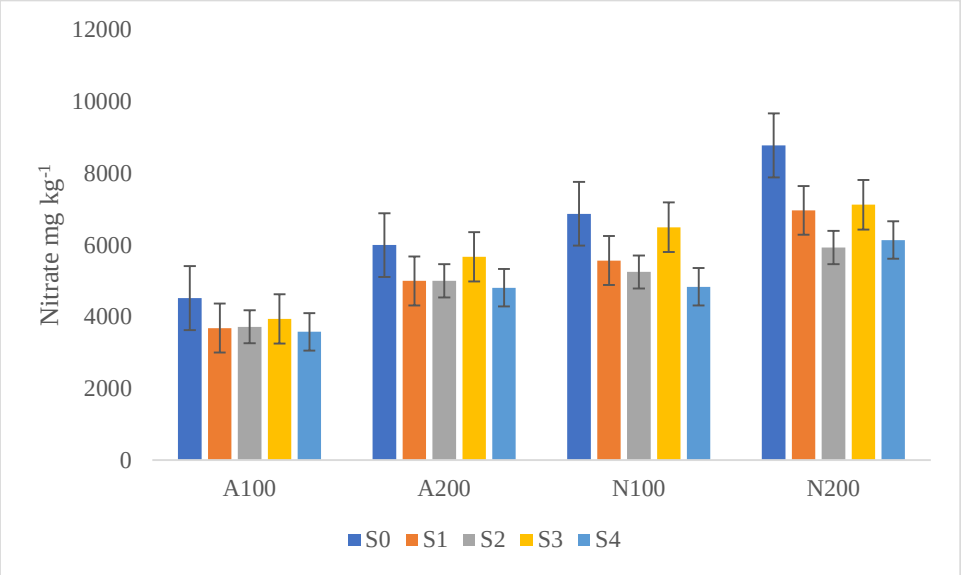

Figure 4. The results for nitrate levels in spinach leaves are shown for each potassium nitrate and *Streptomyces* strain combination. SO was the negative control.

The concentration of the measured minerals (N, P, K and Fe) in leaves of spinach are presented in Table 1. A summary of the error is presented in Table 3 and Table 4. The nitrogen concentration in the leaves enhanced with increasing nitrogen fertilizer dose. There was a significant difference between the sources of applied N- fertilizer. The P, K and Fe contents showed the same trends when plants were grown using different sources and doses of nitrogen fertilizer. It is worth noting that the macro and micronutrient content was increased when plants were grown in *Streptomyces* inoculated soils.

Table 3.

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Source of Variation	df	Nitrate	Protein	Chlorophyll	NR	N
Bacteria (B)	4	6680443	4.03819	1939.35	63.858	40.785
Fertilizers (F)	3	2.446E+07	6.61704	1491.11	112.289	103.522
B×F	12	458502	0.13556	40.92	4.051	2.795
Error		1708.63	0.00013	0.36	0.012	0.864

Table 4.

Source of Variation	df	P	K	Fe	FW	DW
Bacteria (B)	4	3.06625	7.9917	617.358	14.92	0.23
Fertilizers (F)	3	2.36239	34.0980	283.622	8.09	0.37
B×F	12	0.04669	0.5780	6.803	0.1497	0.01
Error		0.00500	0.0047	0.467	0.04	0.00012

4. Discussion

The results of this study revealed a significant difference among the four N-fertilizer treatments in various measured growth properties of spinach. We illustrated that 4 different species of *Streptomyces* were able to increase spinach yield compared to the control. A positive correlation between nitrate-N supply in the soil and NRA in the leaves was observed; by increasing nitrate fertilizer rate, Nitrate reductase activity increased and, as a result, nitrate content in the leaves decreased significantly over the control. Our results showed more growth and yield in spinach treated with nitrate-N in comparison to ammonium-N. Studies have shown that N in the form of nitrate is preferred to N- ammonium in most leafy vegetables, so applying nitrate fertilizers tends to increase their growth [26, 38]. Some experiments have illustrated that when growing plants with N-ammonium fertilizers as the only source of nitrogen, plants were smaller in comparison to the same plants fertilized by N-nitrate [9; 27].

Conversely, improvements in yield and growth in plants have been reported when plants were fertilized mainly by N- nitrate fertilizers [28; 29]. Some mechanisms have been suggested to explain ammonium's toxicity to plants. One of them might be the effect of ammonium in reducing cell elongation and division which lead to smaller plants [30]. Our results indicate that plants grown in presence of nitrate had greater wet and dry weight along with better biochemical properties.

As mentioned above, nitrate is important in plant growth. However, nitrite in some vegetables such as spinach could lead to nitrate accumulation. This accumulation could decrease the beneficial value of spinach. There are several studies which illustrated PGPR can alter and regulate enzyme activity in plants in response to biotic and abiotic stress [31]. The results showed that there were significant differences in nitrate reductase activity and nitrate content in the leaves of spinach among the four nitrogen supplies and rates. The observed changes in NRA appeared to be triggered by *Streptomyces* strains, when compared to the control. While nitrate content in the spinach leaves decreased, protein content as well as nutrients increased significantly in the plants. Studies have shown NR is a substrate-induced enzyme, so by increasing nitrate concentration in

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plants, NR activity might increase as well; there was positive correlation between them [9]. These changes indicated the plant growth promoting effects of these strains increased quality as well as yield in spinach.

Application of potassium nitrate fertilizers enhanced nitrate uptake and stimulated the activity of the nitrate reductase enzyme, thus improving the yield and quality of the plants which studies of Anjana et al revealed application of potassium led to increase in nitrogen assimilation in spinach [32]. This may be due to increase in the activity of enzymes such as NR, which is responsible for nitrate assimilation and facilitating water and nutrient uptake [33]. Additional studies are required to confirm the effects of *Streptomyces* on the nitrate reductase enzyme activity in various plants and under different nitrogen fertilizers. Our study demonstrated that increased nitrate in the soil and plants led to nitrate accumulation which could enhance NRA, similar to the study by Liu et al [9] and Koyama et al [34]. In our study we that illustrated four different *Streptomyces* species were able to increase NRA in comparison to the control, which is aligned with other studies [35; 31; 36] which illustrated alteration of enzyme activity by PGPR.

Author Contributions: Conceptualization, M.S., H.A.N., and N.E.; formal analysis, M.S.; investigation, M.S., S.S.; resources, S.S., N.R.; writing—original draft preparation, M.S.; writing—review and editing, S.S.; supervision, H.A.N., N.E., N.R., and S.S. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement: Raw data is available from the authors by request.

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Conflicts of Interest: The authors declare no conflict of interest.

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