

Effects of organic and inorganic fertilization with bio-inoculants on the sustainable management of plant-parasitic nematodes infesting okra (*Abelmoschus esculentus*)

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Abstract

Root-knot nematodes are believed to be amongst the biological constraints causing severe damage and a great reduction in the productivity of okra. The purpose of this study was to apply organic matter and non-symbiotic nitrogen-fixing bacteria to minimize the addition of chemical fertilizers that constantly pollute the environment. Experimental studies were conducted in the field for two summers to determine the effect of inoculations of non-symbiotic nitrogen-fixing bacteria, such as *Azotobacter chroococcum* and *Azospirillum brasilense* singly and in combinations, with different recommended doses of inorganic nitrogen as well as organic matter such as neem seed cake on the growth, yield, and organic parameters of the okra crop towards the management of plant-parasitic nematodes. The results show a significant reduction in nematode multiplication through soil application of nitrogen-fixing bacteria and neem seed cake along with different recommended doses of nitrogen inoculated plants. *Azotobacter* was found to be less effective than *Azospirillum* in agronomic parameters and nematode control. The most pronounced increases were observed in the yield and growth parameters such as plant height, fresh as well as dry weights, fruit weights/plant, number of total fruits/plant and primary branches, chlorophyll content, and ascorbic acid content when *A. chroococcum* and *A. brasilense* were added concomitantly in various combinations. Agronomic parameters such as NPK content in the plant as well as in residual soil increased considerably in almost all the combinations irrespective of these biofertilizers and neem seed cake. In conclusion, the combined application of a 100% recommended dose of nitrogen fertilizer along with *Azospirillum* and neem seed cake is recommended for better growth and yield of okra with better control of nematodal population.

Keywords: *Azospirillum*; *Azotobacter*; management; neem-seed cake; plant-parasitic nematodes

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Introduction

A common vegetable plant known locally as Bhindi is Okra (*Abelmoschus esculentus* L.), cultivated during the summer in both the northern and southern states of India. It is also used for cleaning sugarcane juice as a replacement for paper and a source of vitamins A and B. India is considered the first in the world for okra production by 6075.80 tons (NHB, 2018). In particular, India's productivity is much less than that of other countries, which could be primarily due to insufficient and inadequate fertilizer supplies, the occurrence of diseases, and the inability to implement modern improved development technologies. Nutrient management plays an important role in crop productivity by reacting well to higher levels of chemical fertilizers, as the crop needs a high level of nutrient requirements. On the other hand, pests can reduce the world's crop yields by an annual average of more than 20%, but individual fields can suffer losses from one or more pests by 50-100% (Khan *et al.*, 2012; Mohamed and Abd-El Hameed, 2014; Ashry *et al.*, 2018). Infestation of okra plants with the root-knot nematode, *Meloidogyne incognita* caused a reduction in yield losses of more than 10-29% (Manunatha *et al.*, 2017; Ahmad *et al.*, 2021 a, b). The infected plants were investigated and showed signs of a lack of nutrients and a significant swelling of both primary and secondary roots (Helmi and Mohamed, 2016; Abdel-Sattar *et al.*, 2020).

Root-knot nematodes are believed to be among the biological constraints causing severe damage and a great reduction in the productivity of okra (Safiuddin *et al.*, 2011, Mukhtar *et al.*, 2013; Hussain and Mukhtar, 2019). In order to overcome this menace, the use of agrochemical fertilizers, wide-ranging pesticides may help in the management of deleterious pathogens. Chemical fertilizers and pesticides have probably continued to be the most effective control strategies to date. However, their detrimental effects are a cause for public concern that calls for rationalized use of nematicides and reorientation of protection strategies towards ecologically safe and sound management of pathogens. The widespread and persistent use of agrochemicals has resulted in the creation of resistance to pesticides (Chattopadhyay *et al.*, 2017; Mohamed *et al.*, 2018a). This has resulted in the direct toxicity of predators, pollinators, the population of fish, man and animal products (Choudhary *et al.*, 2018), and has had harmful effects on environmental and soil health, and low soil condition, fertility, and productivity (Singh *et al.*, 2007; Meena *et al.*, 2020; El-Sheshtawy *et al.*, 2021). There has been immense pressure to control plant pathogens and the nutrient requirements of plants by farmers around the world, using modern environmentally sustainable techniques. Policymakers and scientists are currently assessing the use of biological materials as alternative sources of nutrients to enhance soil productivity and crop production instead of chemical fertilizers (Ullah *et al.*, 2018). It is because organic farming leads to good food without any damage to the soil and consumer health (Mamia *et al.*, 2018). Organic fertilizers are used for sustainable agriculture as an alternative nutrient source to conventional fertilizers (Zhu *et al.*, 2018).

Chemical pesticides have dominated the successful management of plant diseases, but they also pose a number of hazards to humans and the environment. In addition to the target pest, they also kill a lot of beneficial microorganisms in the rhizosphere, contaminate soil and water, and accumulate in plant parts. We must explore and exploit other methods of disease management (Eroa, 2015). The use of biofertilizers as an environmentally friendly alternative to chemical fertilizers, to improve soil fertility and to enhance crop production through their biological activity in the rhizosphere, has been widespread for more than a decade, and is expected to continue (Rizvi *et al.*, 2015). Biofertilizers such as farm manures, botanical compost like neem seed cake, manure and plant growth-promoting bacteria are good sources of N, P, and K (Timsina, 2018) which not only improved crop productivity (Kamran *et al.*, 2018) but also had a beneficial effect on soil properties (Tully and McAskill, 2020) besides cutting down on the expenditure on costly fertilizers (Sofy *et al.*, 2021a). In order to maintain the required crop productivity, an integrated application of the various nutrients including biofertilizers is needed nowadays. Biofertilizers are known to indirectly impact soil microbiological activities, helping the plant to achieve better results in improved productivity through nitrogen fixation, phosphorus mobilization, and potassium solubilization (Fasusi *et al.*, 2021). A stronger solution to the rapid action would be the foliar application of biofertilizer which contains hormones, vitamins, and micronutrients

that play a vital role in plant growth and plant-pathogen reduction (Verma *et al.*, 2000; Abd El- Rahman and Mohamed, 2014).

The best biocontrol measures have been the microorganisms that grow in the rhizosphere. Under natural conditions, these organisms provide the frontline defense against pathogens. Among the various microorganisms, nitrogen fixing bacteria like *Azotobacter* and *Azospirillum* live in close proximity to the root system of crop plants, and they harvest atmospheric nitrogen, which is then made available to plants or released into the soil over time. As an alternative to nitrogenous chemical fertilizers, these biofertilizers are not only environmentally friendly but also economically viable (Khan *et al.*, 2012). In addition to fixing nitrogen, biological nitrogen fixers produce hormones, vitamins, and other growth factors that are necessary for plant growth and development, solubilize phosphorus, and suppress plant pathogens (Khan *et al.*, 2012). Among a wide variety of organic matters that have been tested as organic amendments to soil for managing plant-parasitic nematodes are oil seed cakes. Oil seed cakes are a byproduct of plant seed oil-processing industries and may suppress plant-parasitic nematodes in economically important crops (Rizvi *et al.*, 2015). Neem cake is an organic byproduct obtained in the process of cold processing of neem tree fruits and kernels, and the solvent extraction process for neem oil cake. Neem cake has an adequate quantity of NPK in organic form for plant growth. Being a totally botanical product, it contains 100% natural NPK content and other essential micronutrients such as N (2-5%), P (0.5-1%), K (1-2%), Ca (0.5-3%), Mg (0.3-1%), S (0.2-3%), Zn (15-60 ppm), Cu (4-20 ppm), Fe (500-1,200 ppm), and Mn (20-60 ppm) (Rizvi *et al.*, 2015). It reduces alkalinity in the soil, improves the organic matter content of soil, helps improve the soil texture, water holding capacity, and the soil aeration for better root development. It contains salannin, nimbin, azadirachtin, and azadiradione as major components.

So, the use of biofertilizers with the organic matter has proved to be a viable option for sustaining crop production (Wahane *et al.*, 2020). With short supply and uncertainty in the price of inorganic fertilizers and increasing awareness in favour of adopting biofertilizers is lacking in many crops including okra. Thus, a comprehensive approach to the supply of nutrients through chemical fertilizers along with organic materials and bio-fertilizers has become more essential, reducing the use of inorganic fertilizers and providing environmental protection. *Azotobacter* and *Azospirillum*, for instance, are free-living nitrogen fixers that live in the root soil of cultivated plants and fix atmospheric nitrogen which is available to the plant or released into the soil in due course (Aasfa *et al.*, 2021).

Okra production in India seems to be lower due to the root-knot nematode infestations and the nutrient supply imbalance. Therefore, the present work aimed to study the influence of different recommended doses of nitrogen, neem cake as organic matter and biofertilizers such as *Azotobacter* and *Azospirillum* in various combinations on plant growth parameters such as plant height, fresh weight as well as dry weights, fruit yield plant⁻¹, chlorophyll content, ascorbic acid content, soil fertility in terms of NPK content and subsequently on the multiplication of root-knot nematodes and development of root-galls caused by *M. incognita*.

Materials and Methods

Preparation of experimental field

A field experiment has been carried out at the Aligarh Muslim University, Agricultural Research Farm to determine the effect of inoculation of *A. chroococcum* CBD15 and *A. brasilense* Sp245, individually or in combination with neem cake and different recommended doses of inorganic fertilizer on the growth, yield and agronomic parameters of okra (*Abelmoschus esculentus* L.) cv. 'Arka Anamika'. The field was thoroughly prepared with the help of agricultural devices and well protected with fencing wire. The field was infested with a population of plant-parasitic nematodes including root-knot nematode, *M. incognita* and some other microbial populations. The experimental beds of 6m² were prepared to leave a 0.5m buffer zone between them for irrigation purposes.

Composition of soil and treatments

The field soil was sandy loam in texture, having pH of 6.2 and 6.5, organic carbon of 1.03 and 1.10% and electrical conductivity (0.022ds/m) with an available water holding capacity of 151mm at one meter soil depth (Table 1). The field soil has available N of about 132.40 and 178.64, P of about 43.54 and 46.75 and K about 55.29 and 65.48 kg/ha respectively for the two respective years. The experiment was conducted with three replications in a randomized block design. The beds of 6m² and each treatment in beds with three replications. For analysis of data, we observed the five plants from each bed (treatments). Neem cake and biofertilizers such as *Azotobacter* and *Azospirillum* were the organic sources of nutrients and fertilizer urea for nitrogen was the inorganic source applied in different treatments. Each bed except those of 100% RDF of NPK at the rate of 100kg of nitrogen fertilizer hectare⁻¹ and untreated one received neem cake of 110 kg N/ha incorporated uniformly into the soil at least 15 days before sowing to ensure proper decomposition of this cake before the sowing of seeds of okra. The detail of the experimental treatments is given as under

100% Recommended dose of fertilizer (RDF) + *Azotobacter* + Neem cake (A)

75% RDF + *Azotobacter* + Neem cake (B)

50% RDF + *Azotobacter* + Neem cake (C)

100% RDF + *Azospirillum* + Neem cake (D)

75% RDF + *Azospirillum* + Neem cake (E)

50% RDF + *Azospirillum* + Neem cake (F)

100% RDF + *Azotobacter* + *Azospirillum* + Neem cake (G)

75% RDF + *Azotobacter* + *Azospirillum* + Neem cake (H)

50% RDF + *Azotobacter* + *Azospirillum* + Neem cake (I)

Azotobacter + Neem cake (J)

Azospirillum + Neem cake (K)

Azotobacter + *Azospirillum* + Neem cake (L)

Neem cake alone (M)

100% Recommended dose of NPK (N)

untreated control (O)

Preparation of inoculum of biofertilizers and treatments of seed

Pure cultures of biofertilizers such as *A. chroococcum* CBD15 and *A. brasilense* Sp245 were transmitted and cultivated for four days respectively on the Jensen and the N-free malate medium. The biomass was then centrifuged, washed in sterile distilled water twice and immersed in a phosphate pH 7.0 buffer of 0.15%. The seeds of okra were surface sterilized, air-dried and then inoculated with biofertilizers such as *Azotobacter* and *Azospirillum* in a slurry prepared by dissolving 800 g of brown sugar in 1000 mL of water and then warring it for 15 min at 40 °C. The seeds were thoroughly mixed with the culture of these biofertilizers separately. The seeds were then dried in the shade for 24 h and sown individually and/ or mixed with various levels of N such as 50%, 75% and 100% of the recommended doses. After 15 days of sowing, the plants were thinned to keep a plant 30 cm apart. Nitrogen has been applied as urea according to the treatments in split doses, i.e., half of it as a basal dressing, and half of it in two identical splits 3 and 5 weeks after germination, while at least one dose of N has been applied after the first irrigation. Phosphate and potash were added in two doses: one as basal and the other 5 weeks after germination in all beds, at an equivalent dose of 60 kg/ha in a single superphosphate as basal and muriate. In the four months of the growing period, all the cultural activities appropriate for watering and weeding were performed.

Table 1. Effect of *A. chroococcum*, *A. brasilense* and neem cake along with different doses of nitrogen fertilizers singly and in various combinations on physico-chemical and biological characteristics of okra

S. No	Treatments	Bulk density (mg/m ³)	Water holding capacity (%)	Porosity (%)	pH	EC (ds/m)	OC (%)	Soil biological properties	
								Azotobacter ($\times 10^6$ cfu/g soil)	Azospirillum ($\times 10^6$ cfu/g soil)
A	100% recommended N+ Azotobacter+ Neem cake	1.26 ^{fg}	65.93 ^{de}	53.28 ^c	6.50 ^g	0.25 ^f	2.05 ^f _g	6.19 ^{d-g}	7.70 ^{c-b}
B	75% recommended N + Azotobacter+ Neem cake	1.27 ^{ef}	63.57 ^e	52.18 ^{cd}	6.47 ^h	0.28 ^{ef}	1.91 ^g	5.68 ^{e-h}	7.25 ^{c-f}
C	50% recommended N + Azotobacter+ Neem cake	1.29 ^{def}	59.04 ^f	51.94 ^d	6.43 ⁱ	0.31 ^{de}	1.76 ^h	5.30 ^{fh}	6.77 ^{d-g}
D	100% recommended N+ Azospirillum+ Neem cake	1.20 ^{hij}	70.50 ^e	55.78 ^b	6.61 ^d	0.18 ^{gh}	2.33 ^c _d	7.70 ^{a-d}	8.97 ^{a-c}
E	75% recommended N+ Azospirillum+ Neem cake	1.22 ^{ghi}	68.40 ^{cd}	55.15 ^b	6.57 ^e	0.19 ^{gh}	2.23 ^{de}	7.12 ^{b-e}	8.73 ^{a-d}
F	50% recommended N+ Azospirillum+ Neem cake	1.24 ^{gh}	66.56 ^d	54.26 ^b	6.54 ^f	0.23 ^{fg}	2.15 ^e _f	6.63 ^{c-f}	8.23 ^a
G	100% recommended N Azotobacter+ Azospirillum+ Neem cake	1.15 ^k	77.48 ^a	57.13 ^b	6.77 ^a	0.12 ⁱ	2.56 ^a	9.10 ^a	9.98 ^a
H	75% recommended N Azotobacter+ Azospirillum+ Neem cake	1.17 ^{jk}	75.07 ^{ab}	56.20 ^a	6.70 ^b	0.14 ^{hi}	2.48 ^a _b	8.62 ^{ab}	9.62 ^{ab}
I	50% recommended N Azotobacter+ Azospirillum+ Neem cake	1.18 ^{ijk}	73.34 ^b	55.95 ^{ab}	6.64 ^c	0.16 ^{hi}	2.39 ^{bc}	8.11 ^{a-c}	9.30 ^{ab}
G	Azotobacter+ Neem cake	1.37 ^{bc}	51.66 ^h	49.30 ^b	6.33 ^e	0.39 ^e	1.32 ⁱ	4.71 ^{gh}	5.44 ^{fi}
K	Azospirillum+ Neem cake	1.33 ^{cd}	54.80 ^g	50.11 ^h	6.37 ^k	0.36 ^{de}	1.44 ⁱ	4.90 ^{gh}	5.90 ^{ci}
L	Azotobacter+ Azospirillum+ Neem cake	1.31 ^{de}	56.82 ^{fg}	51.47 ^d	6.40 ^j	0.33 ^e	1.63 ^h	5.16 ^{fh}	6.50 ^{d-h}
M	Neem cake	1.46 ^a	47.32 ^j	47.80 ^f	6.27 ⁿ	0.44 ^b	1.05 ^j	4.29 ^h	4.80 ^{hi}
N	100% recommended NPK	1.40 ^b	49.74 ^{hi}	48.19 ^f	6.30 ^m	0.41 ^{bc}	1.19 ^j _k	4.60 ^{gh}	5.20 ^{gi}
O	Untreated Control	1.50 ^a	43.62 ^j	47.32 ^f	6.24 ^o	0.58 ^a	0.88 ^l	4.19 ^h	4.55 ^j

Each value is a mean of five replicates and different letters on column indicate significant difference at $p \leq 0.05$ according to Duncan's test

Determination of growth parameters and yield attributes

Different growth parameters and yield attributes such as plant height, fresh as well as dry weights, the number of branches plant⁻¹, green fruit yield plant⁻¹, the number of fruits plant⁻¹, ascorbic acid content and chlorophyll content were determined at the end of harvesting the experiment from untreated as well as treated beds with biofertilizers and neem cake as well as 100% of the recommended dose of fertilizers.

Estimation of chlorophyll and ascorbic acid content

Okra leaves were used to estimate the chlorophyll as described by Hiscox and Israelstam (1979) method. A vial containing 7 mL of dimethyl Sulfoxide (DMSO) was held in one hundred mg okra-leaf sections, with a 60 min injection of chlorophyll content extracted into the fluid. This extract was then converted and immediately tested into a completed tube. Using a spectrophotometer, the chlorophyll extract was read at 645 and 663 nm against a DMSO blank.

For the estimate of ascorbic acid, samples of okra fruit of 10g per replicate were taken and crushed with a mortar and pestle separately, and then 50 mL of water was added to the extract beakers. The solutions resulted were centrifuged at 10,000 rpm. The supernatant has been screened carefully and filtered extract with

Whatman filter papers to determine vitamin C. The value of ascorbic acid was determined by 2, 6 dichlorophenol indophenol colouring using the titration method (Rangana, 1976).

Estimation of N, P and K in plants and soil

According to the IITA (1975) protocol, nitrogen content in plants and soil was estimated. The Lindner (1944) method was used to determine the phosphate and potash content of these plants. The content of phosphate and potash in the soil was also calculated by the methods (Olsen *et al.*, 1954; Jackson, 1973), respectively.

Determination of nematode population

Before treatment and after the test, soil samples with Cobb's sieving and decanting method and decanting method along with Baerman's funnel were processed. The population of pathogenic nematodes was counted from each bed (Southey, 1986). The number of nematodes was observed and counted in 5 counts. *M. incognita* was also counted separately for eggs and root-galls plant⁻¹ caused by root-knot nematodes.

Statistical analysis

Data was analysed by using IBM SPSS 17, for Duncan's test, Sigma Plot 11 for graph, and standard deviation and standard error were analysed by MS Excel.

Results

Growth parameters and yield attributes

The results in Figure 1 showed that the inoculation of *Azotobacter* and *Azospirillum* along with organic matter as neem cake in different combinations caused significant increases in crop growth and yield attributes in the presence of various recommended inorganic nitrogen doses. All treatments significantly improved plant height, fresh and dry weights, number of primary branches, fruit yield plant⁻¹, and fruit plant⁻¹ when compared to the untreated control. The plant height differed significantly among the treatments and the most pronounced increase in plant height (123.49 cm) was observed in 100% recommended dose of N + *Azotobacter* + *Azospirillum* + neem cake but the minimum value was observed in the untreated control (56.94 cm). However, 75% and 50% recommended N with *Azotobacter* and *Azospirillum* in combination with neem cake also caused significant increases in plant height. Application of neem cake and 100% recommended NPK alone showed a non-significant effect as compared to control plants.

Azotobacter and *Azospirillum* inoculation along with various recommended doses of N along with neem cake also greatly improved the fresh weight of the okra plant. The highest fresh weight was noted (235.81 g) in 100% recommended dose of N and the two biofertilizers along with neem cake and the lowest value (84.64 g) was recorded in the untreated control. A similar trend was also noted in dry weight. There was a considerable improvement in the number of fruits plant⁻¹ and fruit yield plant⁻¹ across all treatments. The highest fruit yield plant⁻¹ (303.82 g) was recorded in beds receiving 100% recommended N + *Azotobacter* + *Azospirillum* + neem cake, followed by 75% recommended N + *Azotobacter* + *Azospirillum* + neem cake (288.33 g) and 50% recommended N + *Azotobacter* + *Azospirillum* + neem cake (280.33 g). This parameter was found highest in such treatments in comparison with sole use of inorganic nitrogen (100% recommended dose NPK) and neem cake and either of the biofertilizers. The better growth in the number of primary branches and fruits plant⁻¹ follow the same pattern as described earlier.

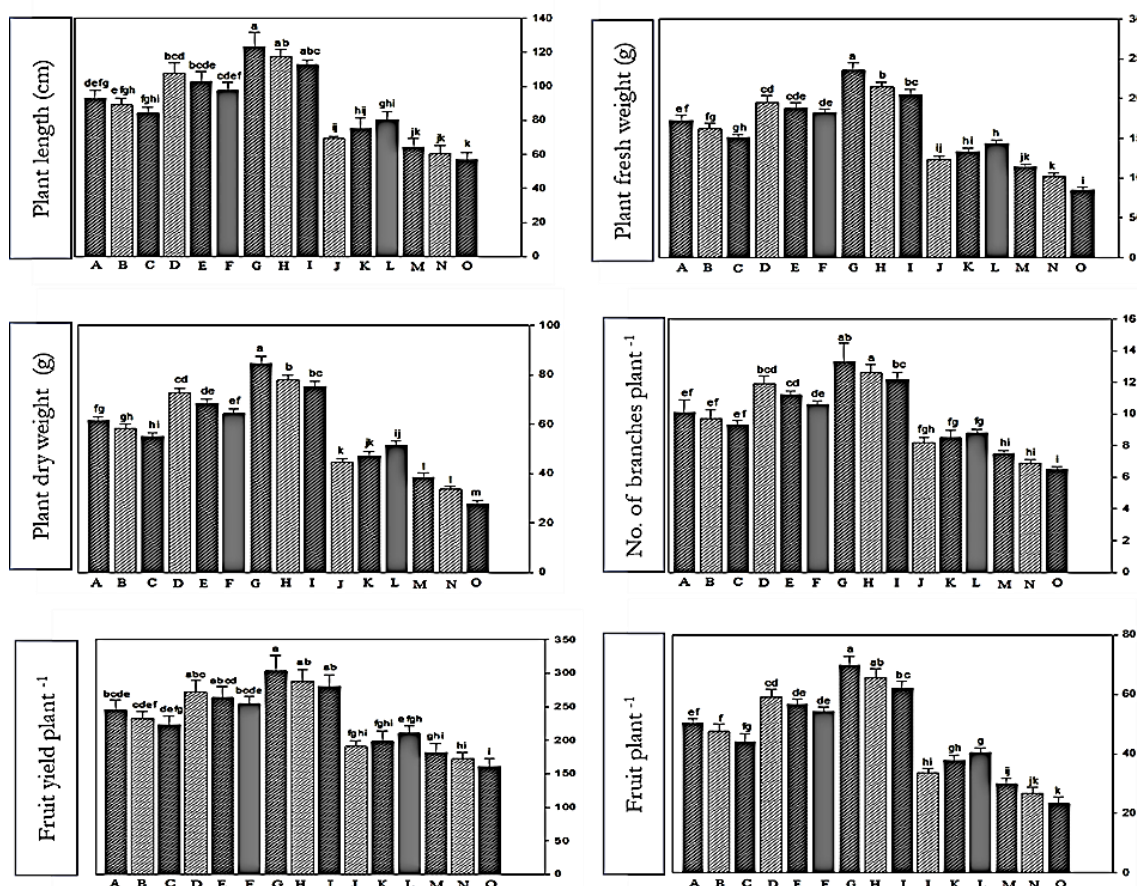


Figure 1. Effects of *A. chroococcum*, *A. brasilense* and Neem cake along with different doses of nitrogen fertilizer singly and in various combinations on plant length, fresh as well dry weights, number of branches plant⁻¹, fruit yield plant⁻¹ and fruit plant⁻¹ of okra

Each value is a mean (\pm SD) of five replicates and different letters on bars indicate significant difference at $p \leq 0.05$ according to Duncan's test.

A= 100% recommended N+ *Azotobacter*+ Neem cake, B= 75% recommended N+ *Azotobacter*+ Neem cake, C= 50% recommended N+ *Azotobacter*+ Neem cake, D= 100% recommended N+ *Azospirillum*+ Neem cake, E= 75% recommended N+ *Azospirillum*+ Neem cake, F= 50% recommended N+ *Azospirillum*+ Neem cake, G= 100% recommended N+ *Azotobacter*+*Azospirillum*+ Neem cake, H= 75% recommended N+ *Azotobacter*+ *Azospirillum*+Neem cake, I= 50% recommended N+ *Azotobacter*+ *Azospirillum*+ Neem cake, J= *Azotobacter*+ Neem cake, K= *Azospirillum*+ Neem cake, L= *Azotobacter*+ *Azospirillum* +Neem cake, M= Neem cake, N= 100% recommended NPK, O= Untreated Control.

Chlorophyll content

In comparison with the untreated control, both individual and combined inoculation significantly improved the chlorophyll content of leaves. The most pronounced increases in chlorophyll content (4.027 mg/g) were observed in okra plants treated with *Azotobacter* and *Azospirillum* inoculation with 100% recommended dose of nitrogen and neem cake, when compared to untreated plants, which had a lower chlorophyll content (2.465 mg/g). Neem cake alone also caused significant improvement, but not more than either of the biofertilizers along with the neem cake (Figure 2). Ascorbic acid content was also recorded at the highest value (94.28) in dual inoculation and the minimum again in the untreated control (32.47). Soil amendment of neem cake along with biofertilizers also improved chlorophyll content and ascorbic acid content in various combinations, but to varying extents (Figure 2).

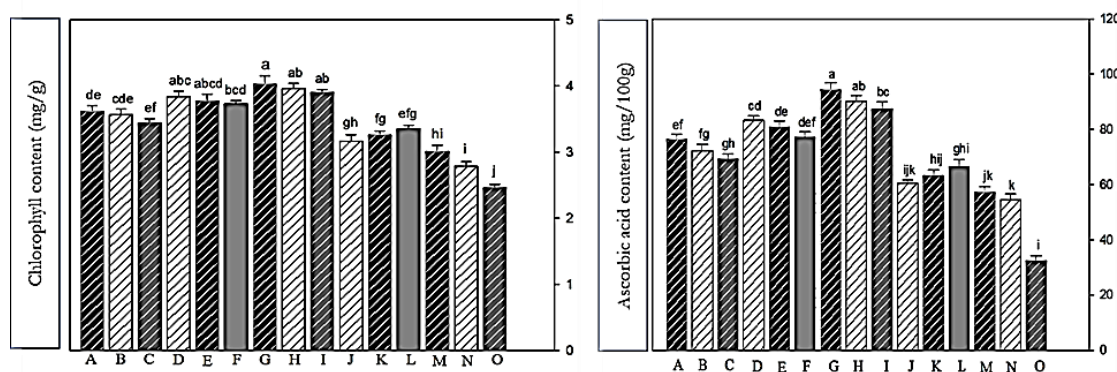


Figure 2. Effects of *A. chroococcum*, *A. brasilense* and Neem cake along with different doses of nitrogen fertilizer singly and in various combinations on chlorophyll content and ascorbic acid content of okra. Each value is a mean (\pm SD) of five replicates and different letters on bars indicate significant difference at $p \leq 0.05$ according to Duncan's test.

A= 100% recommended N+ Azotobacter+ Neem cake, B= 75% recommended N+ Azotobacter+ Neem cake, C= 50% recommended N+ Azotobacter+ Neem cake, D= 100% recommended N+ Azospirillum+ Neem cake, E= 75% recommended N+ Azospirillum+ Neem cake, F= 50% recommended N+ Azospirillum+ Neem cake, G= 100% recommended N+ Azotobacter+ Azospirillum+ Neem cake, H= 75% recommended N+ Azotobacter+ Azospirillum+ Neem cake, I= 50% recommended N+ Azotobacter+ Azospirillum+ Neem cake, J= Azotobacter+ Neem cake, K= Azospirillum+ Neem cake, L= Azotobacter+ Azospirillum+ Neem cake, M= Neem cake, N= 100% recommended NPK, O= Untreated Control.

Nutrient content and uptake in plants

Individual and combined inoculation of neem cake and biofertilizers along with different doses of inorganic nitrogen significantly increased the absorption of nutrient capability by the plant roots, however, to varying degrees (Figure 3). The maximum nutrient contents of N (99.52 mg/kg), P (89.93 mg/kg), and K (79.20 mg/kg) were found in those beds receiving dual inoculation of *Azotobacter* and *Azospirillum* with neem cake and 100% recommended dose of inorganic nitrogen as compared to the untreated control which recorded lower amounts of N (40.25 mg/kg), P (27.37 mg/kg), and K (18.39 mg/kg). Significant enhancement in nutrient content was also observed in all the treatments where either of the biofertilizers were added along with the neem cake. The treatment having 50% RDF of nitrogen and biofertilizers along with neem cake was found to be less effective as compared to other treatments and where inorganic nitrogen was not given as a supplement. The result was more pronounced in the presence of *Azospirillum* than in *Azotobacter*.

Soil fertility status

The available soil nutrients such as NPK were influenced significantly after the end of the experiment, and the analysis of soil samples seems to be through the organic sources of nitrogen such as organics and bio-organics, along with inorganic nitrogen levels during the time of the study. The maximum availability of NPK in the soil was observed in those beds receiving neem cake biofertilizers such as *Azotobacter* and *Azospirillum* along with a 100% recommended dose of inorganic nitrogen, where N (220.57 kg/ha), P (77.84 kg/ha), and K (95.35 kg/ha) were determined as compared with other treatments and untreated control. In the untreated control, the fertility status of the soil was noted as N (122.40 kg/ha), P (24.20 kg/ha), and K (52.24 kg/ha). Although the other treatments also significantly improved the available nutrient content of soil, and they were found in such beds where either of the bio-fertilizers were added along with neem cake and different doses of nitrogen fertilizer. The recommended dose of 100% was found almost effective with either of the biofertilizers or neem cake for improving soil fertility. Combined treatments of *Azospirillum* and different nitrogenous fertilizer levels along with neem cake were found to be more pronounced than the combined treatments with *Azotobacter* (Figure 3). Similarly, at higher doses of nitrogen application, the available N content in the soil

was observed to be more than the lower dose of inorganic nitrogen. The available P and K contents in the soil were significantly influenced by varying nitrogen doses also.

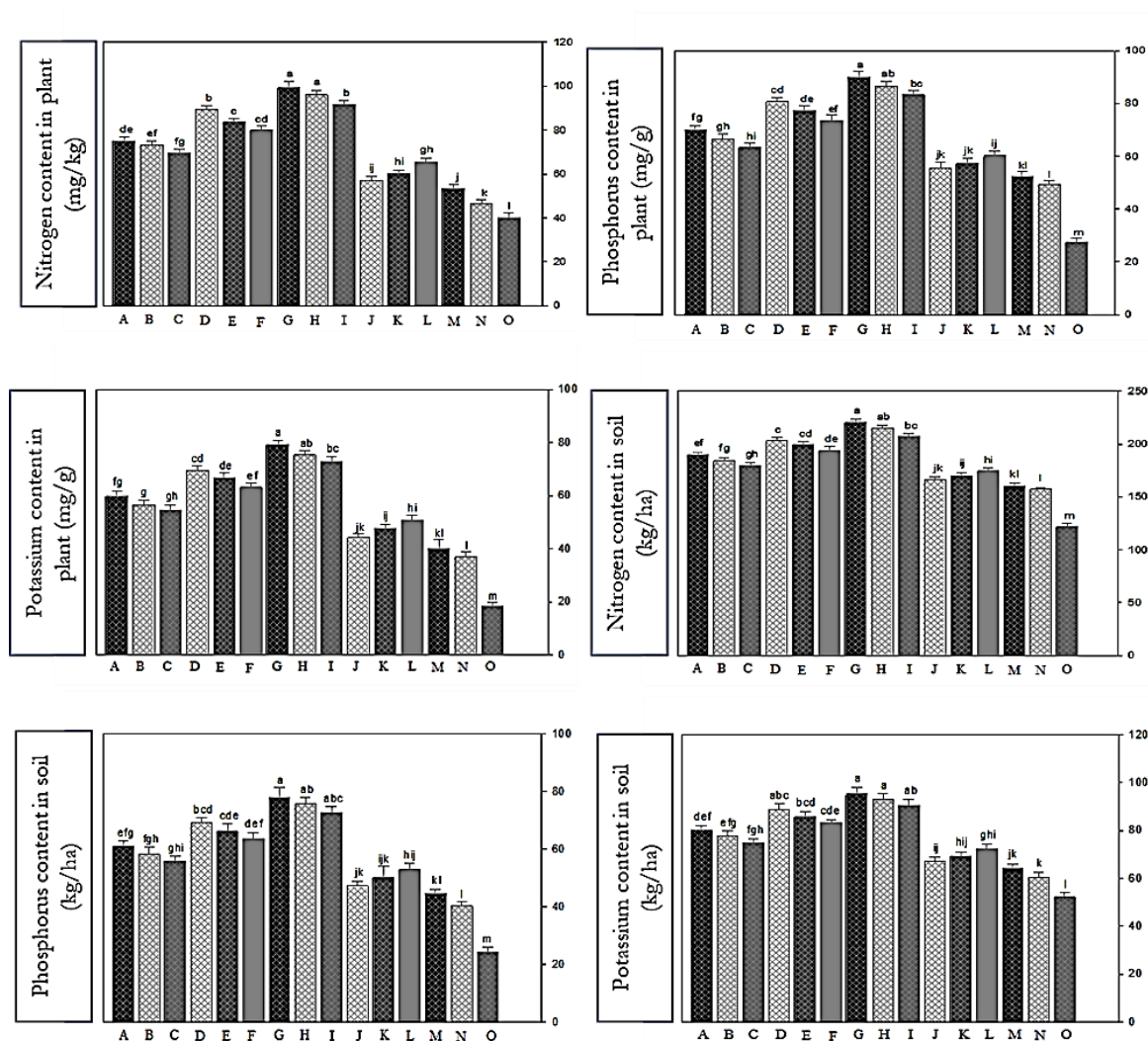


Figure 3. Effects of *A. chroococcum*, *A. brasilense* and Neem cake along with different doses of nitrogen fertilizer singly and in various combinations on nutrients uptake and soil fertility of okra

Each value is a mean (\pm SD) of five replicates and different letters on bars indicate significant difference at $p \leq 0.05$ according to Duncan's test.

A= 100% recommended N+ *Azotobacter*+ Neem cake, B= 75% recommended N+ *Azotobacter*+ Neem cake, C= 50% recommended N+ *Azotobacter*+ Neem cake, D= 100% recommended N+ *Azospirillum*+ Neem cake, E= 75% recommended N+ *Azospirillum*+ Neem cake, F= 50% recommended N+ *Azospirillum*+ Neem cake, G= 100% recommended N+ *Azotobacter* +*Azospirillum*+ Neem cake, H= 75% recommended N+ *Azotobacter* + *Azospirillum* +Neem cake, I= 50% recommended N+ *Azotobacter*+ *Azospirillum*+ Neem cake, J= *Azotobacter*+ Neem cake, K= *Azospirillum*+ Neem cake, L= *Azotobacter*+ *Azospirillum* +Neem cake, M= Neem cake, N= 100% recommended NPK, O= Untreated Control.

Disease incidence

The infected plants have shown steep growth and root galls caused by *M. incognita*, a root-knot nematode. After extraction with various mesh size sieves, the existence of other nematodes was verified. In these beds, treatments, with neem cake and biofertilizers, such as *Azotobacter* and *Azospirillum*, together with recommended doses of inorganic fertilizers, caused a significant decrease in plant-parasitic nematodes. However, the maximum reduction in nematode population (408 per 200 g soil) was observed in beds treated with neem cake, *Azotobacter*, and *Azospirillum* together with a 100% recommended dose of inorganic

nitrogen as compared to beds with no treatment where it was determined as (4219 per 200 g soil). The initial nematode population (1537 per 200 g soil) was also recorded before the initiation of the experiment. Nematode multiplication was affected in the beds treated with neem cake and either biofertilizers along with different recommended doses of nitrogen fertilizer, but the 100% recommended dose proved to be more detrimental to the nematode population. Further, combined inoculation of *Azospirillum* and different doses of nitrogen fertilizer along with neem seed cake was found to be more efficacious than the combined treatments with *Azotobacter* (Figures 4 and 5).

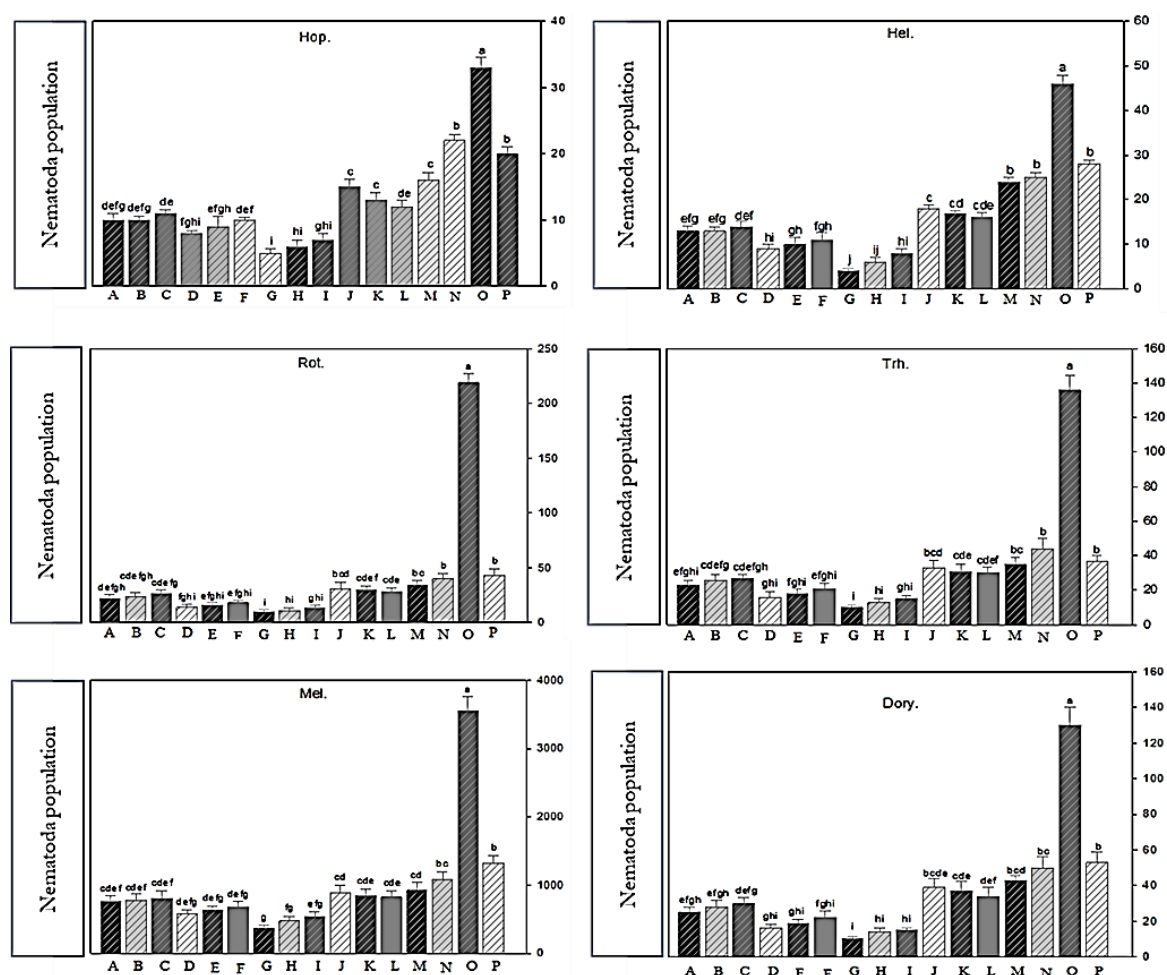


Figure 4. Effects of *A. chroococcum*, *A. brasilense* and Neem cake along with different doses of nitrogen fertilizer singly and in various combinations on population of plant-parasitic nematodes infesting okra Hop. = *Hoplolaimus indicus*, Hel. = *Helicotylenchus indicus*, Rot. = *Rotylenchulus reniformis*, Trh. = *Tylenchorhynchus*, Mel. = *Meloidogyne incognita*, Dorylaim = *Dorylaims*. Each value is a mean (\pm SD) of five replicates and different letters on bars indicate significant difference at $p \leq 0.05$ according to Duncan's test.

A= 100% recommended N+ *Azotobacter*+ Neem cake, B= 75% recommended N+ *Azotobacter*+ Neem cake, C= 50% recommended N+ *Azotobacter*+ Neem cake, D= 100% recommended N+ *Azospirillum*+ Neem cake, E= 75% recommended N+ *Azospirillum*+ Neem cake, F= 50% recommended N+ *Azospirillum*+ Neem cake, G= 100% recommended N+ *Azotobacter*+*Azospirillum*+ Neem cake, H= 75% recommended N+ *Azotobacter*+ *Azospirillum*+Neem cake, I= 50% recommended N+ *Azotobacter*+ *Azospirillum*+ Neem cake, J= *Azotobacter*+ Neem cake, K= *Azospirillum*+ Neem cake, L= *Azotobacter*+ *Azospirillum* +Neem cake, M= Neem cake, N= 100% recommended NPK, O= Untreated Control, P= Initial Population.

Root-knot development was observed in terms of the number of eggs and root-galls per root system in all the treatments. The highest reduction in the number of eggs (49.95 per 5 g roots) and root-galls (20.22 per 5 g roots) was noted in those beds treated with 100% of the recommended dose of N, *Azospirillum* and *Azotobacter* together with neem cake as compared to the untreated control where they were noted as 277.50 and 140.40 respectively. The other treatments also caused significantly decreased but not at par with the individual as well as in lower doses of N fertilizer (Figure 5).

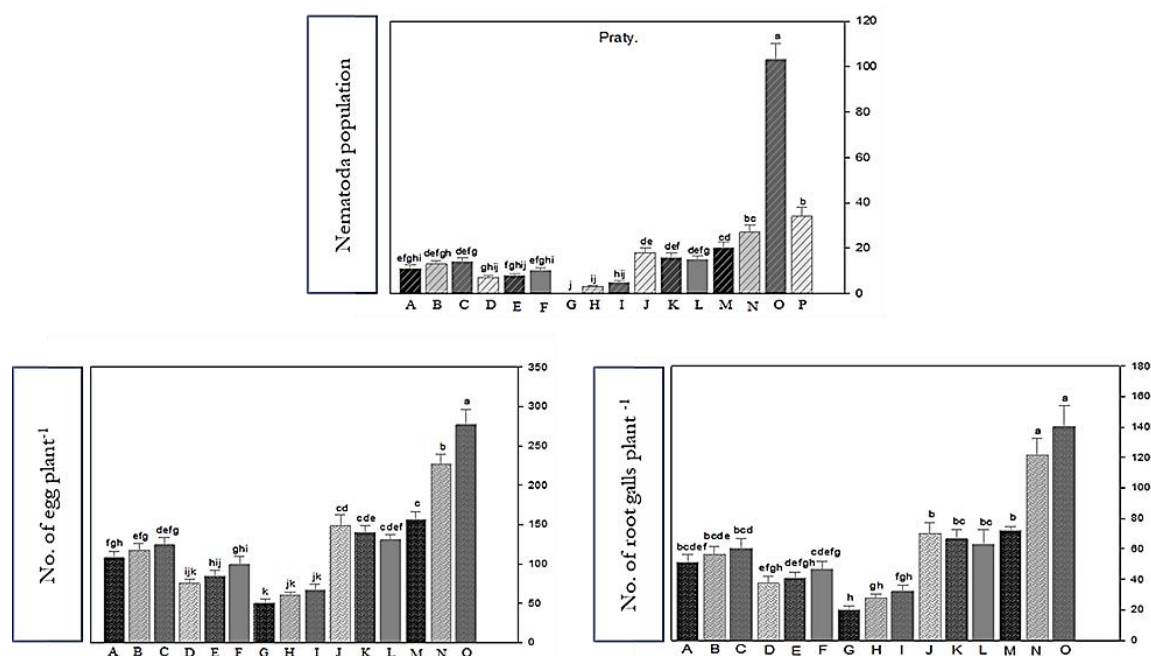


Figure 5. Effects of *A. chroococcum*, *A. brasilense* and Neem cake along with different doses of nitrogen fertilizer singly and in various combinations on the population of *Pratylinchus* spp. and root-knot development of okra

Each value is a mean (\pm SD) of five replicates and different letters on bars indicate significant difference at $p \leq 0.05$ according to Duncan's test.

A= 100% recommended N+ *Azotobacter*+ Neem cake, B= 75% recommended N+ *Azotobacter*+ Neem cake, C= 50% recommended N+ *Azotobacter*+ Neem cake, D= 100% recommended N+ *Azospirillum*+ Neem cake, E= 75% recommended N+ *Azospirillum*+ Neem cake, F= 50% recommended N+ *Azospirillum*+ Neem cake, G= 100% recommended N+ *Azotobacter*+*Azospirillum*+ Neem cake, H= 75% recommended N+ *Azotobacter*+ *Azospirillum*+Neem cake, I= 50% recommended N+ *Azotobacter*+ *Azospirillum*+ Neem cake, J= *Azotobacter*+ Neem cake, K= *Azospirillum*+ Neem cake, L= *Azotobacter*+ *Azospirillum* +Neem cake, M= Neem cake, N= 100% recommended NPK, O= Untreated Control, P= Initial Population.

Discussion

The use of organic substances like neem cake, biofertilizers like *Azotobacter* and *Azospirillum* influenced the growth characteristics of the okra plant. Soil amendment of organic matter like neem cake might have helped in different metabolic functions by supplying these micro-nutrients during the early stage of growth that might have promoted vigorous okra plant growth. Our results are in agreement with those of Sachan *et al.* (2017); Singh and Ram (2018) who found that the application of neem oil cake along with chemical fertilizer significantly increased the number of fruits per plant, fruit weight, which resulted in increasing yield. This is due to the better availability and uptake of plant nutrients for a longer time of crop growth due to neem cake as compared to other combinations of fertilizer. The application of neem cake not only increased the N status of the soil but also improved the rate of multiplication of beneficial microorganisms,

which in turn helped in the decomposition of applied manures. Crop growth and output have improved significantly due to the impact of neem cake and inorganic fertilizers at various recommended doses because organic matter and fertilizers help in the addition of elements to the soil, which increases soil fertility, which leads to a boost in okra biomass production, the number of fruits and other growth attributes (Aly *et al.*, 2012; Wani and Ali, 2013). The higher production of okra yield was likely due to the higher photosynthesis associated with vegetative growth like fresh and dry weights, the number of primary branches, and green fruit yield. The present findings are corroborated by the findings of Ghuge *et al.* (2015), Anand *et al.* (2016).

The combined effect of chemical fertilizers along with neem oil cake helped to absorb nutrients which were utilized for early initiation of the flowering buds and ultimately developed more flowers within the shortest possible period. The present findings are in conformity with the reports of Kumar *et al.* (2017). The availability of nutrients helps the plant to bear a greater number of flowers and reduces the chances of flower drop, resulting in a greater number of fruits per plant (Mishra *et al.*, 2020). The synergistic association between neem cake as organic matter and biofertilizers like *Azotobacter* and *Azospirillum* has been attributed to improving the production of growth-promoting substances such as gibberellic acid, indole acetic acid, and dihydrozeatin that have an essential role in the physiological activity of plants and have improved the green fruit yield and biomass and consequently the fruit weight. These results were recorded in different plants (Reza *et al.*, 2016). The increased yield can be attributable to improved root proliferation, increased absorption of nutrients and water, plant growth, photosynthesis, and food accumulation (Abd El-Aal and Salem, 2018; Mohamed *et al.*, 2018b; Sofy *et al.*, 2021b). Besides, Khan *et al.* (2012) showed that *Azotobacter* and *Azospirillum* and neem cake improved root elongation, improved water, mineral absorption, and produced plant hormones that could be responsible for the plant growth of chili plants.

Various treatments were also found to enhance ascorbic acid content. The enhancement of ascorbic acid content in okra crops treated with organic matter like neem cake and biofertilizer could be due to slowly but continuously providing both macro-and micronutrients that could have contributed to the assimilation of carbohydrates that help in the synthesis of ascorbic acid (Bahadur *et al.*, 2009; Santos *et al.*, 2019; El-Beltagi *et al.*, 2019; 2020). Moreover, Abd El-Aal and Salem (2018) showed that *Azotobacter chroococcum* EMCCN1458, *Bacillus megaterium*, *Bacillus circulans* and *Azospirillum lipoferum* D178 inoculation enhanced the total phenolics, flavonoids and ascorbic acid contents in the moringa plant. These increases must be due to the direct result of increasing both photosynthesis rate and efficiency.

The highest content of chlorophyll, ascorbic acid, plant nitrogen, phosphate and potash, and soil nitrogen residual and potash were observed in the dual inoculation of *Azotobacter* and *Azospirillum* with a 100% dose of nitrogen and neem cake. More nutrients, especially P and K, that are supplemented by neem cake as well as biofertilizers, have been preserved in the soil as a result of increased nutrient content. *Azotobacter* is able to convert atmospheric nitrogen to ammonia, which in turn is taken up and utilized by the plants (Prajapati *et al.*, 2008). Such bacteria are immensely resistant to oxygen during nitrogen fixation due to the respiration protection of nitrogenase (Prajapati *et al.*, 2008; Sofy *et al.*, 2021 c, d). Our results are in agreement with those of Velmourougane *et al.* (2019) and Arora *et al.* (2018). Non-symbiotic nitrogen fixers who live near the root systems of crop plants harvest atmospheric nitrogen that, in due course, is made available to the plants or released into the soil (Roper and Gupta, 2016). The marked improvement in N, P, and K content of plants under the influence of the combined application of neem cake, inorganic fertilizers, and biofertilizers seems to be due to the greater availability of these nutrients in the soil. The greater availability of such nutrients leads to better development and more absorption of soil and efficient translocation in the plant system (Mohamed, 2011; Reza *et al.*, 2016). Dual inoculation of biofertilizers and 100% recommended dose of N, P, and K besides neem cake noticed a significant enhancement in nutrient content. The N level is essential for increasing biomass, and growth regulators such as auxins and cytokinin can be explained as nitrogen compounds related to plant nitrogen metabolism, resulting in increment growth resulting from higher growth promoter concentrations than growth inhibitors. Other workers (Wani *et al.*, 2016) also supported our findings in some other crops.

The application of potash and phosphorus results in an increase in dry matter could be explained by the overall improvement in plant growth and vigour owing to increased availability, absorption, and translocation of nutrients in plants. This is in agreement with those of Bhattacharya *et al.* (2004). Similarly, in another report, Oosterhuis *et al.* (2014) revealed that synthesis and accumulation of carbohydrates in shoots increased biological processes like root development, photosynthesis, and energy transfer reactions due to the application of potassium. The positive yield response to NPK application was also reported in potatoes by Gondwe *et al.* (2020). The inoculation of biofertilizers such as *Azotobacter* and *Azospirillum* with neem cake along with different levels of nitrogen fertilizer significantly boosts the NPK content in plants as compared to uninoculated control and other treatments (Gao *et al.*, 2020; Cassan *et al.*, 2020). However, large amounts of N uptake by the okra crop were observed with *Azospirillum* inoculation than that of *Azotobacter* singly and along with different levels of nitrogenous fertilizers. The increase in nutrient uptake with neem cake as the organic matter was due to the increased availability of nutrients to the plants. It also improved the soil's physical properties as well as the soil, which enhanced the profuse rooting system, resulting in better absorption. Inorganic fertilization enhanced the contents of N, P, and K in vegetative plant parts at different treatments, including at various recommended doses of nitrogenous fertilizer. Khan *et al.* (2021) also observed an increase in N, P, and K uptake by N application. This seems to be due to the nitrogen fixation carried out by the inoculation of these biofertilizers. It might be because nitrogen uptake is directly correlated with yield. Also, Datta *et al.* (2009) also worked with different types of organic matter and correlated with the production of yield attributing characters. The growth parameters of okra are enhanced due to the availability of nutrients. Organic matter like neem cake on decomposition releases the nutrients and thus makes them available to the plant roots. Their availability, acquisition, mobilization, and the influx into plant tissues increased, and this improved yield components and finally the yield. The further physiological role of nitrogen in enhancing dry matter accumulation might have led to increased yield attributes and thereby yield of the crop at higher rates of nitrogen (Kumar *et al.*, 2011).

Microbial activity has also been reported to provide the necessary requirements of nutrients. The combined application of neem cake with biofertilizers could stimulate the uptake of nutrients due to increased microbial activity and continuous availability of native nutrients from the soil to crops due to soil amendment of organic matter as neem cake has a vast reservoir of nutrients (Bairwa *et al.*, 2009; Ayito and Iren, 2018). In addition, Chauhan *et al.* (2017) showed that the key ecosystem variable in microbial communities has a critical role in the biochemical transformation of nutrients, for example, the fixation of N_2 . Thus, N will become usable without N fertilization due to biological fixation for plant growth for many years. In order to preserve fertility in many habitats, this process catalysed by N_2 -ase is required. The ability to repair N_2 is widespread in many ecosystems in several bacterial classes. The introduction of biofertilizers like *Azotobacter* and *Azospirillum* greatly improved the nutrient content of the soil. It might be due to the application of neem cake and enhanced microbial activity through free-living nitrogen-fixing organisms *Azotobacter* and *Azospirillum*, which fix atmospheric nitrogen and also convert organically bound nitrogen to inorganic form. Higher microbial activity leads to better decomposition, which ultimately releases essential nutrients in the soil required for both plant and microbial growth (Elnasikh *et al.*, 2011; Eifediyi *et al.*, 2015). The organic matter and biofertilizers in the present study increase the carbohydrate content, which acts as an energy source for these biofertilizers and other microbes. Our findings are in agreement with those of Mina *et al.* (2008).

The beneficial effects of neem cake and biofertilizers not only favour the greater availability of nutrients throughout the crop growth but fertilization at different levels resulting in significant improvement in nutrient content and uptake. Our results are in close conformity with those of Khan *et al.* (2009) and Zhang *et al.* (2010). Improvements in nitrogen, phosphorus, potassium, and others significantly at various treatments which influenced physiological parameters further promoted the uptake of plant nutrients and increased supply of nutrients through the enhanced proliferation of roots essential for more uptake of nutrients (Mohamed *et al.*, 2016; Moustafa-Farag *et al.*, 2020; Abu-Shahba *et al.*, 2021). Similarly, Kumar *et al.* (2015) have also observed the total soluble solids (TSS), total sugars, and juice percentage of strawberries were recorded

the highest by the application of vermicompost + PSB followed by treatment comprising of vermicompost + *Azospirillum*.

The higher amount of available P was due to the application of neem cake and biofertilizers, which might reduce the fixation of water-soluble P, increase the mineralization of organic P owing to microbial activity, and thus enhance the availability of phosphorus. A similar enhancement in phosphorus after organic matter addition was also noticed by Prasad *et al.* (2010). Significant improvement was also seen in the case of the K amount in residual soil due to the decomposition of organic matter like neem cake and the action of microbes present in the soil, which may be attributed to the direct addition of potassium to the available pool of the soil. Soil amendment of organic matter and its residue subsequently increased the soil organic carbon level in the soil and, in turn, released more amounts of nutrients (Mina *et al.*, 2008). Significant variation in available N, P, and K contents in soil was determined with each different dose of N level and being the highest at 100% recommended N. An increase in the level of nitrogen also assured the availability of these nutrients to the crop in an adequate amount and remained in the soil in substantial quality after fulfilling the crop requirement that ultimately improved the overall soil fertility.

The multiplication rate of plant-parasitic nematodes was found to decrease in those beds of okra plants inoculated with organic matter like neem seed cake and free-living nitrogen-fixing biofertilizers such as *Azotobacter* and *Azospirillum* along with recommended doses of inorganic fertilizers. Dual inoculation of biofertilizers and neem cake along with a 100% recommended dose of nitrogenous fertilizer brought about a greater reduction in root-knot development as well as the multiplication of plant-parasitic nematodes. Akram *et al.* (2016) found that disease incidence by the root-knot nematode *Meloidogyne incognita* was significantly reduced when *A. chroococcum* was applied to chickpea plants. Several mechanisms can be implicated in the management strategies used by bacteria for the control of plant diseases. These may include the production of siderophores, antimicrobial substances, toxins, and also growth hormones like auxins, gibberellins, and cytokinin. Organic matters like neem seed cake are rich in minerals and upon decomposition release organic acids, phenols, aldehyde, ammonia, etc. thereby absorbing ions and releasing them slowly over the entire growth period, which act as nematicidal substances (Khalil, 2013; Ntalli *et al.*, 2020). Neem seed cake contains nimbidin, thionimone and azadirachtin which have been reported to be highly nematicidal and nematostatic. These compounds significantly decreased the nematode population (Oka, 2010; Ntalli *et al.*, 2020). Olabiyi and Ayeni (2015) also reported that the active principles of neem significantly decreased the nematode population. The organic acid is also found to be toxic to plant-parasitic nematodes in vitro conditions (Taba *et al.*, 2006). Organic soil amendments may increase the microbial activity in amended soil. Choudhary *et al.* (2010) were of the opinion that the rise in the population of saprophytic as well as antagonistic organisms ultimately suppresses the growth of pathogenic forms. The soil microbial population plays a very important role in organic matter decomposition and nutrient mobilization. These microorganisms are an important component of the soil environment. Various sources of nutrients exert influence on the multiplication rate of the nematode population (Laishram *et al.*, 2013). Soil amendment with organic matter is well known to activate the biocontrol capability of various antagonists as well as N-fixing organisms like biofertilizers. Kaur and Mohan (2013) reported that amendment with organic matter enhanced the biocontrol potential of antagonistic organisms which effectively suppress soil-borne pathogens.

The indirect effects of biofertilizers on soil microbial activity are known to aid the plant in its turn to improve and ultimately to induce resistance to these soil pathogens, as stated by Fasusi *et al.* (2021) and Nosheen *et al.* (2021). Biofertilizers further help to enhance soil fertility by biological nitrogen fixation, besides solubilization of phosphorus and reduction of the growth of phytopathogenic fungi (El-Nagdi *et al.*, 2019), thus supplemental nitrogen greatly affects the nematode population. *Azotobacter*, *Azospirillum*, and other bioinoculants are important free-living N-fixing organisms that have detrimental effects on the nematode population (El-Haded *et al.*, 2011; Youssef and Eissa, 2014). Soliman *et al.* (2011) also reported that many PGPR like *Azotobacter*, *Azospirillum*, and *P. fluorescens* inhibited egg hatching and killed juveniles by producing a wide variety of antibiotics, siderophores, and organic compounds.

Improvement in N, P and K content was observed in all the treatments except the untreated control, but the highest was in those plants that received neem cake, 100% of the recommended dose of N fertilizer, and biofertilizers. This increase may be due to intense microbial activity. Sen (2003) also observed the enhancement in N, P, and K content in the soil, thereby improving soil health, which ultimately affects the pathogenic population. The improvement in P and K content due to the application of organic matter, inorganic fertilizer, and biofertilizers make the soil environment unfavourable for pathogens that might affect the nematode population (Laxminarayan and Patiram, 2006; Sharaf *et al.*, 2009).

Conclusions

The findings of this study showed that the application of neem seed cake, biofertilizers and various recommended doses of nitrogen fertilizer had a major and essential impact on the growth and yield attributes of okra. In addition, they influence the multiplication of plant-parasitic nematodes and the production of root-knots in okra. Integrated nutrient management is crucial for maintaining or improving soil fertility and the supply of plant nutrients to the optimal degree to sustain desired crop production through integrated optimization of the benefits of all sources of nutrients. The benefits to sustainable crop production may be attributed to enhanced biological activities in the rhizosphere and to improved soil structure and increased available nutrients for the benefit of organic matter and inorganic fertilizer. On the other hand, biofertilizers are cheap and environmentally friendly and have a huge potential for nutrient supply that can decrease the use of chemical fertilizers by 25-50%. Better soil health and consequently a lower population of pathogenic species below the inoculum threshold is indicated by increased nitrogen availability when bio fertilization is applied. Soil is a valuable natural resource that is depleted through the indiscriminate use of chemical fertilizers, but organic farming components such as organic and biological fertilizer or microbial inoculants are in a balanced proportion to the possible replacement of chemical fertilizer. Long-term use of such components increases soil carbon dioxide and NPK, thus maintaining soil health and cultivation.

Authors' Contributions

SA, GA, AEH, RR, SAT, IM: Conceptualization, Methodology, Data curation, Writing - original draft of data, Visualization, Investigation, SA, GA, AEH, RR, SAT, IM: Conceptualization, Methodology, Data curation, Writing - original draft of molecular data, HIM: Validation, review and editing.

All authors have read and approved the manuscript

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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