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RESEARCH ARTICLE

Synergy of biochar and biofertilizers to improve bell pepper fruit biochemical quality with increased soil carbon, *Azospirillum* population and mycorrhization

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Abstract

Sustainable crop production depends more on maintaining good soil health. Biochar significantly improves the soil's physical, chemical, biological and nutritional properties while aiding in carbon sequestration. Biofertilizers, which promote nutrient cycling, heavy metal chelation and the production of plant growth substances are crucial to sustainable agriculture. While the individual benefits of biochar and biofertilizers are well-documented, their combined effect in vegetable farming is less studied. The synergistic application of biochar and biofertilizers can improve soil health and nutrient dynamics, enhancing crops' biochemical properties. In a pot culture experiment, *California Wonder* bell peppers were treated with biochar and biofertilizers, including *Azospirillum*, Vesicular Arbuscular Mycorrhiza (VAM), and *Serendipita indica*. These were applied directly to the soil during transplanting. Results showed that plants treated with biochar + *Azospirillum* had higher flavonoid, total soluble protein and ascorbic acid content. Biochar + VAM treatment resulted in higher polyphenol content, total dry matter and root volume. *S. indica* reduced the vegetative period and induced earlier flowering. Biochar also increased soil organic carbon (2.89 %), improved the rhizosphere *Azospirillum* population and enhanced fungal endophytes' root colonization. Combining biochar and biofertilizer improves bell pepper plant performance, enhances biochemical parameters and improves soil health.

Keywords

Azospirillum; biochemical; organic carbon; *Serendipita indica*; VAM

Introduction

Chilli *(Capsicum annuum)* is an essential commercial crop in India, cultivated throughout the country. Some varieties are classified as chilli peppers due to their pungency, while less pungent bell peppers are grouped as "sweet peppers". In India, bell pepper production was recorded at 518.16 metric tons in 2021-2022 (1). Bell peppers are rich in vitamins, antioxidants and various carotenoids, which help reduce the risk of chronic diseases and promote eye health. They also offer protection against cancer, diabetes, cataracts and cardiovascular diseases. Due to the presence of beta-cryptoxanthin and high levels of ascorbic acid, bell peppers lower the risk of arthritis and other inflammatory ailments.

Sustainable crop production depends on maintaining good soil health. Biochar, a solid organic residue produced from biomass pyrolysis, contains plant nutrients and ash, which, combined with its large surface area, increased porosity and potential to support soil-microbe interactions, improves soil properties by reducing tensile strength and soil compaction while enhancing nutrient absorption (2). Biochar undergoes a chemical change during heating, resulting in an aromatic structure that is resistant to microbial degradation and remains stable for up to 100 or 1000 years. It can be produced from various feedstock, such as agricultural wastes, rice husks, coconut husks, paper products, wood chips, animal manure, bagasse and urban green waste. In rice-producing countries, rice husk, a byproduct, is abundant and its high silica content enhances nutrient retention, turgidity and plant structure. Converting rice husk into biochar benefits energy generation, sustainable waste recycling, carbon sequestering, improving soil quality and promoting plant growth and productivity (3).

Biofertilizers improve soil health and crop yields across diverse agro-ecological conditions. They enhance the soil environment by fixing nitrogen, releasing plant growth-regulating substances, solubilizing or mineralizing phosphate and potassium, synthesizing antibiotics and breaking down organic matter (4).

Azospirillum is one of the most extensively studied and commercially used plant growth-promoting rhizobacteria. Its benefits are linked to nitrogen fixation and its ability to enhance stress tolerance by increasing phytohormone levels. Studies on various plants (e.g., wheat, rice, *Arabidopsis thaliana*) have shown that *Azospirillum* inoculation promotes growth and nutrient uptake (5). Vesicular arbuscular mycorrhizal (VAM) fungi have been reported to colonize roots in various agroecosystems (6). VAM forms stable soil aggregates, promotes a macroporous soil structure that allows water and air penetration and absorbs nutrients like phosphorus, zinc and copper, supplying them to the host plant. VAM can meet 20-25 % of a plant's phosphorous requirement (7). *Serendipita indica* is a well-known symbiont that promotes plant development, boosts disease resistance and increases tolerance to drought, extreme temperatures and salinity. It also provides systemic resistance to toxins and heavy metals, promoting growth and seed production (8).

Rice husk biochar, with its high silica content and nutrient retention capacity, is an effective adsorptive material that enhances soil fertility and fertilizer efficacy when combined with other fertilizers. Studies have shown that biochar made from rice straw can act as a carrier for rhizobia, improving bacterial inoculant colonization and survival. This biochar-based inoculant increased both roots and shoots' nodulation, nutrient uptake and biomass (9). By altering nutrient availability and the soil environment, biochar influences microbial populations and overall soil health. Proper application rates of biochar, combined with bioinoculants, can boost nutrient input in agricultural systems.

While the benefits of biochar and biofertilizers have been well-documented in agriculture, their combined effect in vegetable farming has not been extensively studied. Improving the biochemical quality of bell peppers with these inputs could enhance their nutritional value, bioactive compounds and therapeutic effects. This study aims to evaluate the biochemical attributes of bell peppers under the influence of biochar amended with different biofertilizers. The synergistic effects of biochar and biofertilizers resulted in enhanced biochemical properties due to improved soil health and nutrient dynamics.

Materials and Methods

2.1 Experimental materials

The experiment was conducted as a pot study at the College of Agriculture, Vellayani, Kerala, India, situated at 8°5' N latitude and 76°9' E longitude, with an altitude of 29 m above mean sea level, during April - August 2022.

The "California Wonder" bell pepper variety seeds were collected from Namdhari Seeds Pvt-Ltd, Ramnagara Taluk, Karnataka and sown in trays. Biochar was produced using rice husk as raw material through pyrolysis in an anaerobic biochar unit at the Organic Farm, Department of Soil Science, College of Agriculture, Vellayani. The rice husk was burned at 500 °C for 5 h during the pyrolysis.

Azospirillum and Vesicular Arbuscular Mycorrhiza were collected as biofertilizer packages from the Department of Microbiology, College of Agriculture, Vellayani. Vesicular Arbuscular Mycorrhiza contained a consortium of mycorrhizal species, such as *Glomus fasiculatum, Glomus etunicatum, Glomus mosseae, Glomus gigospora* and *Acaulospora mellea,* with a spore count of 120 spores/g. *Azospirillum* contained more than 5×10^7 colony-forming units/g of *Azospirillum lipoferum* strain CRT1 (NCBI Accession number DQ438997.1). *Serendipita indica*, provided by Dr Ajith Varma, former Professor at Jawaharlal Nehru University, New Delhi, India, was also available from the Department of Microbiology, College of Agriculture, Vellayani. The culture was grown on a potato dextrose agar medium and incubated for 10 days at 26 °C. The grown *S. indica* was transferred to potato dextrose broth and cultured at 110 rpm in a shaker at 26 °C for consistent growth. After 10-15 days, the mycelial mat was separated using a muslin cloth (10). A talc formulation of *S. indica* was prepared by mixing talcum powder (particle size: 50-80 mm) with the fungal mat on a 1 % w/v basis (8).

2.2 Biochar and biofertilizer treatments

Seeds of the "California Wonder" bell pepper variety were sown in trays (top diameter: 33 mm, bottom diameter: 22 mm, height: 38 mm) containing a potting mix of coir pith and vermicompost in a 1:1 ratio. Five-week-old seedlings were transplanted into 12-inch plastic pots containing 5 kg of potting mix (1:1:1 ratio of soil, coir pith compost and farmyard manure). The pots were subjected to respective treatments in triplicate. Biochar was applied at a rate of 0.5 % (w/w), with 25 g of biochar added to each pot containing 5 kg of potting mix. A 500 g *Azospirillum* solution was prepared in 750 mL water and used for seedling root dip. VAM and *S. indica* were applied at 20 g and 50 g per pot respectively, at the time of transplanting. The plants were maintained using the organic

practices recommended by Kerala Agricultural University (11). The roots were dipped in a 2 % *Pseudomonas* biofertilizer solution before transplanting. Neem oil spray (3 %) was applied at 20, 40 and 60 days after transplanting (DAT) and vermicompost (5 g) was top-dressed every 10 days.

2.3 Flavonoid content

The total flavonoid content was determined with the aluminium chloride colourimetric assay. A 0.5 g fruit sample was macerated in 80 % ethanol at 40 °C and centrifuged at 4500 rpm for 15 min. The supernatant was collected and 1 mL of aliquot was mixed with 4 mL of distilled water. After 5 min, 0.3 mL of 5 % sodium nitrite solution and 0.3 mL of 10 % aluminium chloride were added. After 5 min, 2 mL of 1 M sodium hydroxide was added and the volume was increased to 10 mL. The resulting yellow-orange mixture was measured at 510 nm using a spectrophotometer (Model-ELICO SL 218, Double Beam UV-VIS, India). The total flavonoid content was expressed as mg/g (12).

2.4 Polyphenol content

The total phenolic content was determined using Folin-Ciocalteu's method. A 0.5 g fruit sample was macerated in 80 % ethanol at 40 °C and centrifuged at 4500 rpm for 15 min. One mL of the supernatant was mixed with 5 mL of distilled water, followed by adding 0.5 mL of Folin-Ciocalteu's reagent and 1.5 mL of 20 % sodium carbonate. The final volume was adjusted to 10 mL with distilled water. The mixture was incubated for 2 h at room temperature to develop a deep blue colour and absorbance was measured at 750 nm using a spectrophotometer. The total polyphenolic content was expressed as mg/g (12).

2.5 Total Soluble Protein

The total protein content was estimated using Bradford's method (13). Bovine serum albumin (BSA) was used as a standard. A 500 mg fruit sample was homogenized in 10 mL phosphate buffer solution (PBS). After centrifugation at 3000 rpm for 5 min, 0.1 mL of the supernatant was diluted to 3 mL with PBS and 5 mL of Coomassie brilliant blue dye was added. Absorbance was measured at 595 nm using a spectrophotometer and the total soluble protein content was expressed as mg/g.

2.6 Ascorbic acid

Ascorbic acid (vitamin C) content was estimated by titration with 2,6- dichlorophenol-indophenol dye (14). A sample of 0.5 g of fruit was extracted using 4 % oxalic acid and the volume was adjusted to 100 mL. After centrifugation, 5 mL of the supernatant was mixed with 10 mL of 4 % oxalic acid and titrated against the dye solution. The ascorbic acid content was expressed in mg/100 g, calculated using the following formula:

Ascorbic acid content = (Titre value x dye factor x volume made up)/(Aliquot taken x weight of sample)*100

2.7 Beta carotene

It was measured using the petroleum ether-acetone extraction method. One gram of fruit sample was homogenized in 10 mL acetone and extracted with petroleum ether. The absorbance of the coloured layer was measured at 453, 505, 645 and 663 nm using a spectrophotometer. Beta carotene content was calculated using the following equation (15) and expressed in µg/100 g:

$$\text{Beta carotene} = 0.216A_{663} - 1.22A_{645} - 0.304A_{505} + 0.452A_{453}$$

2.8 Days to first flowering and fruiting

The number of days from transplanting to the appearance of the first open flower and first fruit was recorded.

2.9 Root volume

Root volume was measured using the water displacement method (16). Roots were immersed in water and the difference between the initial and final water levels was used to determine root volume expressed in cm^3.

2.10 Total dry weight

The entire plant was uprooted and dried at 60 °C until a constant weight was achieved and the total dry weight was recorded using a digital balance.

2.11 The organic carbon content of the soil

Soil organic carbon was estimated using the chromic acid wet digestion method (17) and expressed as %.

2.12 Population of *Azospirillum*

The population of *Azospirillum* in the soil was determined using the most probable number (MPN) method. One gram of soil was serially diluted (10^-3, 10^-4 and 10^-5) and inoculated into nitrogen-free semi-solid malate medium culture tubes. The tubes were incubated for 24 h. Microaerophilic growth was identified by the appearance of a white subsurface pellicle and the media turning blue indicated the presence of *Azospirillum*. The population of *Azospirillum* was then quantified using the MPN method based on the MPN index and dilution factor. The results were expressed as colony-forming units/g of soil (cfu/g) (18).

2.14 VAM and *Serendipita indica* colonization

Fungal colonization was assessed using the root staining method (10). Roots were cut 15 days after inoculating the seedlings with microorganisms. Following a thorough wash with water to remove residual soil particles, the roots were chopped into one cm-long pieces. The root pieces were boiled in 10 % KOH for 5 to 10 min, then rinsed with water 3 times and treated with 1 N HCl for 3 min. The acid was drained and the root bits were immersed in Trypan blue stain for 10 min. After staining, the root bits were transferred to a lactophenol solution for destaining. The presence of mycelial networks and vesicles was examined to confirm VAM colonization, while the presence of double-walled spherical chlamydospores was assessed for *S. indica* colonization.

Statistical analysis

A completely randomized design was employed and the statistical analysis was conducted using "GRAPES" software (19). Data were analyzed using one-way analysis of variance (ANOVA) to determine significant differences among the treatments. The tables present the statistical differences as critical difference (CD) values.

Results

3.1 Flavonoid content

Flavonoid content was significantly influenced by the applied treatment (Table 1). The therapy with *Azospirillum* increased the flavonoid content by 35.44 % (4.28 mg/g) compared to the control, followed closely by VAM, which enhanced the flavonoid content by 33.86 % (4.23 mg/g) compared to the control. Furthermore, adding biochar along with *Azospirillum* resulted in a remarkable 54.11 % increase (4.87 mg/g) in flavonoid content compared to the control. This increase was statistically comparable to the biochar with VAM treatment, which produced a 41.45 % increase (4.47 mg/g).

3.2 Polyphenol content

Significant variation in polyphenol content was observed across all treatments studied (Fig. 1). Among the biofertilizer treatments, VAM exhibited the highest polyphenol content at 21.80 mg/g, representing a 24.14 % increase compared to the control and was comparable to *Azospirillum*, which had a polyphenol content of 21.47 mg/g (22.26 % higher than the control). Additionally, combining biochar with biofertilizers significantly enhanced the polyphenol content of bell pepper fruits. Specifically, an increase of 38.61 % was observed in the biochar + VAM treatment compared to the control fruits, resulting in a polyphenol content of 24.34 mg/g).

3.3 Total soluble protein

The total soluble protein content in bell pepper fruits varied significantly across all treatments studied (Fig. 1). Among the 3 biofertilizer treatments, *Azospirillum* and VAM demonstrated higher total soluble protein contents of 20.22 mg/g and 20.08 mg/g respectively. Both biofertilizers, *Azospirillum* (38.58 % increase compared to control) and VAM (37.62 % increase compared to control) showed comparable effects in enhancing total soluble protein content. In contrast, combining biochar with *Azospirillum* led to a significant increase of 62.23 %. The combination of biochar and VAM resulted in a 45.85 % increase in total soluble protein content compared to the control. The biochar + *Azospirillum* treatment yielded the highest total soluble protein content in bell pepper fruits at 23.67 mg/g, statistically comparable to the biochar + VAM treatment, which had a 21.28 mg/g content.

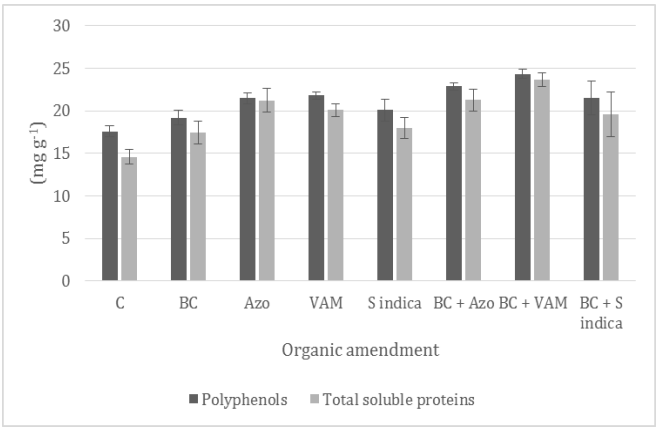

Fig. 1. Effect of biochar and biofertilizers on polyphenols and total soluble proteins in bell pepper fruits (var. California Wonder) C- Control; BC- Biochar; Azo - *Azospirillum*; VAM- Vesicular Arbuscular Mycorrhizae; P indica- *Serendipita indica* ; BC + Azo- Biochar + *Azospirillum*; BC + VAM- Biochar + Vesicular Arbuscular Mycorrhizae; BC + P indica- Biochar + *Serendipita indica* . Values are the average of 3 replicates means. Mean ± standard error (SE) is indicated by error bars. (One-way ANOVA, Least Significant Difference test $p < 0.05$).

3.4 Ascorbic acid

Applying biochar amended with biofertilizers significantly influenced the ascorbic acid content of bell pepper fruits (Table 1). Among the 3 biofertilizer treatments, *Azospirillum* had the highest ascorbic acid content (24.84 %), followed closely by VAM treatment (23.98 %). Specifically, *Azospirillum* produced fruits with an ascorbic acid content of 125.73 mg/100 g, making it the most effective treatment for enhancing ascorbic acid levels in bell pepper fruits. In comparison, VAM fungi yielded 124.87 mg/100 g. Adding biochar further increased ascorbic acid content, with the combination of *Azospirillum* and biochar resulting in an increase of 29 % compared to control fruits (129.92 mg/100 g). The treatment with VAM and biochar also showed a significant increase of 26.98 % over control fruits, measuring 127.90 mg/100 g).

3.5 Beta carotene

The results indicated no significant differences among the treatments regarding the beta carotene content in bell pepper fruits (Table 1). However, it was noted that when biofertilizers were combined with biochar, the beta-carotene content increased compared to the sole application of biofertilizers.

3.6 Days to first flowering and fruiting

Statistical analysis revealed no significant variation among the treatments regarding the days to first flowering and fruiting (Table 2). However, the application of *Serendipita indica* reduced the duration of the vegetative period by 7 to 8.2 %. It induced earlier flowering and fruiting in treated plants, resulting in advances of 5.2 to 7.7 % compared to the control plants.

Table 1. Effect of biochar and biofertilizers on flavonoids, ascorbic acid and beta carotene content of bell pepper fruits var. California Wonder.

Treatment	Flavonoid content (mg g^{-1})*	Ascorbic acid (mg 100 g^{-1})*	Beta carotene (µg 100 g^{-1})
Control	3.16^{e}	100.71^{e}	35.81
Biochar	3.77^{d}	119.25^{d}	35.96
Azospirillum	4.28^{bc}	125.73^{b}	36.53
VAM	4.23^{bcd}	124.87^{bc}	36.81
S. indica	3.88^{cd}	121.48^{d}	35.29
Biochar + *Azospirillum*	4.87^{a}	129.92^{a}	37.92
Biochar + VAM	4.47^{ab}	127.89^{ab}	37.53
Biochar + *S. indica*	3.97^{cd}	122.33^{cd}	36.07
SEm(±)	0.17	1.11	0.69
CD (0.05)	0.500	3.353	NS

*The values represent average of 3 replicate means. Statistical significance is indicated by letter differences between treatments (One-way ANOVA, Least Significant Difference test $p < 0.05$). Similar letters represent no significance, while a different letter is an indication of a significant difference, indicating the significance between them.

Table 2. Effect of biochar and biofertilizers on days to first flowering and days to first fruiting bell pepper var. California Wonder.

Treatment	Days to first flowering	Days to first fruiting
Control	56.67	64.67
Biochar	54.33	63.67
Azospirillum	54.67	64.00
VAM	54.00	62.33
S. indica	52.67	61.33
Biochar + *Azospirillum*	55.67	62.67
Biochar + VAM	53.67	62.00
Biochar + *S. indica*	52.00	59.67
SEm(±)	0.94	1.16
CD (0.05)	NS	NS

3.7 Root volume

A significant difference in root volume of bell pepper was observed across all treatments (Table 3). The fungal endophytes (VAM and *S. indica*) improved root characteristics more effectively than the other treatments. Among the three biofertilizer treatments, VAM produced the highest root volume at 2.8 cm^3, comparable to the 2.7 cm^3 root volume generated by *S. indica*. Applying biochar further enhanced root volume, combining biochar and VAM, resulting in the highest root volume of 3.1 cm^3 among all 8 treatments.

3.8 Total dry matter

A significant difference in the total dry weight of bell peppers was observed across all treatments (Table 3). Among the three biofertilizer treatments, the highest dry weight of 34.10 g was recorded in the VAM treatment. The application of biochar in combination with biofertilizers further enhanced this increase. Specifically, the biochar + VAM treatment achieved the highest total dry matter at 36.15 g, representing a 46.7 % increase compared to the control plants.

3.9 The organic carbon content of the soil

The organic carbon content of the soil varied significantly across all treatments (Fig. 2). Compared to the control, all 3 biofertilizers similarly increased the organic carbon content. VAM was the most effective, resulting in a 32.43 % increase over the control, followed by *S. India* with a 24.32 % increase and *Azospirillum* with a 19.81 % increase. When applied alone, VAM increased the soil's organic carbon content by 1.47 %, which increased to 2.62 % when combined with biochar.

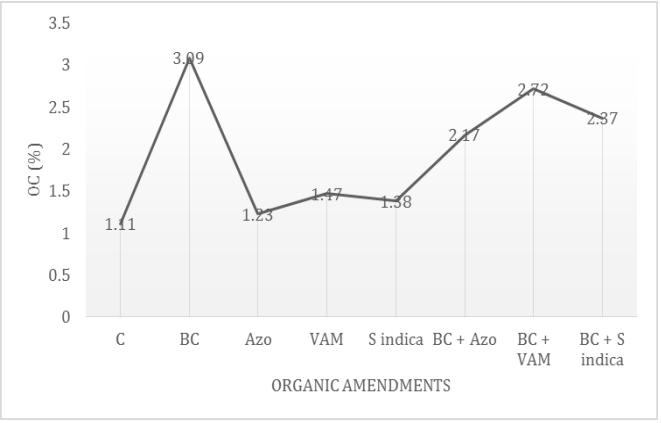

Fig. 2. Effect of biochar and biofertilizers on the organic carbon content of soil after the experiment. C- Control; BC- Biochar; Azo- *Azospirillum*; VAM- Vesicular Arbuscular Mycorrhizae; P indica- *Serendipita indica* ; BC + Azo - Biochar + *Azospirillum*; BC + VAM- Biochar + Vesicular Arbuscular Mycorrhizae; BC + P indica- Biochar + *Serendipita indica* . Values are the average of 3 replicates means. Mean ± standard error (SE) is indicated by error bars. (One-way ANOVA, Least Significant Difference test $p < 0.05$).

Additionally, biochar application significantly elevated the soil organic carbon content, with the highest level of 2.98 % recorded in the biochar treatment, representing a 160.36 % increase compared to the control soil.

3.10 Population of *Azospirillum*

The population of *Azospirillum* was recorded at the 50 % flowering stage and harvest. The 50 % flowering stage was determined by counting the days after transplanting (DAT) until 50 % of the plants had at least one open flower, which was observed to occur at 55 DAT. The bacterial population increased throughout the experiment from the 50 % flowering stage to the harvest stage (Table 4). A white subsurface pellicle was observed, indicating a positive result for the MPN test (Fig. 4).

Additionally, adding biochar significantly enhanced the *Azospirillum* population compared to the sole application of *Azospirillum* alone, resulting in increase of 40 % at the 50 % flowering stage and 40.74 % at harvest. At the 50 % flowering stage, the biochar + *Azospirillum* treatment (T6) recorded a population of 2.8×10^3 cfu/g, while the *Azospirillum* treatment alone (T3) reported 2.0×10^3 cfu/g. Furthermore, at harvest, the biochar + *Azospirillum* treatment (T6) recorded 3.8×10^3 cfu/g, compared to 2.7×10^3 cfu/g in the *Azospirillum* treatment (T3).

Table 3. Effect of biochar and biofertilizers on root volume and total dry matter of bell pepper var. California Wonder.

Treatment	Root volume(cm^3)*	Total dry matter (g)*
Control	1.83^{e}	24.63^{e}
Biochar	2.03^{e}	27.73^{d}
Azospirillum	2.43^{d}	31.37^{c}
VAM	2.80^{b}	34.10^{ab}
S. indica	2.70^{bc}	30.55^{c}
Biochar + *Azospirillum*	2.48^{cd}	32.31^{bc}
Biochar + VAM	3.10^{a}	36.15^{a}
Biochar + *S. indica*	2.77^{b}	31.78^{bc}
SEm(±)	0.09	0.859
CD (0.05)	0.272	2.598

* The values represent the average of 3 replicates mean. Statistical significance is indicated by letter differences between treatments (One-way ANOVA, Least Significant Difference test $p < 0.05$). Similar letters represent no significance, while a different letter is an indication of a significant difference, indicating the significance between them.

Table 4. Population of *Azospirillum* in rhizosphere soil of *Azospirillum* treatment and *Azospirillum* with biochar treatment.

Treatment	Population of *Azospirillum* (*10^3 cfu g^{-1})	
	50 % flowering (55 DAT)	Harvest
Azospirillum	2.0	2.7
Biochar + *Azospirillum*	2.8	3.8
Control	Not detected	Not detected

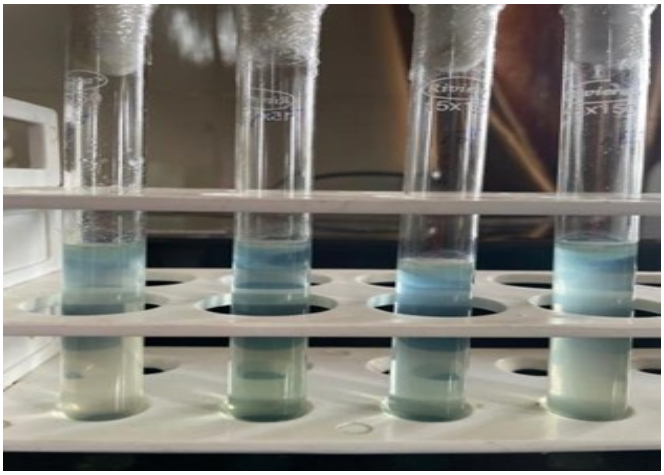

Fig. 4. *Azospirillum* culture tubes with white subsurface pellicles taken from soil of biochar + *Azospirillum* treatment.

3.11 VAM and *Serendipita indica* colonization

Root colonization percentages were recorded at the 50 % flowering stage and harvest (Table 5). Mycelia and vesicles were observed in the root samples (Fig. 3). At the 50 % flowering stage, VAM (T4) exhibited a root colonization rate of 68 %, while the biochar + VAM treatment (T7) achieved 72 % root colonization. In contrast, the uninoculated control plants showed only 30 % colonization. Similarly, VAM (T4) recorded an 80 % colonization rate at harvest and biochar + VAM (T7) reached 86 % colonization, while the uninoculated control plants exhibited 34 % colonization. A progressive decrease in root colonization percentage was noted from the 50 % flowering stage to the first harvest stage. However, biochar enhanced VAM colonization percentages in the roots by 7.5 % and 5.88 % at both stages respectively. Chlamydospore-like structures resembling *S. indica* were observed in the cortical region of the root samples (Fig. 5). However, this observed colonization was insignificant among the plants treated with *S. indica*.

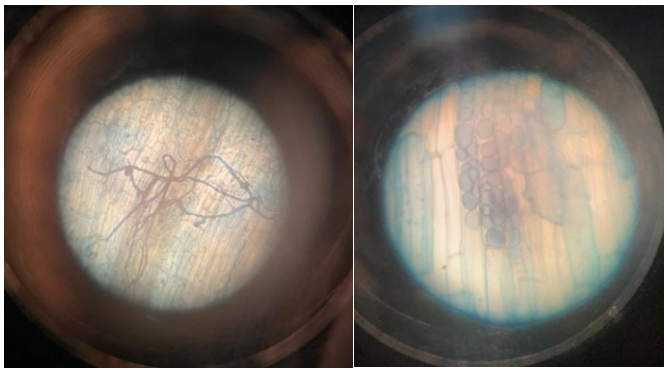

Fig. 3. VAM mycelia (40X) and vesicles (100X) in bell pepper root bits under biochar + VAM treatment.

Table 5. Root colonization percentage of VAM in plants grown under VAM treatment and VAM with biochar treatment.

Treatment	VAM colonization (%)	
	50 % flowering (55 DAT)	Harvest
VAM	80	68
Biochar + VAM	86	72
Control	34	30

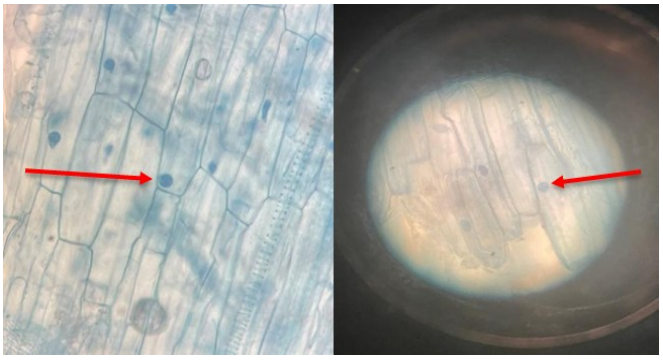

Fig. 5. Chlamydospores structure (100X) similar to *S. indicia* in bell pepper root bits.

Discussion

Biochemical parameters are essential indicators of photosynthetic activity, closely related to the growth status of vegetation. In the present study, the biochemical parameters recorded included flavonoid, polyphenol, beta carotene, total soluble proteins and ascorbic acid. All biochemical parameters, except for beta carotene were significantly influenced by the treatments applied.

Beta carotene content was not affected by the application of biochar and biofertilizers. Although biofertilizers enhance nutrient availability, the specific nutrients required for beta-carotene synthesis may already be sufficient in the soil, limiting the biofertilizers' impact. Additionally, the particular strains of microbes present in the biofertilizers may not directly influence beta-carotene synthesis or may compete with other soil microorganisms involved in carotenoid production. The effect of microbial inoculants on secondary metabolite content can vary significantly depending on the crop (20).

Biofertilizers and biochar significantly enhanced the flavonoid content in bell pepper fruits. In our study, treatment with *Azospirillum* increased flavonoid content, followed by VAM treatment. It was also found that a concentration of 1×10^{-8} cfu/mL of *Azospirillum* increased tomato flavonoid content (21). Additionally, research has shown that flavonoid content increased in cultivating *Capsicum annuum* with PGPR because the bacteria utilize quercetin and kaempferol as carbon sources (22). Furthermore, adding biochar alongside *Azospirillum* and VAM was observed to increase the flavonoid content of the fruits further. The variations in the physical and chemical properties of the feedstock may have improved growth conditions, thereby enhancing the content of secondary metabolites, which contributed to the higher expression of flavonoids in tomatoes cultivated in substrates treated with different biochars (23). Positive changes in the phenolic profile and flavonoid content were also reported in *Guadua angustifolia* following *Azospirillum* inoculation (24).

Polyphenols are becoming increasingly significant due to their beneficial effects on health. Higher concentrations of phenolic compounds were observed under biofertilizer treatments, which can be attributed to improved nutrient availability and enhanced enzymatic activity, including phenylalanine ammonia-lyase, 4-coumaryl-CoA and cinnamate 4-hydroxylase (25).

Following AMF inoculation, hydrogen peroxide, salicylic acid and nitric oxide levels increased in the clover roots, acting as signalling molecules that activate the phenylalanine ammonia-lyase (PAL) pathway. In addition to biofertilizers, biochar also contributed to increased polyphenol content (26). Factors beyond nutrition may be crucial in producing phenolic compounds responding to biochar addition (23). Moderate levels of biochar-borne compounds, such as benzoic acids, phenols and amides, may drive phenolic synthesis, potentially explaining the increase in phenolics observed with biochar.

Total soluble protein content in bell pepper fruits is a crucial indicator of plant physiological status, reflecting stress-related traits. The biofertilizers used in this study were equally effective in increasing total soluble protein content. The amount of nitrogen available in the environment, along with the use of growth-promoting microorganisms, significantly influences protein content. Inoculation with *Azospirillum* may enhance protein biosynthesis through direct nitrogen fixation or indirectly by promoting nitrate accumulation due to increased nitrate reductase activity. Arbuscular mycorrhizal fungi (AMF) assist in activating specific plant genes, accumulating essential nutrients required for various metabolically active compounds, and facilitating amino acid absorption, ultimately increasing protein content in the final product (27). Combining biochar and *Azospirillum* resulted in higher total soluble protein content than the control. The increased protein content is associated with optimal soil nutrient availability, improved plant nutrient uptake and efficient utilization of nutrients by the plants. Enhanced nitrogen use efficiency, facilitated by better soil conditions, enzymatic activities and the potential for nitrogen utilization, likely contributed to the increased soluble protein content (28).

Ascorbic acid serves multiple functions in plant cells, notably as a cofactor in redox reactions and for its antioxidant capacity. It enhances fruit quality and improves stress tolerance. The results of the present study indicated that treatment with *Azospirillum* resulted in fruits with higher ascorbic acid content, followed by VAM treatment. The increase in ascorbic acid levels can be attributed to the microbial inoculant's enhanced ability to fix atmospheric nitrogen and the secretion of growth-promoting chemicals that accelerate physiological processes, including carbohydrate synthesis. The contribution of VAM to this increase may stem from its role in enhancing phosphorus bioavailability. Phosphorus is crucial for the assimilation of photosynthetic carbohydrates and consequently, ascorbic acid synthesis (29).

Furthermore, higher levels of ascorbic acid were observed in the fruits of plants treated with *Azospirillum* plus biochar, with VAM combined with biochar following closely behind. Applying biochar at 10.5 t/ha increased vitamin C content in sweet peppers, likely due to improved water-holding capacity and the release of essential nutrients during its degradation (30). Biochar application enhances soil water retention, facilitating better water movement to the leaves. This increase in water availability positively influences plant metabolism and boosts the production of both primary and secondary metabolites. Additionally, biochar improves nutrient availability and uptake, impacting the plant's biochemical metabolism. Its application significantly influenced growth attributes, metabolic profiles and the antioxidant system in tomatoes (31). When combined with salicylic acid, biochar also improved the biochemical properties of soybean under saline conditions (32).

The increase in flavonoid content, polyphenol content, total soluble protein and ascorbic acid due to biochar and biofertilizer treatments contributes to enhanced antioxidant properties. The improvement in secondary metabolites strengthens plant defence mechanisms and overall health. Higher levels of these compounds can also improve fruits' colour and flavour profile, leading to better market value.

Statistical data analysis on days to first flowering and fruiting showed no significant variation among the treatments. This is likely due to the inherent genetic timelines for flowering and fruiting in different plant varieties, which may not be significantly altered by biochar or biofertilizer applications. Additionally, the timing of biochar and biofertilizer applications may not have coincided with critical developmental phases of the plants, reducing their impact on flowering and fruiting times. Previous studies reported similar findings (33, 34). However, plants treated with *S. indica* showed a noticeable reduction in the days required to produce the first flower and fruit. An explanation for this earliness could be the faster expression of flowering control gene transcripts. *S. indica*-colonized plants exhibited higher expression levels of the PHYA (phytochrome) and CRY1 (cryptochrome) genes. Several critical genes in the gibberellin pathway, such as GA20ox2 (gibberellin 20 oxidase 2), which are responsible for controlling flowering time, also showed significantly increased expression in *Arabidopsis* under the influence of *S. indica* (35).

The present study recorded parameters such as root volume and total dry matter and significant differences were observed. The results indicated that the fungal endophytes (VAM and *S. indica*) improved root characteristics more than the other treatments. As reported in tomato crops, these fungal symbionts form an integral part of the root system and interact with other microorganisms in the soil. Plants colonized by endophytes develop a more efficient carbon-use root system, which enhances nutrient absorption. Shoot weight was also higher in VAM-inoculated plants than in the control. The AMF-induced increase in photosynthate synthesis contributed to biomass accumulation below and above ground.

Additionally, the results of this study demonstrated that biochar had an augmentative positive effect on all biofertilizers tested. The co-inoculation of biochar and biofertilizers led to comparatively higher root and shoot weights. This finding is consistent with studies on chickpeas, where biochar was found to significantly and positively influence plant biomass (36). Long-term positive effects of biochar on maize biomass accumulation have also been reported (37).

Soil organic carbon (SOC) is a critical component of soil organic matter and is an essential indicator of soil health. SOC enhances soil aeration, drainage, water and nutrient retention capacity and supports microbial growth. In this study, all 3 biofertilizers were equally effective in increasing soil organic carbon content. Biofertilizers contribute to forming permanent humus compounds, providing evidence of increased soil organic matter stability. Additionally, the presence of extra-radical hyphae, fungal spores and bacterial populations in treated plants contributed to the improved organic carbon content of the soil (38).

It was also evident that biochar significantly elevated soil organic carbon (SOC) content. The higher photosynthetic rate in leaves and increased root biomass production resulting from biochar application may have contributed to this by promoting the translocation of photosynthates to below-ground biomass and enhancing root exudation, which in turn increases SOC (39). Additionally, biochar's stable structure and higher C ratio support the presence of more stable carbon fractions, which makes its carbon stock more resistant to microbial degradation (40). The greater degree of humification observed with biochar application indicates the presence of more stable aromatic carbon forms than easily degradable aliphatic forms. These findings are consistent with results from avocado fields (41) and a meta-analysis that reported a 64.3 % increase in total organic carbon content following biochar amendment (42).

Increasing organic carbon is crucial for maintaining healthy soils, as it enhances nutrient availability, conserves moisture and supports microbial activity. This promotes sustainable agriculture while contributing to environmental health through carbon sequestration and climate change mitigation.

The population of *Azospirillum* was observed to increase as the experiment progressed from the 50 % flowering stage to harvest. The results demonstrated that biochar enhanced the bacterial population in the soil. This effect may be attributed to the carbon fraction in biochar's labile pool, which provides additional carbon for biochemical activity, stimulating increased microbial respiration. This finding is further supported by reports indicating that biochar treatment promotes greater rhizosphere bacterial diversity, mainly due to the recalcitrant structure of biochar's backbone (43). Biochar also offers a suitable habitat for a diverse population of soil microorganisms, owing to its large surface area, as observed in forest soils (44). Applying biochar at various levels and *Azospirillum* greatly enhanced the total diazotroph population in the rhizosphere soil of maize fields (45). This can be explained by biochar's dual role as a source of nutrients and reduced carbon compounds and a habitat for soil bacteria like diazotrophs and *Azospirillum*. Additionally, biochar has been noted to positively affect the colony formation of *Azospirillum* and *Rhizobium* by regulating soil porosity (46).

Biochar enhanced the colonization percentage of VAM in roots at both stages of growth. Volatile compounds in biochar may alter the signalling dynamics in the soil and its increased water-holding capacity supports fungal colonization (47). Additionally, biochar's larger surface area and porosity provide a suitable habitat for AMF hyphae, which can utilize the biochar pores for colonization. These findings align with studies on cacao and pepper plants, where increased mycorrhizal colonization was observed with biochar addition (48, 49). The abundance of carbon substrates consumed by soil microbes also increased with higher biochar levels, contributing to greater genetic diversity and richness (50).

An enhanced soil microbiome through biochar application is crucial for maintaining healthy ecosystems. It improves agricultural productivity by promoting nutrient cycling, suppressing diseases and sequestering carbon, making it a cornerstone of soil health and environmental quality. The graphical representation of the percentage increase in the efficiency of VAM and *Azospirillum* when combined with biochar is shown in Fig. 6 and 7 respectively.

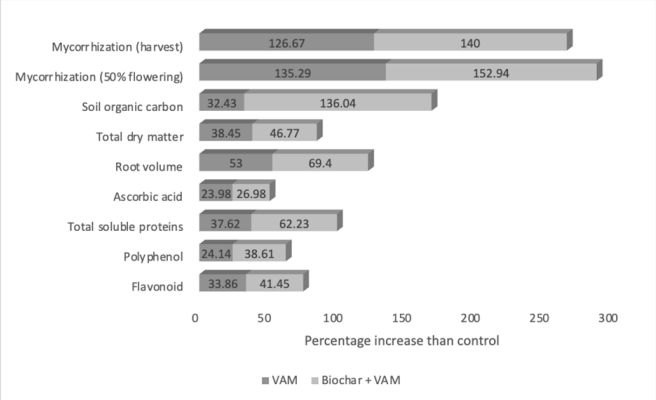

Fig. 6. Percentage increase in the efficiency of VAM when combined with biochar by taking control as the baseline.

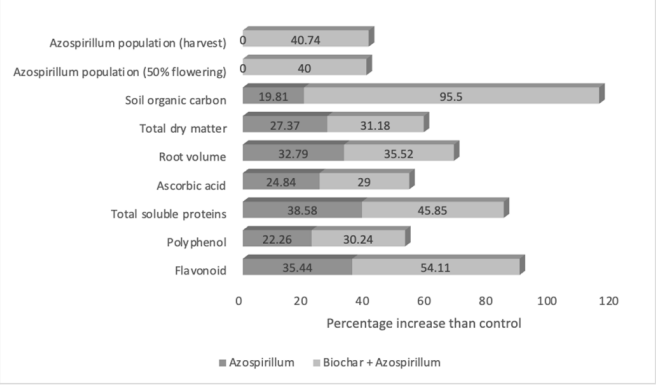

Fig. 7. Percentage increase in the efficiency of *Azospirillum* when combined with biochar by taking control as the baseline.

Conclusion

The present study demonstrated that biochar enhanced the effectiveness of biofertilizers in improving crop production and quality. Among the biofertilizers, VAM and *Azospirillum* were equally effective in promoting bell pepper's growth and biochemical parameters. Additionally, *S. indica* shortened the vegetative period and induced earlier flowering in bell pepper plants. Biochar application also increases the soil's organic carbon content. The population of *Azospirillum* in the rhizosphere and the root colonization percentage of VAM and *S. indica* were further enhanced with biochar application. Therefore, combining biochar and biofertilizers can significantly boost bell pepper plant performance, improving growth and fruit biochemical quality.

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Authors' contributions

VM conceptualized the study. SS performed the work. SS and VM carried out data analysis. VM, MR, AK, NS and BR provided technical guidance and assistance. All authors reviewed and approved the manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical issues: None

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