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Synergistic Bio-Organic Fertilization Enhances Tobacco Antioxidative Defense and Soil Health for Sustainable Agriculture

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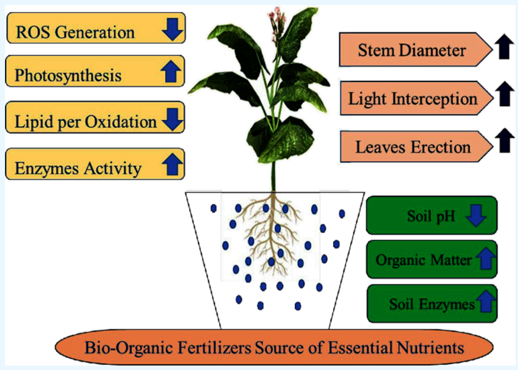
ABSTRACT: The extensive use of chemical/inorganic fertilizer application over the past few decades has significantly enhanced global food production potentials. However, the excessive application of these fertilizers has resulted in environmental issues, soil nutrient imbalances, and poor quality of food. The study aimed to evaluate the impact of various blends of bio-organic fertilizer, farm manure, and compound fertilizer on soil health, focusing on soil nutrient content, soil enzymatic parameters, physio-biochemical attributes, and quality traits of tobacco. The experimental treatments incorporated different combinations of conventional compound fertilizer with organic and bio-organic fertilizers. The findings revealed that the application of these bio-organic fertilizers with various combinations to the soil significantly improved tobacco growth, photosynthetic traits, antioxidant enzyme activity, and soil enzymatic activities. These amendments significantly improved the tobacco leaf quality by limiting the proline accumulation (62.17 and 77.31%) and malondialdehyde content (35.33 and 41.91%) at the reefing and flowering stages, respectively. Soil treated with a combination of bio-organic fertilizer, farm manure, and compound fertilizer (B_4) showed an increased soil enzyme activities with acid phosphatase improving by 19.91%, urease by 40.00%, and catalase activity by 7.41%, which results in enhanced soil nutrient status compared with other treatments. Based on these findings, it can be concluded that combined application of organic amendments resulted in better growth, improved antioxidative defense system, and improved quality of tobacco by reducing the use of compound fertilizer (10%) and activating soil enzymatic attributes, thereby boosting the tobacco productivity in agricultural systems.



1. INTRODUCTION

Soil pollution due to the use of chemical fertilizers is a significant environmental concern with far-reaching consequences.[1] Chemical or conventional fertilizers, while aiding in crop production and increasing agricultural yields, often lead to the contamination of soil ecosystems.[2] The excessive use of conventional fertilizers causes the accumulation of excess nutrients, particularly nitrates and phosphates, which can lead to nutrient imbalances in the soil.[3] Nutrient imbalances disrupt the natural soil ecosystem and can promote the growth of harmful algal blooms in nearby water bodies.[4] The continued use of chemical fertilizers may also alter soil pH levels, leading to increased acidity levels, which can negatively impact the soil microorganisms and reduce overall soil fertility. Furthermore, the accumulation of these fertilizer-derived chemicals in crops may pose health risks to humans if consumed in excess.[5]

Due to the soil health issues caused by chemical fertilizers, there are alternative methods that focus on sustainable, long-term food security while minimizing the negative environmental impacts.[6] Replacing chemical fertilizers with organic alternatives in intensive crop cultivation offers significant benefits for agricultural sustainability and the environment.[7] Organic fertilizers, produced through the biological decomposition of natural materials, improve soil health by enhancing organic matter, soil structure, water retention, and activity of beneficial microorganisms.[8] They release nutrients gradually during decomposition, reducing the chances of nutrient runoff and leaching into groundwater.[9] Unlike chemical fertilizers, organic fertilizers do not contain synthetic chemicals that could harm the environment.[10] Organic fertilizers contain essential nutrients, such as nitrogen, phosphorus, and potassium; organic fertilizers provide a steady supply of nutrients as they are slowly released into the soil. Organic fertilizers, derived

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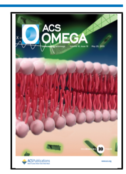

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from natural sources such as compost, animal manure, and plant residues, not only provide vital nutrients but also improve soil structure, water retention, and microbial activity. Farm manure, a traditional organic fertilizer, enriches the soil with organic matter, enhancing soil fertility and promoting healthy plant growth. Bio-organic fertilizers, enriched with beneficial microorganisms, further enhance soil fertility, nutrient cycling, and pest resistance, fostering a balanced soil ecosystem and healthier plant growth. Additionally, beneficial microbes such as *Purpureocillium lilacinum* and Burkholderiaceae play crucial roles in soil health and enhancing plant resilience to stressors.[11] Similarly, the nutrient sources have a direct impact on the soil enzyme activities as they serve as dependable indicators for assessing the impacts, whether beneficial or detrimental, of agro-ecosystems on crop productivity.[12] Numerous studies have shown that organic fertilizers can enhance soil enzyme activities, such as invertase, urease, and catalase. However, the specific enzymes affected may vary depending on the specific conventional or bio-organic fertilizer utilized and the availability of nutrients.[13,14] Similarly, crops grown with organic fertilizers tend to be more resilient to stressors such as drought and pests.[15] This is because organic matter in the soil supports a diverse community of beneficial microorganisms that can help protect plants from diseases.[16] The chemical composition of the soil, environmental conditions, and specific crop being cultivated are crucial factors for optimizing the use of organic fertilizers to achieve the best agricultural outcomes. Numerous studies have evaluated the effects of organic, bio-organic, and synthetic fertilizer in a sole form on the tobacco crop and soil health.[17,18] However, there is limited research on the combined effect of organic, bio-organic, and synthetic fertilizer on tobacco productivity and soil health is limited.

Implementing proper management techniques and application methods is essential for maximizing the benefits of organic fertilizers in crop yield. Soil health, as reflected in the nutrient status and enzymatic activities, can be used to assess changes in soil quality. Incorporating these fertilizers into agricultural practices promotes sustainable soil management, reduces dependence on synthetic chemicals, and supports healthier ecosystems ensuring long-term agricultural viability.[19]

Tobacco holds significant economic value and is widely cultivated across various regions despite differing soil and climatic conditions. The time of harvesting, determined by the maturity period, plays a crucial role in influencing the yield and quality of tobacco leaves.[20] The demand for organic tobacco is increasing as more consumers and producers recognize its advantages over conventional tobacco. Organic tobacco cultivation is becoming popular because it contains lower concentrations of harmful substances, making it a potentially healthier option for consumers. Additionally, organic farming practices enhance soil fertility by utilizing organic fertilizers, which improve the soil structure and nutrient content.[21] Increasing biodiversity, lowering groundwater contamination, cutting production costs (using fewer inputs), and ultimately raising the product's selling price are the major benefits of organic farming. Moreover, the quality traits of tobacco such as its aromatic substances are affected by the organic carbon within organic matter.[22,23]

Overall, transitioning from chemical to organic fertilizers is a good step toward farming that is sustainable and beneficial for the environment.[24] Tobacco can be grown organically on a significant scale, this aspect is crucial, particularly considering the recurring debate about the feasibility of achieving yields comparable to conventional agriculture.[25] It was hypothesized that the prolonged use of inorganic fertilizer would lead to deterioration of soil health by reducing soil nutrients and enzymatic activity, ultimately affecting the overall quality of tobacco. In contrast, the application of bio-organic fertilizers would be more beneficial in enhancing soil enzyme activity and improving the quality of tobacco plants. The objective of this study was to examine the effects of different blends of bio-organic fertilizer, farm manure, and compound fertilizer on soil health, focusing on soil nutrient and soil enzymatic parameters, physio-biochemical attributes, and quality attributes of tobacco. The study aimed to explore the potential for substituting and reducing the use of chemical fertilizers traditionally employed in tobacco cultivation, highlighting the importance of soil fertility and the need for appropriate nutritional strategies.[30,31]

2. MATERIALS AND METHODS

2.1. Study Area, Experimental Treatments, and Design. In Kunming City, Yunnan Province, China, a field trial was conducted between May and October 2022. The experimental site' s coordinates are 102.783° N and 25.338° E, positioned at an elevation of 2100 m above sea level. The area typically experiences an average annual temperature of 14.0 °C and receives an average yearly rainfall of 1100 mm. Before the sowing of the crop, the physiochemical attributes of the soil were determined using the standard protocols. The **soil various bio-organic fertilizers were eva**luated in comparison with the commercial fertilizer applied as ground fertilizer (soil application). The description of experimental treatments is given in Table 1. **Organic Fertilizer: Comprising a mi**nimum of 5% $N+P_2O_5+K_2O$, with an organic matter content of at least 50% and a moisture level not exceeding 30%, *Purpureocillium lilacinum* and Burkholderiaceae are microbial powders containing an effective count of viable bacteria of no less than 1 × 1010 CFU/g. These two microbial powders are to be blended with the aforementioned organic fertilizer in a weight ratio of 1:100 to prepare bio-organic fertilizer. These fertilizers were supplied by Kunming Cuntu Agriculture Technology Co., Ltd. The compound fertilizer contains 47% active ingredients with a ratio of $N:P_2O_5:K_2O$ of 15:8:24 is provided by Yunnan Huayye Investment Co., Ltd. Farm manure is tailored to tobacco farmers. Potassium nitrate fertilizer (KNO_3), with a total nitrogen content of at least 13.5%, potassium oxide of at least 44.5%, and chloride ion content not exceeding 1.2%, is sourced from Yunnan Ouroham Fertilizer Technology Co., Ltd. Potassium sulfate (K_2SO_4) with a water-soluble potassium

Table 1. Description of Experimental Treatments

notations	treatments
B_1	compound fertilizer containing 50% active ingredient at 300 kg ha^{-1}
B_2	organic fertilizer (containing $N+P_2O_5+K_2O \geq 5\%$, organic matter $\geq 45\%$, water content $\leq 30\%$) 1500 kg ha^{-1} + compound fertilizer (containing 47% active ingredient) 240 kg ha^{-1}
B_3	bio-organic fertilizer (contain *Purpureocillium lilacinum*, *Burkholderiaceae* $\geq 1\times 10^8$ CFU/g) 300 kg ha^{-1} + organic fertilizer 1200 kg ha^{-1} + compound fertilizer containing 47% active ingredient, $N:P_2O_5:K_2O$ = 15:8:24 at 240 kg ha^{-1}
B_4	bio-organic fertilizer 300 kg ha^{-1} + farm manure (dead branches and withered leaves for six months7500 kg ha^{-1} + compound fertilizer containing 50% active ingredient 240 kg ha^{-1}

oxide content of at least 52%, a sulfur content of at least 17%, and a chloride content not exceeding 1.5% is acquired from SDI Xinjiang Lop Nur Potassium Salt Co., Ltd. The experiment was designed by using a randomized complete block design (RCBD), replicated three times.

2.2. Crop Husbandry. The field experiment involved plots measuring 60 m each, accommodating 60 plants per plot. The spacing was maintained at 1.2 m between rows and 0.7 m between individual plants. Each treatment was replicated three times, resulting in a total of 12 plots. The specific tobacco cultivar used was Yunyan 87, obtained from Yunnan Tobacco Company. The seeds underwent approximately 60 days of growth in floating polystyrene trays within a greenhouse before being transplanted to the field. All the bio-organic treatments were applied as ground fertilizers according to the treatment plan. Besides that, additional fertilizer in the form of KNO_3 = 150 kg ha^{-1} and K_2SO_3 = 225 kg ha^{-1} was also applied at the time of sowing. Uniform planting density was maintained across all treatments, and both treatments and their repetitions were randomly dispersed throughout the field. Supplementary cultivation procedures were implemented in Kunming, Yunnan Province, China, to meet the technical standards for high-quality tobacco production.

2.3. Data Collection. *2.3.1. Morphological Attributes.* When the tobacco plant entered the flowering period, **topping was performed, which involves removal of the top of the pla**nt to control its growth. As a result, the plant's height and number of leaves remained unchanged from that point onward, typically around 90 days after transplanting. All morphological attributes of the tobacco plant were measured and recorded at this stage as the plant had reached its final growth. Plant height was determined by measuring its height from the soil surface using a meter rod, while leaf length, leaf width, stem girth, and stem diameter were measured using measuring tape. The number of leaves was documented throughout the harvesting stage. The morphological traits were evaluated according to the parameters specified in the document "Investigating and Measuring Methods of Agronomical Character of Tobacco", in accordance with the Tobacco Industry Standard of the People's Republic of China YC/T142-2010.

2.3.2. Photosynthetic Attributes. For each treatment, 1−2 g of young leaves was sampled at two stages: the reefing stage (60 days after transplanting) and the flowering stage (90 days after transplanting) in triplicate. To extract pigments, 5 g of plant leaf samples were crushed in test tubes containing 85% acetone (*v*/*v*) and kept in the dark for 24 h. Subsequently, centrifugation was carried out for 10 min at 4000×*g* at 4 °C. The concentrations of chlorophyll a, chlorophyll b, and carotenoids were determined by taking readings of the supernatant at wavelengths of 470, 647, and 664.5 nm using a spectrophotometer (Halo DB-20/DB-20S, UK), following the procedure described by Lichtenthaler (1987).[33] The total chlorophyll content was estimated by summing the quantities of chlorophyll a and chlorophyll b.

2.3.3. Enzymatic Antioxidants and Lipid per Oxidation Attributes. Understanding the activity levels of SOD, POD, and CAT enzymes provides valuable insights into the antioxidant defense system of plants and their ability to cope with oxidative stress, thereby forming strategies for improving plant resilience and productivity. Young leaves (1−2 g) were sampled (at eight leaves) after 60 and 90 days after transplanting, and samples were taken in triplicate. To assess enzyme activity, the supernatant was obtained from a leaf sample weighing 1 g, extracted using 50 mM phosphate buffer, and then centrifuged for 10 min at 15,000×*g*. The activities of peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) were determined following the procedures outlined by Velikova et al. (2000)[34] and Beauchamp and Fridovich (1971),[35] respectively. Malondialdehyde activity (MDA) was assayed using the thiobarbituric acid reaction method.[29,36]

2.3.4. Osmolyte Attributes. A fresh leaf sample weighing 0.5 g taken after 60 and 90 days after transplanting was grounded with a pH 7.2 buffer. Protease inhibitors at a concentration of 1 μM were added to a saline phosphate buffer to create a consistent mixture. For the saline buffer solution, 1.37 mM NaCl, 2 mM KH_2PO_4, 2.7 mM KCl, and 10 mM Na_2HPO_4 were dissolved in 1 L of deionized water, with pH adjustment using HCl, followed by autoclaving. The extract was then centrifuged at 12,000×*g* for 5 min to separate the supernatant. The determination of proline content was conducted using the method published by Maehly and Chance (1954),[37] while the assessment of soluble sugars and soluble proteins was performed using the methodologies indicated by Giannakoula et al. (2008)[38] and the Bradford test (Bradford, 1976),[39] respectively.

2.3.5. Plant Defense Enzyme Attributes. The leaf samples collected at the reefing and flowering stages were cut into 5 mm pieces and ground using a mortar and pestle in liquid nitrogen. The activity of polyphenol oxidase (PPO) was assessed following the method outlined by Jockusch (1966),[40] while phenylalanine ammonia lyase (PAL) activity was determined according to the procedure detailed by Okey et al. (1997).[41]

2.3.6. Leaf Quality Attributes. At the curing stage, for each treatment, 2−4 g of leaves were sampled in the triplicate form. About 1.0 g of leaf sample was collected and frozen in liquid nitrogen. The alkaloid content was extracted from dried samples and analyzed using gas chromatography−mass spectrometry (GC/MS) following the method outlined in Goossens et al. (2003),[42] employing nicotine (Sigma-Aldrich) as the internal standard. Total and reducing sugar concentrations were determined according to the procedure described by Somogy (1952).[43] For potassium (K) and chloride (Cl) concentration determination, the leaf samples were oven-dried, weighed, and ashed at 550 °C for 8 h in a muffle furnace. Flame photometry (PFP7, Jenway, UK) was utilized to determine K concentration, while Cl concentration was assessed via titration with $AgNO_3$ following Jaiswal (2004).[44] The total nitrogen (N) content was determined by digesting a sample of approximately 0.3 g and performing distillation and titration using the semimicro Kjeldahl method.[38,45]

2.3.7. Soil Enzymes and Soil Chemical Attributes. After harvesting, soil samples weighing 100 g were collected from a depth of 0−15 cm across each treatment in triplicate. Immediately after collection, the samples were air-dried at room temperature (21 °C). Subsequently, paper bags were used to store the dried samples until further analysis. By preparing water/soil suspensions in a 2:1 ratio, following the procedures outlined by Ryan et al. (2001),[46] soil pH and electrical conductivity (EC) were determined. According to GB9834-1988, method soil organic matter (OM) content was measured, while using flame atomic absorption spectrophotometry (method GB 9836-1998), available potassium (AP) was determined. The sodium hydrogen carbonate solution-Mo-Sb antispectrophotometric method (HJ/T 704-2014) was used to measure soil available phosphorus (AP). Hydrolytic

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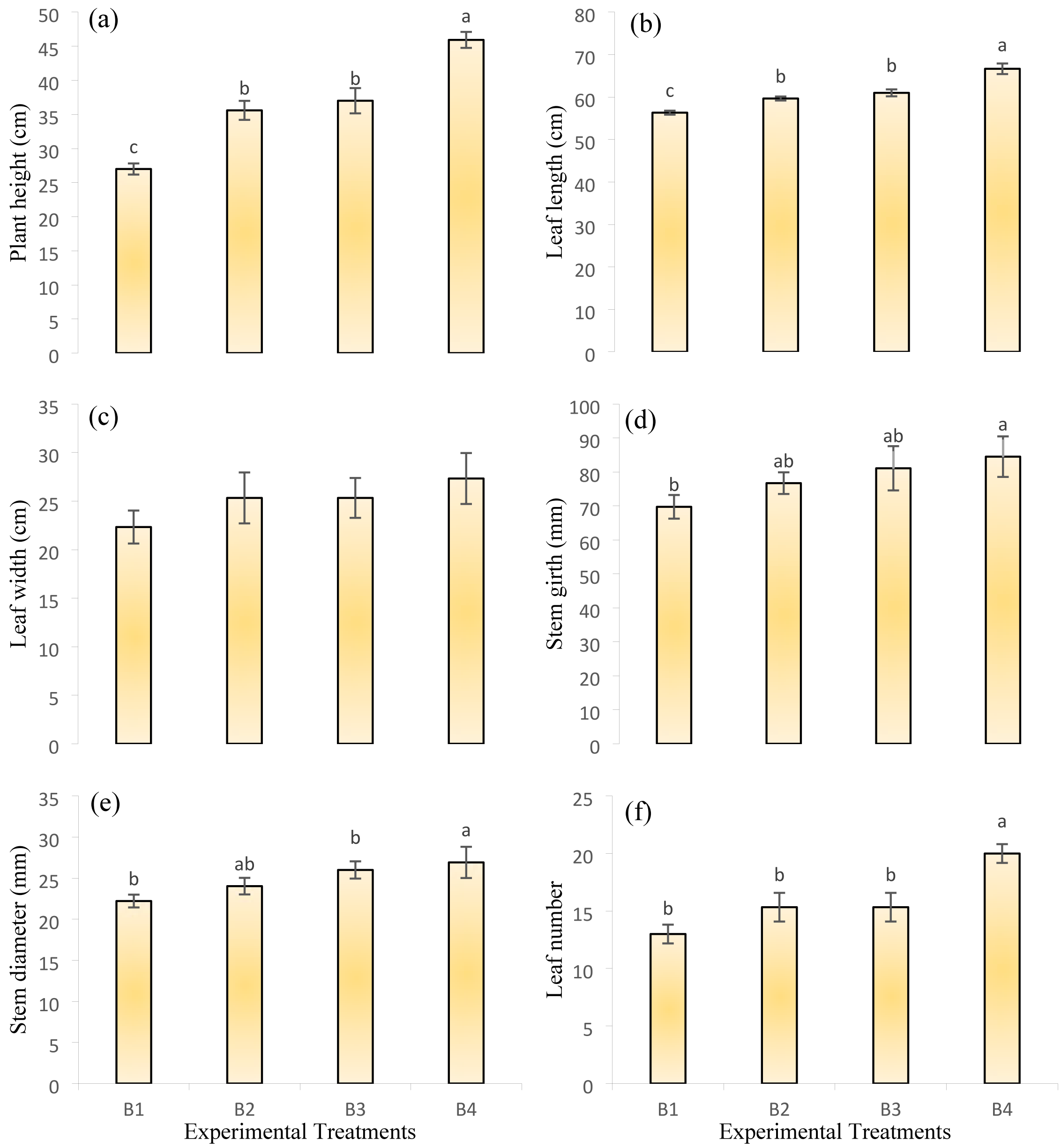

Figure 1. Effect of various bio-organic fertilizers on the morphological attributes of tobacco. The statistics illustrate the means with standard deviation (SD) and were replicated three times, the letters in the bars indicate significant differences between treatment means at $p \leq 0.05$ using a least significant difference test (LSD), and figures without lettering showed nonsignificant response: (a) plant height, (b) leaf length, (c) leaf width, (d) stem girth, (e) stem diameter, and (f) leaf number.

nitrogen was quantified using the Kjeldahl method, following the procedures detailed by the Institute of Soil Science, Chinese Academy of Sciences (Nanjing, China) in the Procedures for Soil Analysis, 2002. The hydrolyzed nitrogen in the soil samples was analyzed using the procedures established by Yunnan Sanbiao Agriculture and Forestry Technology Co., Ltd. in China.

The soil enzymes' activity was assessed in each rhizosphere soil sample using a TECAN Infinite M200 spectrophotometer (G10S UV–vis, Thermo Fisher, USA) in conjunction with methylumbelliferone (MUB)-linked model substrates, utilizing fluorometric methods. The activities measured in this study were soil acid phosphatase, soil urease (following the method described by Emami et al., 2013),[47] soil cellulase (according to the procedure outlined by Kizilkaya et al., 2012),[48] soil sucrase, and soil catalase activities (as per the method described by Allison and Jastrow, 2006).[49] The detection wavelengths were configured to be 450 nm for S-CAT and 365 nm for the other enzymes. The estimate of aryl-acylamidase was conducted according to the techniques described by Nakamura et al. (1990),[50] utilizing acetanilide (AAN) as the substrate. The protocols for ß-1,4-glucosidase were modified from Dick (2011),[51] with color intensity measured at a wavelength of 400 nm. Soil dehydrogenase enzymatic activity was evaluated using the methods outlined by Min et al. (2001).[52]

2.4. Statistical Analysis. An analysis of variance (ANOVA) was conducted on the data set to explore potential statistically significant differences among the bio-organic fertilizer treatments. The bars represent the treatment means with standard deviation (SD) and were replicated three times; the letters in the bars indicate significant differences between treatment means at $p \leq 0.05$ using the least significant

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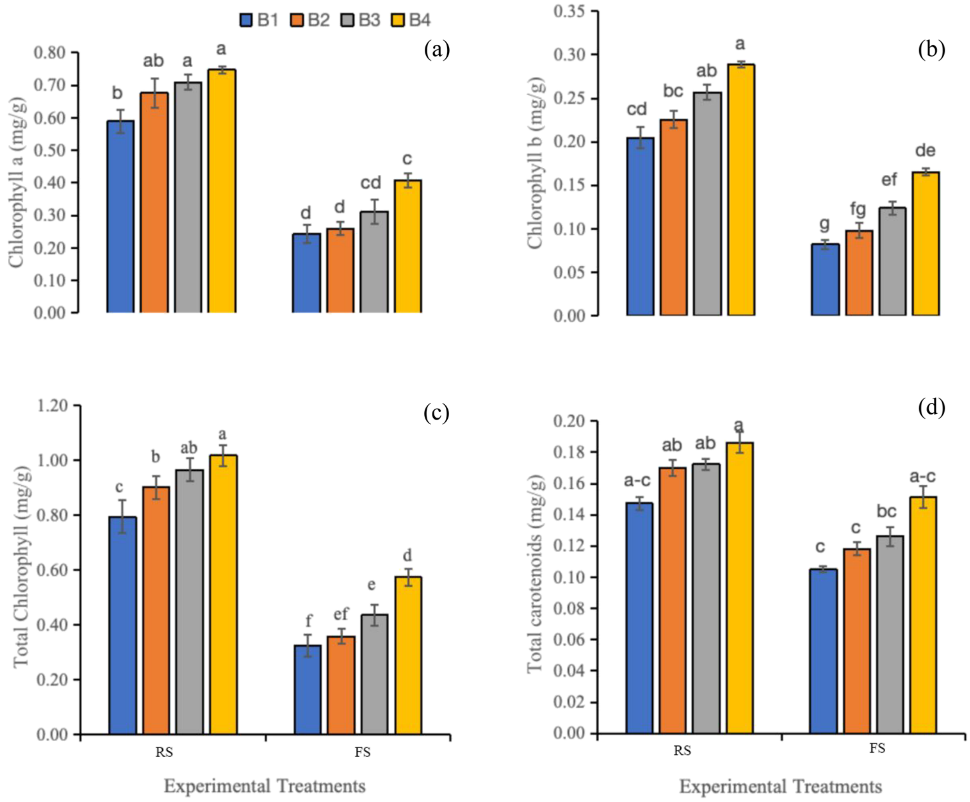

Figure 2. Effect of various bio-organic fertilizers on the photosynthetic attributes of tobacco. The statistics illustrate the means with standard deviation (SD) and were replicated three times, the letters in the bars indicate significant differences between treatment means at $p \leq 0.05$ using a least significant difference test (LSD), and figures without lettering showed nonsignificant response; RS = reefing stage and FS = flowering stage; (a) chlorophyll a, (b) chlorophyll b, (c) total chlorophyll, and (d) total carotenoids.

difference test (LSD). Pearson's correlation analysis was employed to examine the relationships among the variables. The statistical software package Statistics 8.01 was used to analyze the data. Principal component analysis and correlation analysis were computed using Minitab-19.

3. RESULTS

3.1. Morphological Attributes. The addition of bio-organic fertilizer in various combinations significantly ($p \leq 0.05$) affected the morphological attributes of tobacco plants (Figure 1a–f). Various forms of bio-organic fertilizer improved the morphological attributes in comparison with control. The addition of bio-organic fertilizer combined with farm manure and compound fertilizer (B_4) increased the plant height (70.11%), leaf length (18.35%), leaf width (22.39%), stem diameter (21.15%), stem girth (21.17%), and leaf number (53.84%) as compared to the control where only compound fertilizer was used (B_1). The treatment B_3 with bio-organic fertilizer, organic fertilizer and compound fertilizer, proved to have a better response as compared to B_2. The decreasing pattern in terms of all of the morphological attributes was noted as $B_4 > B_3 > B_2 > B_1$.

3.2. Photosynthetic Attributes. Various combinations of bio-organic fertilizers at various growth stages significantly improved the synthesis of photosynthetic pigments in tobacco over control treatment (Figure 2a–d). The reefing stage proved to have the maximum accumulation of photosynthetic attributes compared to the flowering stage. However, it was noted that treatment B_4 had a considerable impact on the improvement of chlorophyll content. The reefing stage of the tobacco plants proved the highest photosynthetic attributes as compared to the flowering stage. The B_4 treatment increased the content of Chl a (27.11 and 70.83%), Chl b (45 and 112.80%), total Chl (29.11 and 78.12%), and carotenoids contents (26.66 and 50.00%) at the reefing and the flowering stages respectively, as compared to the control.

3.3. Enzymatic Antioxidants and Lipid Peroxidation Attributes. The data depicted in Figure 3a–d represent the impact of various combinations of bio-organic, organic, and compound fertilizers on the enzymatic antioxidants and lipid peroxidation attributes of tobacco at various growth stages. Application of bio-organic fertilizer decreased the enzymatic antioxidants and lipid peroxidation parameters. Except for peroxidase activity, all the other enzymatic antioxidants and lipid peroxidation attributes showed better responses at the flowering stage as compared to the reefing stage. The treatment B_4 decreased the catalase activity (46.55 and 35.04%), superoxide dismutase activity (17.62 and 19.95%), peroxidase activity (42.62 and 37.98%), and malonaldehyde contents (35.33 and 41.91%) in comparison with the control at the reefing and flowering stages, respectively. The treatment B_3 with bio-organic fertilizer, organic fertilizer, and compound fertilizer proved to have a better response as compared to B_2.

3.4. Plant Defense Enzyme Attributes. Data on plant defense enzymes shown in Figure 4a,b exhibited that both PPO and PAL activities showed better responses at the flowering stage as compared to the reefing stage. However, the addition of various treatments of bio-organic fertilizers also improved the activities of plant defense enzymes as compared to control. The treatment B_4 enhanced the PPO (225.09 and

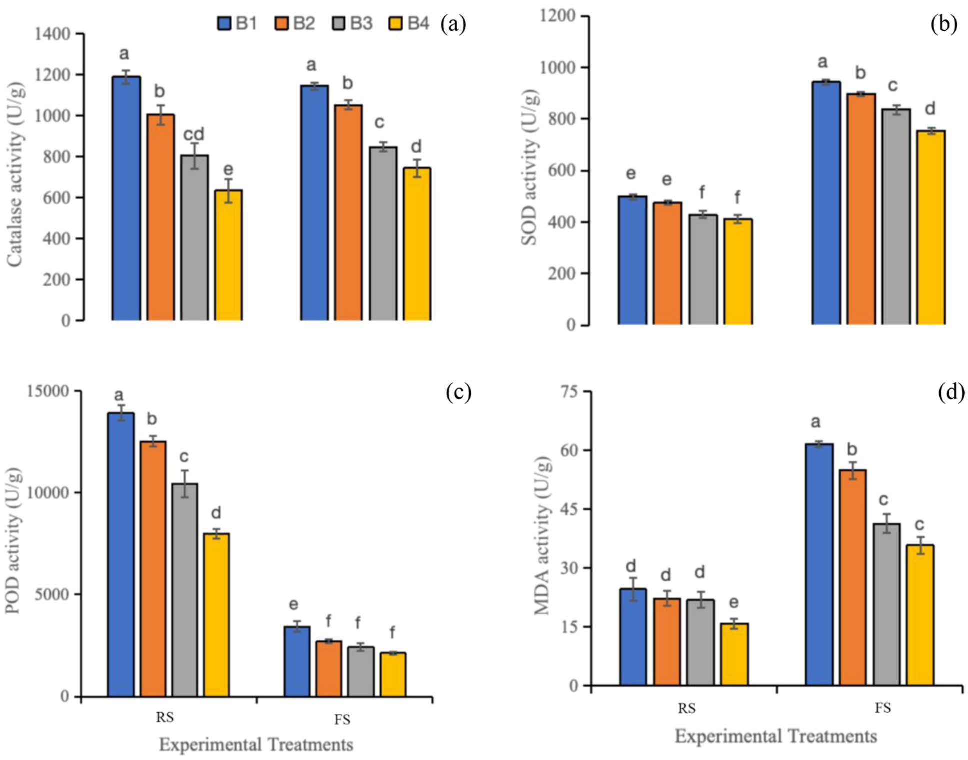

Figure 3. Effect of various bio-organic fertilizers on the enzymatic and lipid peroxidation attributes of tobacco. The statistics illustrate the means with standard deviation (SD) and were replicated three times, the letters in the bars indicate significant differences between treatment means at $p \leq 0.05$ using a least significant difference test (LSD), and figures without lettering showed nonsignificant response; RS = reefing stage and FS = flowering stage; (a) catalase activity, (b) SOD activity, (c) POD activity, and (d) MDA activity.

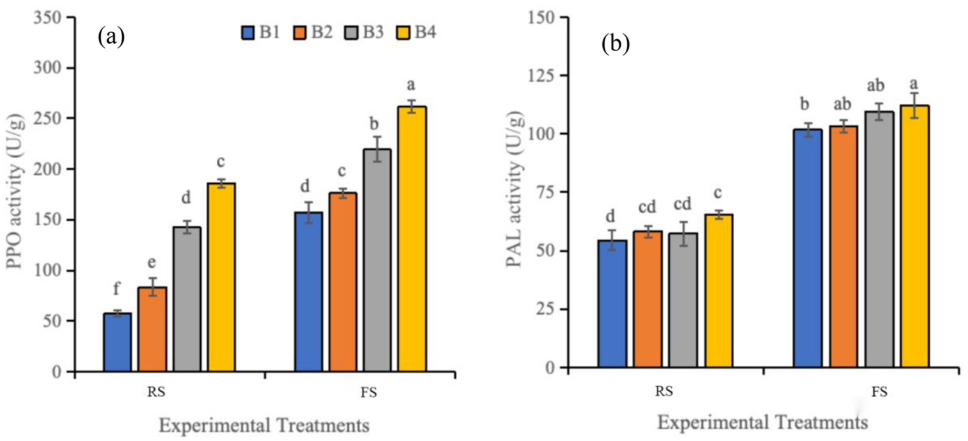

Figure 4. Effect of various bio-organic fertilizers on the plant defense enzyme attributes of tobacco. The statistics illustrate the means with standard deviation (SD) and were replicated three times, the letters in the bars indicate significant differences between treatment means at $p \leq 0.05$ using a least significant difference test (LSD), and figures without lettering showed nonsignificant response; RS = reefing stage, FS= flowering stage; (a) PPO activity, (b) PAL activity.

66.64%) and PAL activities (20.23 and 10.17%) compared to B_1.

3.5. Osmolyte Attributes. The tobacco plants subjected to various combinations of bio-organic fertilizers, organic fertilizer, and compound fertilizers showed a linear decrease in the osmolyte attributes. More accumulation of osmolyte attributes was noticed at the flowering stage as compared with the reefing stage. The B_4 treatment showed an increase in soluble sugars (44.73 and 99.53%), soluble proteins (59.96 and 18.29%), and a decrease in proline accumulation (62.17 and 77.31%), at the reefing and flowering stages, respectively (Table 2).

3.6. Leaf Quality Attributes. The addition of bio-organic fertilizer in various combinations affected ($p \leq 0.05$) the leaf quality attributes of tobacco plants (Figure 5a−f). Various treatments of bio-organic fertilizer increased the quality attributes by limiting the chloride ion accumulation as compared to the control. The addition of bio-organic fertilizer combined with farm manure and compound fertilizer (B_4) increased the total sugar (35.13%), reducing sugars (45.61%), total nitrogen (17.43%), total alkaloids (50.34%), total

Table 2. Effect of Various Bio-Organic Fertilizers on the Osmolyte Attributes of Tobacco

stages	treatments	soluble sugars (mg g^{-1})	soluble protein (mg g^{-1})	proline (μg g^{-1})
reefing stage	B_1	20.52 ± 1.55 g	46.01 ± 3.11 d	730.19 ± 54.76 b
	B_2	25.52 ± 1.64 f	49.88 ± 4.83 d	665.54 ± 62.74 bc
	B_3	25.50 ± 1.89 f	50.12 ± 2.96 d	628.99 ± 47.14 c
	B_4	29.70 ± 1.73 e	73.60 ± 3.16 a	276.21 ± 13.80 e
flowering stage	B_1	43.35 ± 0.63 d	52.36 ± 2.87 cd	979.88 ± 43.43 a
	B_2	51.78 ± 2.33 c	58.38 ± 2.02 bc	646.86 ± 16.50 c
	B_3	56.28 ± 2.54 b	59.86 ± 2.48 b	451.10 ± 12.45 d
	B_4	86.50 ± 2.59 a	61.94 ± 2.64 b	222.33 ± 15.21 e
LSD value ($p \leq 0.05$)		**4.15**	**6.59**	**81.74**

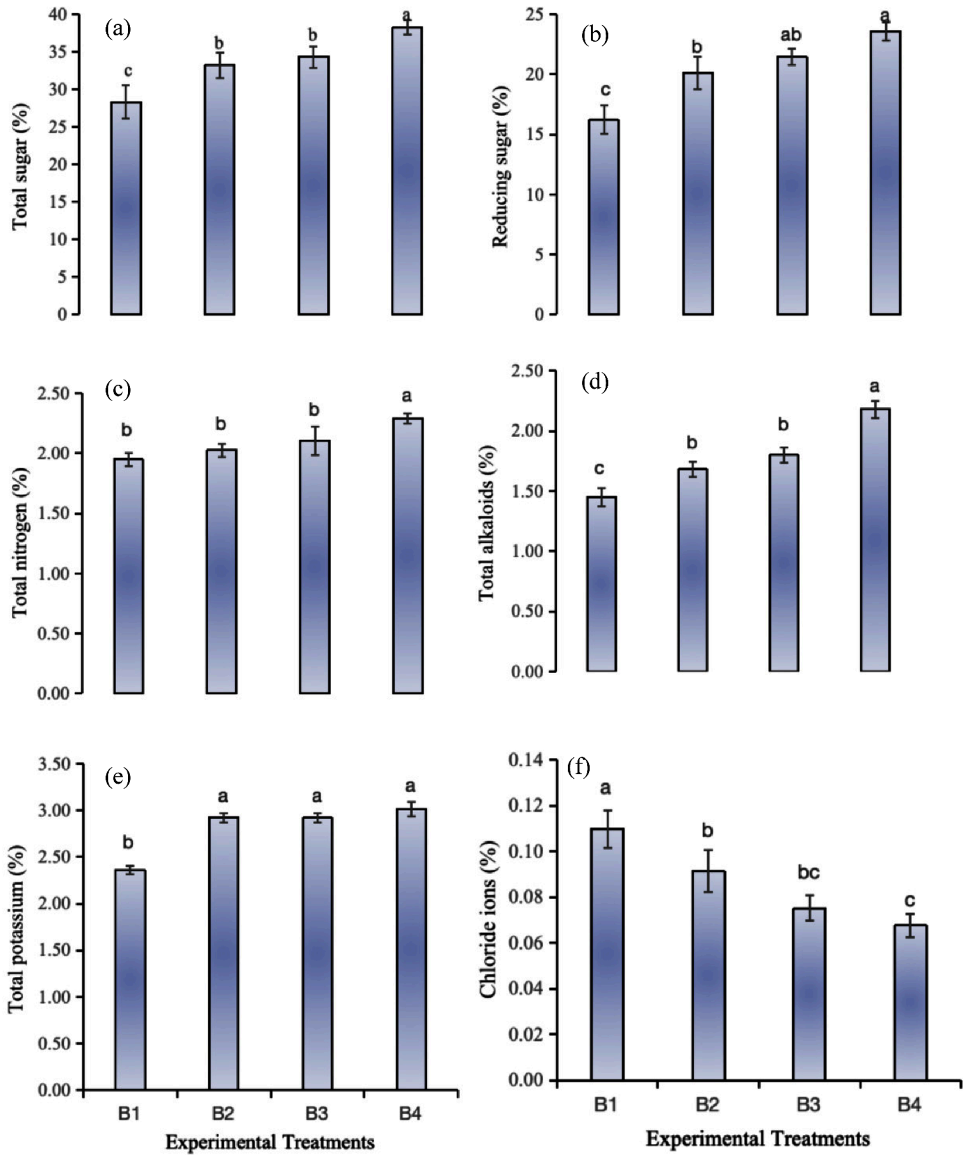

Figure 5. Effect of various bio-organic fertilizers on the leaf quality attributes of tobacco. The statistics illustrate the means with standard deviation (SD) and were replicated three times, and the letters in the bars indicate significant differences between treatment means at $p \leq 0.05$ using a least significant difference test (LSD); (a) total sugar, (b) reducing sugar, (c) total nitrogen, (d) total alkaloids, (e) total potassium, and (f) chloride ions.

potassium (27.54%), and decreased the chloride contents (36.36%) as compared to B_1. The treatment B_3 proved to have a better response compared to B_2.

3.7. Soil Physico-Chemical Attributes. The findings of the current study revealed that utilizing bio-organic fertilizers in combination with various amendments led to enhancements in soil nutrient status and other soil indicators (Figure 6a–f). The application of soil-applied bio-organic fertilizers resulted in a notable decrease in electrical conductivity by 45.28% and a slight shift toward a more neutral pH by 4.21%. The treatment

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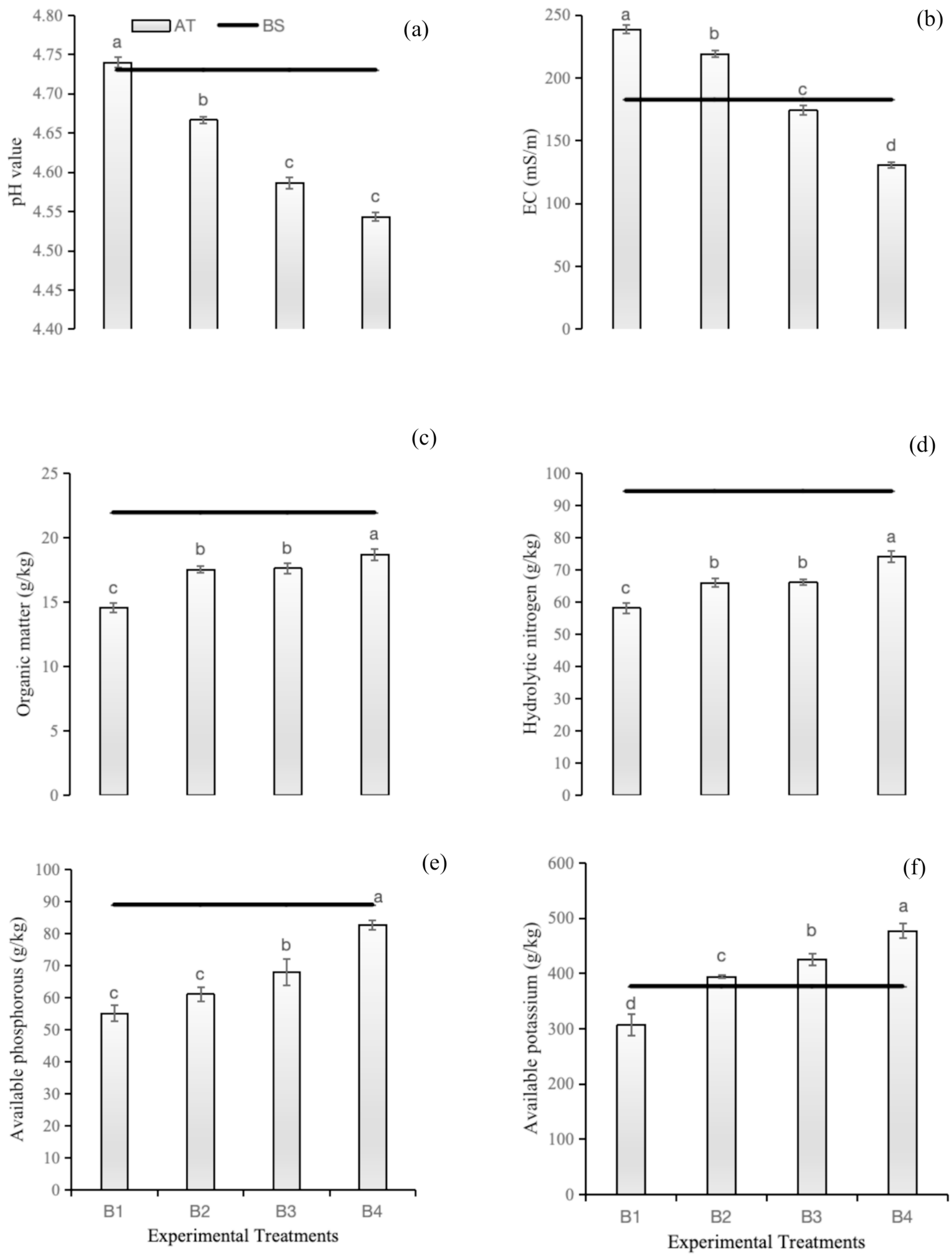

Figure 6. Effect of various bio-organic fertilizers on the physico-chemical attributes of soil under tobacco cultivation. The statistics illustrate the means with standard deviation (SD) and were replicated three times, and the letters in the bars indicate significant differences between treatment means at $p \leq 0.05$ using a least significant difference test (LSD); AT = At harvesting, BS = Before sowing; (a) pH value, (b) electrical conductivity, (c) organic matter, (d) hydrolytic nitrogen, (e) available phosphorus, and (f) available potassium.

B_4 showed the most significant improvements, the maximum increase in organic matter by 28.48%, hydrolyzable nitrogen by 27.35%, available phosphate by 50.03%, and available potassium by 55.52% compared to the B_1 treatment.

3.8. Soil Enzymatic Indices. The combined use of bio-organic fertilizer, compound fertilizer, and organic fertilizer in tobacco fields showed a consistent and noteworthy linear increase in soil enzymatic attributes (Figure 7a–h). Among these, the treatment involving B_4 exhibited the most substantial improvements: β-glucosidase increased by 338.76%, dehydrogenase increased by 129.28%, cellulase increased by 18.38%, sucrase increased by 6.31%, acid phosphatase increased by 19.91%, urease increased by 40.00%, aryl-acylamidase increased by 52.26%, and catalase increased by 7.41% compared to the B_1 treatment.

3.9. Principal Component Analysis. The PCA1 and PC2 accounted for about 89% of the variability of the determined variables (Figure 8). The flowering stage is associated with peroxidase activity, chlorophyll (a and b), and carotenoid concentrations, and the harvesting stage showed a close association with the measured parameters of soluble sugars, malonaldehyde, superoxide dismutase activity, PAL, and PPO. The parameters of catalase activity, proline accumulation, and soluble proteins were plotted away from the reefing and flowering stages. The control samples were also plotted away from the bio-organic fertilizers (B_2 to B_4). Notably, MDA and PPO (polyphenol oxidase) showed significant influence along

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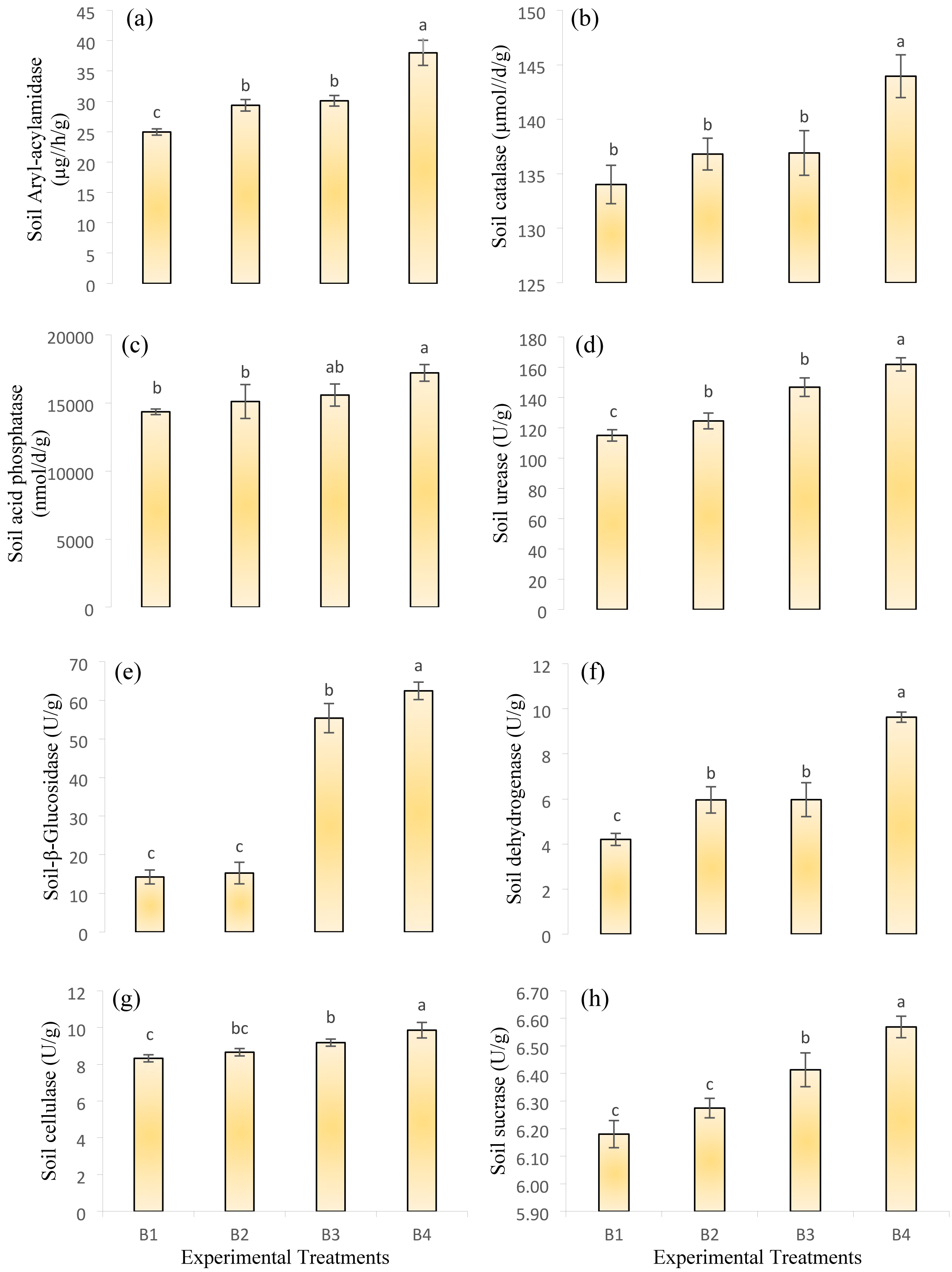

Figure 7. Effect of various bio-organic fertilizers on the soil enzymatic activities under tobacco cultivation. The statistics illustrate the means with standard deviation (SD) and were replicated three times, and the letters in the bars indicate significant differences between treatment means at $p \leq 0.05$ using a least significant difference test (LSD); (a) soil aryl-acylamidase, (b) soil catalase, (c) soil acid phosphatase, (d) soil urease, (e) soil-β-glucosidase, (f) soil dehydrogenase, (g) soil cellulase, and (h) soil sucrase.

PC1, indicating its role in the oxidative stress response. Bio-organic fertilizer (B_4) stood with distinct separation in the plot, suggesting its significant effect on enhancing tobacco quality and improving soil health compared to other treatments. The plot also suggests that the growth stage plays an important role in differentiating the samples, with HS samples showing more variability than other. This analysis highlights the complex interactions among fertilizers, growth stages, and biochemical markers.

3.10. Correlation Matrix. The Pearson correlation matrix of various biochemical parameters, i.e., antioxidant enzyme, chlorophyll content, proline, MDA, and carotenoids, is shown

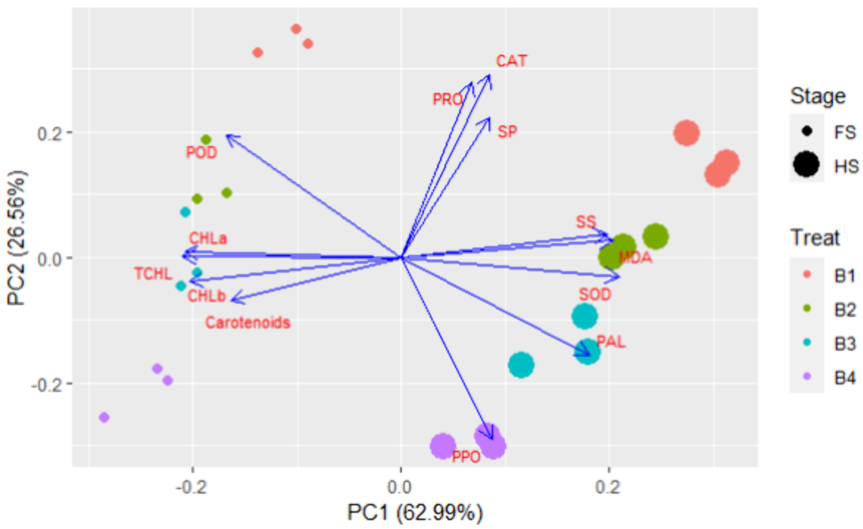

Figure 8. Principle component analysis of various attributes of a tobacco plant under the influence of various combinations of bio-organic fertilizer at various growth stages; CAT = catalase activity, PRO = proline activity, SP = soluble protein, POD = peroxidase activity, SS = soluble sugar, CHL a = chlorophyll a, CHL b = chlorophyll b, MDA = malonaldehyde contents, TCHL = total chlorophyll, PPO = polyphenol oxidase, and PAL = phenylalanine ammonia lyase.

in Figure 9. Positive correlations (closer to +1) were represented in red, and negative correlations (closer to −1)

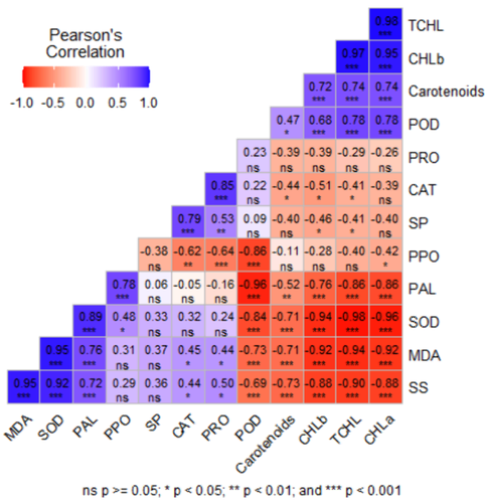

Figure 9. Pearson correlation matrix of various attributes of a tobacco plant under the influence of various combinations of bio-organic fertilizer; CAT = catalase activity, PRO = proline activity, SP = soluble protein, POD = peroxidase activity, SS = soluble sugar, CHL a = chlorophyll a, CHL b = chlorophyll b, MDA = malonaldehyde contents, TCHL = total chlorophyll, PPO = polyphenol oxidase, and PAL = phenylalanine ammonia lyase.

were represented in purple in the color scale. It also showed strong positive correlations between MDA and SOD, PAL, and SOD (r = 0.76−0.95) that imply linear association between oxidative stress markers and antioxidant defense mechanisms. Also, MDA and SOD correlate closely with POD (0.79 and 0.78), indicating a role of POD in the oxidative stress response. Chlorophyll and carotenoids correlated strongly (TCHL and CHL b with carotenoids) but negatively with MDA that indicated a possible opposite relation between the MDA increase (oxidative stress) and chlorophyll decrease. On the other hand, PPO and SP did not have strong correlation with other variables, i.e., have weaker correlations with other variables, less indirect, or less consistent with the variables that have been measured. In addition, the matrix displayed the significant and not significant correlations to distinguish the statistical strength of the relations, and some of the relationships have been noted as highly significant ($p < 0.001$).

4. DISCUSSION

Bio-organic fertilizer has proven to be a valuable tool in enhancing the growth parameters of tobacco plants.[46] The results of the current study clearly demonstrated that the addition of bio-organic fertilizer improved the plant growth attributes of tobacco plants (Figure 1). This improvement in the growth of tobacco plants might be due to the presence of organic matter, such as compost, plant residues, and beneficial microorganism, which promote the overall health and productivity of tobacco crop.[47] The microorganisms in bio-organic fertilizers, especially mycorrhizal fungi and nitrogen-fixing bacteria, establish symbiotic relationships with the tobacco roots, enhancing nutrient uptake and improving soil structure.[48] This leads to better nutrient availability for the plants, resulting in improved growth and development. Additionally, bio-organic fertilizers release nutrients gradually, which reduces the risk of overfertilization and minimizes environmental impacts. The microbes in these fertilizers produce growth-promoting substances like phytohormones and enzymes that stimulate root growth and nutrient absorption in crop plants. Moreover, the organic matter in bio-organic fertilizers enhances soil structure and water retention capacity, creating optimal conditions for root development and nutrient uptake, which ultimately supports the improved growth and minimizes the risk of disease. Similar findings were noticed that tobacco plants grown with bio-organic fertilizer exhibit improved growth rates, larger leaf sizes, improved resistance to diseases and pests, and higher yields.[49] The organic matter in bio-organic fertilizers serves as a slow-release nutrient source, providing a consistent supply of nitrogen, phosphorus, and other essential elements for the tobacco plant's metabolic processes, including photosynthesis.[50] The gradual changes observed in the photosynthetic

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attributes of the tobacco plant as depicted in Figure 2a–d might be due to the slow nutrient release, which helps to prevent the nutrient imbalance or excess that inhibits photosynthesis. The enhanced soil microbial activity driven by the presence of these microorganisms' aids supports the decomposition of organic matter and the conversion of nutrients into the forms readily available to the plants, which may explain the improvement in the photosynthetic attributes. The overall effect of these processes in the tobacco plant with enhanced chlorophyll content, increased carotenoid levels, expanded leaf area, and improved nutrient utilization efficiency. A significant positive correlation was observed between the growth and photosynthetic attributes, indicating that these improvements contribute to healthier plant growth and photosynthetic capacity and higher productivity. This makes bio-organic fertilizers a valuable tool for sustainable agriculture leading to improved photosynthetic rates, higher biomass production, and ultimately increased tobacco yield and quality.[51]

The organic matter in bio-organic fertilizers can stimulate the upregulation of the plant's antioxidant enzyme systems at various growth stages. The results of the current study clearly showed that addition of bio-organic fertilizer can upregulate enzymatic antioxidants, limiting lipid peroxidation and improving the osmolyte attributes. For example, the application of organic fertilizer can activate the expression of genes associated with antioxidant enzymes, resulting in increased enzymatic activity, similar findings were also reported by Rm and LP.[52] Increased SOD, CAT, and POD activity helps tobacco plants to combat oxidative stress by scavenging harmful reactive oxygen species (ROS) produced during photosynthesis and other metabolic processes.[53] Moreover, the balanced nutrient supply and promotion of healthy soil ecosystem provided by bio-organic fertilizers reduce environmental stress on tobacco plants, likely due to improved soil health, enhanced nutrient availability, and better soil enzymatic attributes, which ultimately lead to healthier plants with improved quality. This process helps to minimize oxidative stress and the production of ROS. Additionally, the organic matter in these bio-organic fertilizers improves the soil structure and water-holding capacity, ensuring a steady water supply, to the tobacco plants, which is crucial in preventing drought-induced oxidative stress.[54] Lipid peroxidation, a damaging process that affects the cell membranes and structures through the oxidative degradation of lipids, is mitigated by the increased activity of antioxidants enzymes. This helps to interrupt the chain reaction of lipid peroxidation, preventing the formation of lipid peroxides as observed in our study. By promoting balanced nutrient availability, bio-organic fertilizers indirectly reduce oxidative damage to lipids in plant cells, enhancing overall plant health and resilience.[55]

Adequate water availability is crucial for the accumulation of osmolytes in plant cells. In the current study, variation in the osmolyte contents was observed by the application of bio-organic fertilizers combined with various amendments (Table 2). Nutrients such as nitrogen, phosphorus, and potassium are required for osmolyte production.[56] This improvement in the osmolyte status in this study may be attributed to the addition of bio-organic fertilizers, which provides optimum moisture and nutrients that establish symbiotic relationships with tobacco plants. The presence of beneficial microorganisms and organic matter in bio-organic fertilizers can stimulate these osmolytes to help the plant cope with osmotic stress by stabilizing proteins and cell structures and maintaining turgor pressure.[48] By assisting plants adapt to adverse conditions, bio-organic fertilizers indirectly promote the accumulation of osmolytes as a response to stress.[57] Additionally, the use of bio-organic fertilizers positively influences the osmolyte by improving water retention, enhancing nutrient uptake, inducing the production of osmoprotectants, reducing osmotic stress, and lowering the osmotic potential of plant cells.[58] Similar findings were observed in this study, where optimal use of bio-organic fertilizers improved the osmolyte status of tobacco plants.

The addition of bio-organic fertilizers provides a balanced and sustainable nutrient supply, ensuring that tobacco plants receive essential elements for chlorophyll synthesis, photosynthesis, and overall leaf development.[59] The organic matter in bio-organic fertilizers promotes symbiotic relationships with plant roots, enhancing the nutrient uptake efficiency.[60] Additionally, improved soil structure, water-holding capacity, and enhanced microbial activity foster a healthier soil ecosystem, resulting in reduced nutrient leaching and improved nutrient availability for the plants.[26] The gradual nutrient release from bio-organic fertilizers prevents over-fertilization, which can negatively impact leaf quality. These factors collectively contribute to increased leaf size, higher chlorophyll content, and better resistance to diseases and pests, ultimately improving the overall leaf quality of tobacco plants while supporting sustainable and eco-friendly agricultural practices.[23]

The findings of the current study revealed a significant increase in organic matter by 28.48%, hydrolyzable nitrogen by 27.35%, available phosphate by 50.03%, and available potassium by 55.52% compared to the B_1 treatment. These results can be attributed to the rich composition of bio-organic fertilizers, which contain a variety of organic materials and beneficial microorganisms. The increase in organic matter is likely due to the high organic content of these fertilizers, which contributes to the soil's organic carbon pool and improves soil structure and water retention.[17] The rise in hydrolyzable nitrogen can be explained by the microbial activity stimulated by bio-organic fertilizers, which enhances nitrogen mineralization and makes nitrogen more available to plants.[5] Similarly, the substantial increase in available phosphate and potassium can be attributed to the ability of organic amendments to solubilize these nutrients, making them more accessible to plants.[25] Soil enzymatic activities play a crucial role in improving the soil health and fertility status by mediating major biochemical processes that affect the level of soil organic matter, nitrogen, phosphorus, and potassium.[9] These soil enzymes facilitate the decomposition of organic materials, thereby increasing the soil organic matter.[12] Soil enzymes also enhance the nitrogen mineralization, converting organic nitrogen into forms that plants can readily absorb.[13,27,28] The improved soil enzymatic attribute observed in this study likely contributed to the enhanced nutritional status of soil macronutrients. Additionally, soil enzymes involved in the breakdown of organic residues release potassium, which is essential for plant growth, enzyme activation, and photosynthesis. The increase in potassium contents observed in the soil leads to improved plant biomass and photosynthetic activity (Figure 6f). These findings underscore the significant role of bio-organic fertilizers in improving the soil enzyme activity and nutrient cycling, thereby reducing the reliance on chemical/

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inorganic fertilizers and supporting sustainable agricultural practices.

The organic matter in bio-organic fertilizer serves as a food source for soil microbes, enhancing their population and diversity. This microbial community includes various enzyme-producing organisms such as bacteria and fungi. Increased microbial activity results in higher enzyme production and its associated activity.[14] The moderate increase in dehydrogenase activity (129.28%), which is a key enzyme in microbial oxidative processes, indicates an enhanced microbial respiration and overall microbial biomass (Figure 7f). The moderate increases in cellulase (18.38%), sucrase (6.31%), and acid phosphatase activities (19.91%) reflect an improvement in carbon and phosphorus cycling, which are crucial for plant growth and soil fertility. Urease activity, vital for nitrogen cycling, showed a notable increase, highlighting improved nitrogen availability for plants.[17] The significant increase in aryl-acylamidase and catalase activities suggests enhanced soil detoxification processes and microbial adaptation to organic inputs. Enzymes such as cellulases, amylases, and ureases break down complex organic compounds into simpler forms that plants can readily absorb, and they also aid in nutrient cycling. Additionally, the beneficial microorganisms in bio-organic fertilizers facilitate the decomposition of organic matter, releasing nutrients that become more accessible for plant.[29] This synergy between organic matter, microorganisms, and enzyme production promotes improved nutrient availability and soil health, ultimately enhancing the soil's enzymatic activity, boosting nutrient cycling, and supporting plant growth.[32]

5. CONCLUSIONS

In summary, our study demonstrated that employing combinations of bio-organic fertilizer, compound fertilizer, and organic fertilizer notably boosted tobacco productivity, enzymatic activity, and the antioxidant defense system for achieving the goal of improved soil quality and supporting its sustainable use on a long-term basis. The combined application of bio-organic fertilizer combined with farm manure and compound fertilizer resulted in an increase in organic matter (28.48%), hydrolyzable nitrogen (27.35%), available phosphate (50.03%), and available potassium (55.52%) and decreased the pH and EC values up to 4.21 and 45.28-fold as compared to the control, respectively. Improved soil health in terms of better soil enzymatic activation resulted in a remarkable increase in secondary metabolites and activities of PPO (225.09 and 66.64%) and PAL activities (20.23 and 10.17%) at the flowering and harvesting stages. These results provided important information about the combined approach of nutrients and resources for improving the plant defense system and soil health in terms of improved quality production. In short, optimal usage of combinations of bio-organic fertilizer, compound fertilizer, and organic fertilizer had a positive effect on soil health in terms of improved soil enzymatic traits. The findings from greenhouse experiments need field validation as real-world soil conditions and environmental factors may influence results differently. Future research should focus on exploring gene expression changes in NPK transporter pathways and conducting transcriptomic or proteomic analyses to better understand nutrient uptake interactions. Field trials should be conducted to verify the applicability of these findings under diverse environmental conditions. Delving deeper into identifying the key component responsible for signaling systemic alterations in encoding antioxidant enzymes, a more thorough analysis of the oxidative stress response to enhance tobacco quality requires a further comprehensive investigation.

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Notes

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