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Impact of Iron Nanoparticles, Carbon Nanotube, and Biostimulatory Agents Application on Mushroom (*Agaricus bisporus*)

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ABSTRACT

Enhancing A. bisporus L. culture media with nanomaterials and some biostimulants is important for in improving mushroom productivity quantitatively and qualitatively. Magnetic iron nanoparticles (N-FeO), carbon nanotube (CNTs) suspensions, effective microorganisms (EM) bio-fertilisers, and growth stimulants (Atonik) were used individually and in combination to enhance the compost culture media. Quantitative and qualitative traits of the mushroom yield were measured. In a simple oOne-Wway experiment that included 16 treatments —Ssingle agents, two-, three-, and four-way Ccombinations and three replications—, the statistical analysis results of Duncan''s test showed. Tthat the individual impact of the applied study treatments and their combined synergistic effects resulted in a significant increase in the traits of the number of fruiting bodies, fruiting body rate, yield quantity, mushroom biological efficiency ratio, stem length, stem diameter, head diameter, head thickness, carbohydrates content, protein content, ash, and dry matter. The application of Atonik itself resulted in the highest values of fruiting body number, yield, biological efficiency, and stem length, which were respectively were 128.33 body bags^{-1}, 2814 g bag^{-1}, 37.52%, and 3.03 cm, compared to the control of 32.33 body bags^{-1}, 749 g bag^{-1}, 9.98%, and 1.72 cm. The treatments N-FeO+CNT+EM+ATO resulted in a significant increase in the traits of the fruiting body rate, stem length, and carbohydrate content by 32.69 g, 3.40 cm, and 16.78%, respectively, compared to the control of 22.97 g, 1.72 cm, and 8.16%, respectively.

Keywords: *Agaricus bisporus* L.; Compost; Bio-Fertilizer; Growth Enhancers; Iron Nanoparticles; Carbon Nanotubes

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1. Introduction

The mushroom *Agaricus bisporus L.* is experiencing growing significance as a sustainable source of nutrition. It possesses many dietary benefits that may assist in treating various health ailments [1,2]. The mushroom's global production increased from 30.2 million tons in 2010 to 48 million tons in 2017 [3]. China increased its production from 22.6 million tons in 2010 to 38.4 million tons in 2017, representing 75% of global production. Of the 16,000 known species of fungi, approximately 7,000 have varying degrees of edibility, more than 3,000 are edible, and 700 are considered safe mushrooms medically [4–6]. There are approximately 2,000 edible mushroom species, of which 35 are commercially available [7]. Edible mushrooms have health properties and play a pivotal role in supporting the immune system, fighting cardiovascular disease, and preventing cancer risk [8–10].

Enhancing the culture medium with nanomaterials that sustainably increase agricultural production improved the growth, productivity, and development environment of *A. bisporus* L. *and increased its productive traits and nutritional value*. Many nanomaterials, including metals, metal oxides, polymers, carbon nanotubes, and biocatalysts, have demonstrated their promising potential for sustainable agricultural production [11].

Carbon nanotubes (CNTs) are a promising and sustainable technology for increasing the efficiency of agricultural production due to their influence on the regulation of living tissue growth, their ability to penetrate cell walls, and their ability to act as slow-release nutrients and bio-stimulants. Carbon nanotubes are considered a novel nutrient source of carbon to support the growth environment of fungi and improve the quantity and quality of the mushrooms [12]. Carbon nanotubes can be a promising nutrient delivery tool, focusing on two aspects: reducing conventional fertilizer usage and chemicals released into the environment and minimizing damage to living tissues [13].

Magnetic iron oxide nanoparticles (IONPs), in particular, are used in various environmental and agricultural bioactivities due to their high magnetic spin pulses and low toxicity. Continued progress in precisely controlling iron nanoparticles' size, shape, and structural features emphasizes improved methods for the physical, chemical, and biological characterization of iron nanoparticles in nutrition. This approach improves the structure of the culture medium and increases its ability to retain water and essential nutrients for growth. Magnetic iron oxide nanoparticles can be an important nutrient and enzyme regulator to enhance growth and production parameters. They possess a specific property that activates H^+-ATPase in the plasma membrane, increases stress tolerance, and stimulates enzyme synthesis in biochemical reactions [14,15]. They are an important source of iron, characterized by rapid penetration, mobility, and intelligent targeting for absorption within the body compared to conventional sources [16].

Bio-stimulants positively impact the growth and development of living tissue, increase enzyme activity, and enhance the ability to absorb nitrogen [17]. Using bio-stimulants under unfavorable conditions enhances tolerance to abiotic stress in biological systems [18]. Using effective microorganisms (EMs) can enhance growth, and preventive protection against diseases is critical. EMs are beneficial strains of microorganisms that provide natural benefits when applied as bio-stimulants and sustainable bio-fertilizers in the agricultural ecosystem. They increase microbial diversity in the mushroom environment [19]. EMs have been used in the agricultural industry for promising research on enhancing the growth and yield of *A. bisporus*. However, further studies are needed to understand the symbiotic relationship between the mushroom and EMs in the growth environment. Therefore, this study aimed to investigate the impact of N-FeO, CNTs, Atonik, and EMs application as individual treatments and in various combinations on some growth parameters and yield of *A. bisporus*.

2. Materials and Methods

To investigate the influence of using nano-iron, carbon nanotubes, and biostimulants on the quantitative and qualitative yield of the cultivated mushroom *Agaricus bisporus* L., this experiment was conducted at the mushroom production project in Diwaniyah Governorate, Iraq, No. 3541283.97 E501886.60, from Dec 15, 2023, to Jun 2, 2024. The experimental environment (a hall of 2.6 m wide × 8 m long) was sterilized with commercial formalin 37% at a concentration of 4% and left for 72 hours over three days to ensure the elimination of microscopic pathogens 100%. The hall was then ventilated to remove the formalin residue **Table 1**.

The compost culture medium was prepared in the second stage through an open-air fermentation process. The first stage involved preparing the culture medium by moistening the wheat straw for six days. Then, 50% of the poultry manure was added to the wheat straw in two batches after checking the moisture content and seven days after the first batch. The hydration and mixing process was carried out to maintain aerobic fermentation. In the second stage, the produced medium from the first stage was pasteurized at 60 °C for 12 hours. The temperature was then gradually reduced for six days, with the first and second days being between 50–60 °C, the third and fourth days between 45–48 °C, and the fifth and sixth days at 25 °C. The most important

indicators confirming the completion of this stage are measuring ammonia levels, nitrogen content, and moisture content [20]. Random samples were taken for laboratory analysis after the medium was prepared for the fungal inoculum application.

Experimental bags were filled with 18 kg of compost medium, and then 175 g of inoculum was added. After creating suitable conditions of temperature, humidity, oxygen availability, and light inside the farm hall, and after the mycelium had spread throughout the growing medium, a 5 cm thick layer of soil was added, consisting of 70% peat moss, 20% sand, and 10% calcium carbonate [21]. The fungal inoculum was prepared according to the method illustrated by [5,22].

Wheat grains were boiled in water, and then 2% calcium sulfate and 8% calcium carbonate were added based on the dry weight of the grains. The mixture was then appropriately mixed, distributed into glass bottles, and sealed with cotton. The bottles were autoclaved for one hour at 121 °C and 15 psi. The bottles were then left to cool and inoculated with pieces of the parent culture of the Dutch-originated white strain *A. bisporus* under sterile conditions [10,23].

Table 1. Chemical characteristics of the growth medium.

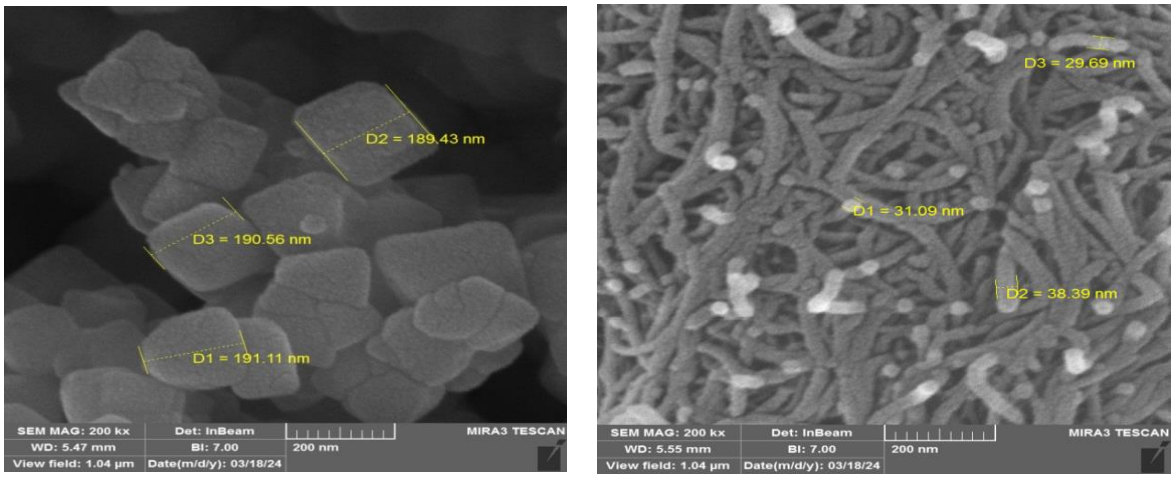
	Qualitative Traits	Value	Measurement Units	Reference
1	C	11.63	%	[24]
2	N	0.45		[25]
3	C/N	18.25		[26]
4	P	0.33		[24]
5	K	0.325		
6	Ca	0.391		
7	Mg	10.08	mg kg $^{-1}$	[27]
8	Fe	28.00		
9	Cu	7.03		
10	Mn	29.12		
11	Zn	28.91		
12	Mo	0.006		
13	Co	4.52		
14	B	1.03		[28]
15	P_H	7.4		
16	Ec	411	μSm^1	

2.1. Study Parameters

The experiment included the following individual and combined treatments:

1. The application of a magnetic nano-iron suspension (N-FeO), whose magnetic nanoparticles possess distinct characteristics compared to other materials due to their large surface area [29],
2. Multi-walled carbon nanotubes (CNTs), **Figure 1** which possess distinct and unique structural characteristics and have a cylindrical nanostructure with a length-to-diameter ratio much higher than any other material [30],
3. Effective Microorganisms (EMs), which consist of 10 genera belonging to 5 different families, including photosynthetic bacteria *Rhodopseudomonas palustrus* and *Rhodobacter sphaeroides*; lactic acid bacteria such as *Lactobacillus plantarum*, *L. casei*, and *Streptococcus lactis*; yeasts such as *Saccharomyces cerevisiae* and Candida utilis; actinomycetes such as *Streptomyces* and *Saccharomyces spp*.; and fermenting fungi. However, lactic acid bacteria, yeasts, and photosynthetic bacteria are the primary components of EM biofertiliser. These organisms coexist and interact in a compatible manner [31].
4. The biostimulant Atonik is an aromatic nitro compound [32] composed of three nitrophenol groups: 0.5% sodium nitrogualacolate, 0.1% sodium para-nitrophenolate, 0.3% sodium ortho-nitrophenolate, and 0.2% sodium ortho-nitrophenolate.

The experiment used different interactions between the individual treatments, with three replicates, according to a Completely Randomized Design (CRD).



(**a**) (**b**)

Figure 1. Scanning electron microscope (SEM) picture: (**a**) magnetic iron oxide nanoparticles, and (**b**) carbon nanotubes.

Nanomaterial suspensions of N-FeO and CNT were prepared using an ultrasonic device and deionized water at 42 °C. Combinations of biostimulants and nanomaterials were used to monitor their impact on mushroom growth and quality. The nanomaterials and biostimulants were applied to the bags by spraying after the covering stage. The materials and stimulants were applied to the culture medium by spraying according to the treatment after each fruiting body harvest time **Table 2**.

Table 2. The experimental treatments and their concentrations.

Treatment	Treatment Concentration
0	Control
N-FeO	magnetic iron oxide nanoparticles (50 mg l^{-1})
CNT	Carbon nanotubes (50 mg l^{-1})
EM	EM Effective Microorganisms (100 ml l^{-1})
ATO	Atonik (0.5 ml l^{-1})
N-FeO+CNT	magnetic iron oxide nanoparticles (50 mg l^{-1}) + Carbon nanotubes (50 mg l^{-1})
N-FeO+EM	magnetic iron oxide nanoparticles (50 mg l^{-1}) + EM (100 ml l^{-1})
N-FeO+ATO	magnetic iron oxide nanoparticles (50 mg l^{-1}) + ATO (0.5 ml l^{-1})
CNT+EM	Carbon nanotubes (50 mg l^{-1}) +EM (100 ml l^{-1})
CNT+ATO	Carbon nanotubes (50 mg l^{-1}) + ATO (0.5 ml l^{-1})
EM+ATO	EM (100 ml l^{-1}) +ATO (0.5 ml l^{-1})
N-FeO+CNT+EM	magnetic iron oxide nanoparticles (50 mg l^{-1}) + Carbon nanotubes (50 mg l^{-1}) + EM (100 ml l^{-1})
N-FeO+CNT+ATO	magnetic iron oxide nanoparticles (50 mg l^{-1}) + Carbon nanotubes (50 mg l^{-1}) + ATO (0.5 ml l^{-1})
N-FeO+EM+ATO	magnetic iron oxide nanoparticles (50 mg l^{-1}) + EM (100 ml l^{-1}) + ATO (0.5 ml l^{-1})
CNT+EM+ATO	Carbon nanotubes (50 mg l^{-1}) + EM (100 ml l^{-1}) +ATO (0.5 ml l^{-1})
N-FeO+CNT+EM +ATO	magnetic iron oxide nanoparticles (50 mg l^{-1}) +Carbon nanotubes (50 mg l^{-1}) + EM (100 ml l^{-1}) + ATO (0.5 ml l^{-1})

2.2. Growth and Yield Parameters

1. Number of fruiting bodies per bag: The number was calculated from the beginning of production to the end.
2. Average fruiting body weight: It was calculated according to the following equation:

$$Average\ fruiting\ body\ weight\ = \frac{Total\ bag\ yield\ (g)}{Number\ of\ fruiting\ bodies\ per\ bag} \quad (1)$$

3. Total yield: It was calculated by adding the cumulative yield of harvests during the experimental period for each harvesting time.

4. Biological efficiency %: It was measured by the following equation [33]:

$$Biological\ efficiency\ \% = \frac{Fresh\ weight\ of\ fruiting\ bodies}{Dry\ weight\ of\ culture\ medium} * 100 \quad (2)$$

5. Morphological characteristics: The morphological characteristics of fruiting bodies, including stem length, stem thickness, fruiting body head width, and thickness, were measured using the fully open method with a digital vernier caliper.
6. Carbohydrate content was measured by taking 0.2 g of the dried, ground fruiting body sample and applying 8 ml of 80% ethyl alcohol. The sample was then placed in water at 60 °C for 30 minutes. The liquid was then centrifuged at 3000 rpm for 15 minutes. This process was repeated three times. A spectrophotometer was used to measure light absorbance [34].
7. Protein content: The protein content of the dried fruiting body was indirectly measured by estimating total nitrogen using the Kjeldahl method. This was achieved by digesting the sample with sulfuric acid according to the following equation, as reported by Pardo et al. [35].

$$Protein\ percentage = Nitrogen\ percentage\ x\ 4.38 \quad (3)$$

8. Ash percentage: It was measured by taking 5 g of dried mushroom powder in a known-weight pellet and placing it in an incinerator at 550 °C for 4 hours until the sample turned to white ash. The pellet was then cooled, and the ash percentage was calculated, as stated, based on the following equation:

$$Ash\ percentage = \frac{100\ \text{x (empty pellet weight} - \text{ash with pellet weight)}}{(\text{sample weight})} \quad (4)$$

9. Dry matter: The dry matter percentage was measured by extracting the dry weight of the fruiting bodies from the wet weight. The fruiting bodies were cut into small pieces, placed in paper bags, and left to dry for three days in an electric oven at 45 °C [36].

2.3. Statistical Analysis

The statistics reported were presented as a mean value for each treatment of the experiment. A normality check was conducted using Kolmogorov-Smirnov and Shapiro-Wilk by SPSS version 27, IBM, New York, the United States. The statistical analysis using Duncan's test showed significant effects of the treatments on yield and quality parameters (GenStat 12th edition, PL20.1m, the United Kingdom). Microsoft Excel 365 was used for data recording [37].

3. Results and Discussion

Effects of nano-iron (N-FeO), carbon nanotubes (CNTs), effective microorganisms (EMs), and the growth stimulant Atonik (ATO) on the growth and yield of *A. bisporus* L.

3.1. Number of Fruiting Bodies (Fruiting Body Bag^{-1}) of the Mushroom

The use of ATO resulted in the highest significant increase in the average number of fruiting bodies 128.33 fruiting bodies bag^{-1}, compared to the control, which resulted in the lowest average number of fruiting bodies, 32.33 fruiting bodies bag^{-1}. However, there were significant differences among the individual treatments (**Table 3**). There were no significant variations among the bilateral interaction treatments. The average number of fruiting bodies decreased with the use of the triple combination N-FeO+EM+ATO by 66.3 fruiting bodies per bag^{-1} compared to the triple combination treatment N-FeO+CNT+ATO by 85.3 fruiting bodies per bag^{-1}. The quadruple combination treatment N-FeO+CNT+EM+ATO resulted in a non-significant but numerical increase in the number of fruiting bodies, 83.33 fruiting bodies per bag^{-1}, for all experimental treatments except the control.

Table 3. The Impact of nano-iron (N-FeO), carbon nanotubes (CNTs), effective microorganisms (EMs), and the Atonik growth stimulant (ATO) on the number of fruiting bodies, fruiting body rate, yield, and biological efficiency of *A. bisporus*.

Treatment \ Traits		Number of * FB		Means of * FB (g)		Yield g bag^{-1}		Biological Efficiency	
1	C	32.33	C	22.97	g	749	D	9.98	d
2	N-FeO	65.33	bc	28.06	cd	1800	C	24.00	c
3	CNT	73.67	bc	25.75	f	1860	C	24.80	c
4	EM	70.00	bc	25.91	ef	1810	C	24.14	c
5	ATO	128.33	A	22.22	g	2814	A	37.52	a
6	N-FeO+CNT	102.00	ab	23.12	g	2161	Abc	28.81	abc
7	N-FeO+EM	95.67	ab	31.14	ab	2474	Abc	32.99	abc
8	N-FeO+ATO	75.00	bc	27.81	cde	2089	Abc	27.85	abc
9	CNT+EM	78.33	B	29.25	c	2272	Abc	30.29	abc
10	CNT+ATO	86.33	ab	31.70	a	2727	Ab	36.36	ab
11	EM+ATO	63.33	bc	31.32	ab	1988	Bc	26.51	bc
12	N-FeO+CNT+EM	93.00	ab	25.70	f	2402	Abc	32.03	abc
13	N-FeO+CNT+ATO	85.3	A	26.63	def	2190	Abc	29.20	abc
14	N-FeO+EM+ATO	91.33	ab	25.54	f	2193	Abc	29.24	abc
15	CNT+EM+ATO	80.67	B	29.70	bc	2303	Abc	30.71	abc
16	N-FeO+CNT+EM+ATO	83.33	B	32.69	a	2735	Ab	36.47	ab

Note: * FB is the fruiting body of the mushroom.

3.2. Average of Fruiting Body (g) of Mushroom

The highest average fruiting body value was achieved with the use of the N-FeO+CNT+EM+ATO combined treatment 32.69 g, outperforming most treatments, including the control 22.97 g. However, its value did not differ significantly from the CNT+ATO, EM+ATO, and N-FeO+EM treatments, which resulted in 31.70, 31.32, and 31.14 g (**Table 3**).

3.3. Total Yield (g bag^{-1}) of the Mushroom

The application of ATO significantly increased the yield compared to the single treatments, with the highest significant increase at 2814 g bag^{-1}, compared to the control at 749 g bag^{-1}. This treatment did not differ significantly from the binary, ternary, and quadruple treatment combinations. At the same time, it outperformed its single counterparts, CNT, EM, and ATO, which were valued at 1860, 1810, and 1800 g per bag, respectively.

3.4. Biological Efficiency of the Mushroom

The highest biological efficiency of the mushroom was achieved with the ATO treatment, 37.52%, compared to treatments of EM, CNT, N-FeO, and the control, which were valued at 24.80, 24.14, 24.00, and 9.98%, respectively. However, it did not differ significantly from the binary, ternary, and quadruple combinations, except for the treatment of EM+ATO, which had a biological efficiency value of 36.36% (**Table 3**).

The significant increase in the number of fruiting bodies, average fruiting body weight, and biological efficiency of *A. bisporus* is attributed to using the quadruple interaction factors N-FeO+CNT+EM+ATO compared to the other combined effects. EM, Atonik, N-FeO, and CNT are remarkable due to their unique characteristics of rapid transport and intelligent nutrient delivery and their ability to improve the content of the compost medium and cover soil, which meets the environmental growth requirements. This positively influences the physical and chemical characteristics of the mushroom, stimulating the mycelium to form fruiting primordia and fruiting bodies, increasing productivity, and reducing toxicity in the yield. The carbon nanotubes are a food source for the mushroom and EM. The growth stimulant ATO plays a role in rapid penetration when sprayed onto the mycelium, as it is characterized by rapid access and stimulation of metabolic processes, mineralization, and stabilization of elements within living tissues. Enhancing the compost medium with nanomagnetic iron oxide particles, which stimulate cell division and increase cell number, also has magnetic properties that help channel nutrients and increase biomass resulting from the application of the EM, which has a symbiotic effect with fungi [38]. This helps achieve high-quality production (clean farming) [39,40].

Applying carbon nanotubes on the mulch soil enhanced the fungi's tolerance to heat stress and low humidity conditions, further enhancing the aforementioned characteristics. The carbon nanotube suspensions and liquid EM biofertilizer act as an available carbon source for the active organisms in the compost, providing the organisms composting the EM compost with a suitable environment for metabolic activity and symbiosis with the fungi. The symbiotic biomass increases at the expense of pathogenic organisms, creating favorable fertile conditions that increase mycelia density, proliferation, and nutrient uptake [41]. The

significant increase is attributed to the individual application of the ATO compared to ATO with 0.1% sodium 5-nitrogualacolate, 0.3% sodium para-nitrophenolate, and 0.2% sodium ortho-nitrophenolate, which are capable of penetrating tissues and reaching sites of biochemical reactions. It is characterized by stimulating metabolic processes by increasing nitrogen fixation, an important element in fungal physiology and metabolism, and achieving high-quality production. It also has a positive effect on the total yield, as spraying Atonik leads to increased inhibition of oxidase, which leads to increased activity of naturally produced auxins, increased permeability of the cytoplasmic membrane, reduced abiotic stresses on the fungus, regulated respiration, increased biological efficiency of the fungus, and increased total yield [42–44].

The significant increase in efficiency achieved with the use of EM+ATO is attributed to its inclusion of probiotic and beneficial strains, including photosynthetic bacteria (*Rhodospirillum, Rhodopseudomonas palustris, Rhodobacter sphaeroides*), lactic acid bacteria (Lactobacillus casei, Lactobacillus delbrueckii, Lactobacillus fermentum), actinomycetes (*Streptomyces* spp.), yeasts (Saccharomyces cerevisiae), phosphorus solubilizers (*Aerobacter, Candida, Penicillium, Xanthomonas*), nitrogen-fixing bacteria (*Azotobacter, Azospirillum, Pseudomonas), and fungi (Penicillium, Aspergillus*). In addition, it stimulates decomposition and transformation processes in the growing medium, contributing to the availability of nutrients, including nitrogen and phosphorus, due to nitrogen-fixing bacteria (Azotobacter, Azospirillum, and Pseudomonas). Nitrogen is an important element in metabolism and the production of growth factors. Other organisms play an important role in modifying the chemical, physical, and fertility conditions of the growing medium, providing safe conditions for mushroom growth and increasing yield, fruiting body weight, and number of fruiting bodies [45,46].

3.5. Stem Length of the Mushroom Fruiting Body (cm)

The treatments N-FeO, CNT, ATO, and EM resulted in significant qualitative increases in the stem length of the mushroom compared to the control, but no significant variations among them, with mean values of 2.83, 2.72, 2.78, 3.03, and 1.72 cm, respectively. The binary combination treatment CNT + N-FeO resulted in the highest mushroom stem height, 4.25 cm, compared to all single and binary treatments except for N-FeO + EM, which had a value of 3.48 cm for the stem length, and the ternary combination ATO + N-FeO + CNT made the value of 3.44 cm. The tetravalent combination CNT + EM + ATO + N-FeO had 3.40 cm (**Table 4**).

Table 4. The influence of application of nanomagnetic iron suspensions, carbon nanotubes, and bio-stimulants on the phenotypic characteristics of *A. bisporus* mushroom.

Traits / Treatments		Morphological Traits							
		Stem Length		Stem Diameter		* MH Diameter		* MH Thickness	
1	C	1.72	C	1.33	f	3.32	E	1.25	E
2	N-FeO	2.83	B	2.05	e	5.14	abcd	1.52	E
3	CNT	2.72	B	2.08	e	4.78	D	1.68	De
4	EM	2.78	B	2.26	cde	5.25	abcd	2.13	Cde
5	ATO	3.03	B	2.11	de	4.93	Cd	1.78	De
6	N-FeO+CNT	4.25	A	2.28	cde	4.78	D	1.70	De
7	N-FeO+EM	3.48	Ab	2.12	de	4.72	D	2.14	Cde
8	N-FeO+ATO	3.22	B	2.13	de	5.50	Abc	3.62	A
9	CNT+EM	3.16	B	2.56	ab	5.77	A	2.74	Abc
10	CNT+ATO	2.77	B	2.64	a	5.32	abcd	2.79	Abc
11	EM+ATO	2.64	B	2.46	abc	5.31	abcd	3.38	Ab
12	N-FeO+CNT+EM	3.15	B	2.10	de	4.98	Bcd	3.56	A
13	N-FeO+CNT+ATO	3.44	ab	2.20	cde	5.34	abcd	3.15	Ab
14	N-FeO+EM+ATO	3.02	B	2.07	e	5.63	Ab	1.73	De
15	CNT+EM+ATO	3.14	B	2.16	de	4.63	D	2.91	Abc
16	N-FeO +CNT+EM+ATO	3.40	ab	2.37	bcd	4.79	D	2.58	Bcd

Note: * MH is the mushroom head.

3.6. Stem Diameter of the Mushroom Fruiting Body (cm)

The binary combination CNT + ATO resulted in the highest significant value for *A. bisporus* stem diameter of 2.64 cm compared to all treatments, including the control, 1.33 cm, except for the binary treatments CNT + EM and EM + ATO, which had 2.56 and 2.46 cm, respectively.

3.7. Head Diameter of the Mushroom Fruiting Body (cm)

The binary combination treatment CNT+EM resulted

in the most prominent head diameter value of 5.77 cm, significantly higher than most treatments, including the control 3.32 cm. All treatments significantly increased compared to the control (**Table 4**).

3.8. Head Thickness of the Mushroom Fruiting Body (cm)

The binary combination treatment N-FeO+ATO and the ternary treatment N-FeO+CNT+ATO resulted in the highest values of head thickness of fruiting body values of 3.62 and 3.56 cm, and they differed significantly from each other. However, none of the single treatments significantly increased this trait over the control, even the binary combinations treatments N-FeO+EM and N-FeO+CNT, which made 2.14 and 1.70 cm, respectively.

There were significant differences in some binary and ternary combinations and single additions due to spraying magnetic iron nanoparticles, carbon nanotubes, EM, and ATO. The increase in the average stem length, stem diameter, head diameter, and head thickness of the CNT+EM, EM+ATO, and CNT+N-FeO binary combinations was attributed to applying carbon nanotubes. The decomposition of these tubes releases carbon, which is used as a nutrient source for the active organisms added due to spraying EM with fungi. They also affect some Gram-negative bacteria, increasing their numbers by increasing biofilm formation and metabolic activity. Carbon nanotubes can penetrate cells and tissues due to their small size and ability to interact with cell components, leading to changes in physiological functions. Bacteria and fungi can absorb and accumulate on their surface due to their large surface area. The carbon nanostructured surface can provide an ideal environment for the growth and proliferation of beneficial bacteria and fungi [47–49]. Magnetic iron oxide nanostructures play a role in regenerating damaged tissues and stimulating enzymes responsible for carbon fixation in metabolic transformations. Due to their unique composition and properties, they work with carbon nanotubes in some binary combinations as a food source for fungi and a nutrient delivery vehicle. They are also among the nanocatalysts that work at the level of stimulating and synthesizing growth and production materials in mushrooms. ATO contains aromatic nitrogenous compounds that penetrate the tissues of the living body, reach metabolic sites, and stimulate the production of additional growth materials, thus increasing production.

The increase in head thickness with the N-FeO+CNT+ATO treatment is attributed to the use of nanomaterials, which increased the availability of nutrients from secondary sources and their uptake by the mushroom. The availability of biofertilizers and biostimulants also contributed to increased biodiversity in the compost medium, thereby increasing the organisms responsible for decomposing organic matter and releasing nutrients such as nitrogen, phosphorus, calcium, potassium, and various micronutrients such as iron, copper, zinc, and boron. In addition, various heterotrophic organisms release organic acids, such as humic and fulvic acids, which form complexes with calcium and phosphorus. These compounds increase oxygen levels, which are essential for mushroom culture management. This stimulates the mycelium to produce fruiting primordia, helps the fungus absorb carbohydrates, increases nutrient availability, and increases biomass activity in the compost. This increases hydrolytic enzymes and protein synthesis, positively impacting the head's thickness and the crop's quantity and quality [50,51].

3.9. The Mushroom Carbohydrate Content (%)

The highest significant mean carbohydrate content was achieved under the impact of the N-FeO+CNT+EM+ATO treatment, 16.78%, compared to the control, 8.16% (**Table 5**). The three-way interactions of the treatments resulted in non-significant differences, except for N-FeO+EM+ATO, 14.72%. The treatment of EM+ATO resulted in a significant increase in the carbohydrate content of the mushrooms, 15.89%. However, the carbohydrate content significantly increased with all individual treatments compared to the control.

Table 5. Influence of magnetic iron nanoparticle suspensions, carbon nanotubes, and biostimulants on the carbohydrate, protein, ash, and dry matter percentages of the mushrooms.

Treatment	Traits	Carbohydrates %		Protein %		Ash %		Carbohydrates %	
1	C	8.16	J	1.66	h	6.30	c	11.88	fgh
2	N-FeO	10.70	I	2.58	gh	13.38	abc	16.87	abcde
3	CNT	12.37	H	4.04	f	11.64	abc	19.53	ab
4	EM	12.68	Gh	5.40	cde	12.28	abc	17.57	abcd
5	ATO	14.36	Cdef	5.61	cde	9.21	abc	14.52	cdefgh
6	N-FeO+CNT	13.46	Efgh	4.37	ef	9.23	abc	19.77	a
7	N-FeO+EM	13.24	Fgh	3.99	f	8.08	bc	15.07	bcdefg
8	N-FeO+ATO	14.06	Defg	3.59	fg	16.50	a	18.63	abc
9	CNT+EM	15.03	Bcde	5.83	cd	10.92	abc	16.21	abcdef
10	CNT+ATO	13.77	Efgh	4.78	def	10.86	abc	10.29	h

Table 5. *Cont.*

Treatment	Traits	Carbohydrates %		Protein %		Ash %		Carbohydrates %	
11	EM+ATO	15.89	Abc	6.77	bc	10.76	abc	13.11	defgh
12	N-FeO+CNT+EM	16.14	Ab	8.44	a	9.32	abc	11.59	fgh
13	N-FeO+CNT+ATO	15.50	Abcd	7.50	ab	12.57	abc	12.85	efgh
14	N-FeO+EM+ATO	14.72	Bcdef	8.00	ab	12.59	abc	12.86	efgh
15	CNT+EM+ATO	15.85	Abc	8.11	ab	16.34	ab	13.40	defgh
16	N-FeO+CNT+EM+ATO	16.78	A	5.42	de	9.01	abc	10.98	gh

3.10. Protein Content of the Mushroom (%)

Applying the N-FeO+CNT+EM treatment resulted in a significant increase in mushroom protein content, 8.44%, compared to all other treatments, including the control at 1.66%. The dual treatment EM+ATO resulted in the highest increase in protein content, 6.77%. However, no significant variations were seen from the single ATO and EM treatments, valued at 5.61% and 5.40%, respectively. The nanomagnetic iron treatment showed no significant effect on mushroom protein content compared to the control.

3.11. Ash Percentage of the Mushroom (%)

The N-FeO+ATO treatment resulted in the highest ash percentage of the mushrooms, 16.50%, surpassing the N-FeO+EM treatment and the control at 8.08% and 6.30%, respectively. The other treatments resulted in non-significant differences for this trait (**Table 5**).

3.12. Dry Matter Percentage in Mushrooms (%)

The treatment of N-FeO+CNT resulted in the highest dry matter percentage, 19.77%, compared to most treatment combinations, including the control at 11.88%. Applying N-FeO, CNT, and EM individually resulted in non-significant effects of 19.53%, 17.57% and 16.87%, respectively, (**Table 5**). The significant effect of the four-way interaction of treatments on carbohydrate content is attributed to the combined synergistic effect of continuous spray applications of nanomagnetic iron, carbon nanotubes, effective microorganisms, and Atonik growth stimulant.

These additives demonstrated their combined effect, enhancing the absorption and availability of microelements. Iron nanoparticles provide a nutrient source that supplies iron to fungi. They stimulate enzyme reactions in biochemical processes due to their penetrating properties and rapid access resulting from their small size and increased surface area. They also stimulate cell division and increase cell number. They possess magnetic properties that help direct nutrients. Iron oxide nanoparticles interact with chemical compounds. These interactions involve exchanging electrons and forming new chemical bonds that aid cell division and growth, enhancing nutrient utilization efficiency. N-FeO particles, combined with CNT, EM, and ATO, increase biomass through the supply and stimulation of metabolic processes, increasing the availability and rapid delivery of nutrients and creating a fertile environment through the decomposition of organic matter, the release of various nutrients and regulating biochemical interactions in mushroom tissues or the culture medium between the biomass produced by the active organisms in EM fertilizer [10,52,53].

The increase in protein, dry matter, and ash content of mushrooms under the influence of the N-FeO+CNT, N-FeO+ATO, N-FeO+EM, and EM+ATO interactions is attributed to the role of liquid EM and its direct role in enhancing the interaction of mycelium with the active organisms that have symbiotic relationships with mycelium, which aid in the decomposition of organic matter from the compost medium and the absorption of nutrients. In other words, iron nanoparticles, biofertilizers, and growth stimulants enhance each other's effectiveness. Nanomaterials provide a rapid and direct supply, while biofertilizers provide a sustained supply, meaning that mushrooms receive nutrients in a balanced and continuous manner.

Spraying with Atonik also regulates growth and stimulates fruiting primordial. It contains chemicals that stimulate metabolic processes and increase enzyme reactions. These chemicals effectively enhance fungi's physiological processes in small quantities. They also increase yields and improve quality[54] Using CNTs is characterized by spraying over a large surface area, high reactivity, compatible pore size, and particle formation, which helps sustainably deliver nutrients to fungi and improve growth, yield, carbohydrate, protein, and dry matter content. The mushroom presented a positive physiological response when carbon nanotubes were added at appropriate concentrations. Since carbon nanotubes are considered smart nanoplatforms (smart nanoplatforms refer to nanomaterials like CNTs that can deliver nutrients efficiently and selectively to fungal tissues due to their nanoscale size, reactivity, and targeting ability) for delivering essential nutrients, various macronutrients or other compounds can be delivered to fungal tissues and cells, and they can be used as

carbon nanotube-based delivery systems [55–57].

4. Conclusions

The effects of some individual treatments and the synergistic effect of magnetic iron nanoparticles, carbon nanotubes, effective microorganisms, and the growth stimulant Atonik significantly influenced mushroom growth and productivity through increased values of the studied traits. Using Atonik increased values of fruiting bodies, yield, biological efficiency, and stem length of the mushroom. The N-FeO+CNT+EM+ATO treatment resulted in the highest mean for mushroom fruiting bodies, stem length, and carbohydrate content. The impact of the EM+ATO treatment was positively significant in increasing the value of biological efficiency, stem diameter, and mushroom content of carbohydrates and protein.

Author Contributions

Conceptualization, M.A., H.A.W.A.-J. and R.A.C.; methodology, M.A., H.A.W.A.-J. and R.A.C.; software, M.A., H.A.W.A.-J. and R.A.C.; validation, M.A., H.A.W.A.-J. and R.A.C.; formal analysis,M.A., H.A.W.A.-J. and R.A.C.; investigation, M.A., H.A.W.A.-J. and R.A.C.; resources, M.A., H.A.W.A.-J. and R.A.C.; data curation, M.A., H.A.W.A.-J. and R.A.C.; writing—original draft preparation, M.A., H.A.W.A.-J. and R.A.C.; writing—review and editing, M.A., H.A.W.A.-J. and R.A.C.; visualization, M.A., H.A.W.A.-J. and R.A.C.; supervision, M.A., H.A.W.A.-J. and R.A.C.; project administration,M.A., H.A.W.A.-J. and R.A.C.; funding acquisition, M.A., H.A.W.A.-J. and R.A.C. All authors have read and agreed to the published version of the manuscript.

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