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Combined effect of nano boron, zinc, bio-inoculum and white fungus waste on *P. aeruginosa* numbers and amidase activity in soil.

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Abstract: An experiment was conducted at an agricultural site affiliated with the Department of Agricultural Research at the Diwaniyah Research Station in Iraq on January 15,2024. The aim was to investigate the effects of three study factors, the first factor, a biofertilizer represented by *P. aeruginosa* bacteria, symbolized as B, applied at two levels (no addition *of P. aeruginosa* B0, addition *P. aeruginosa* B1), the second factor, white mushroom waste, symbolized as Ab, added at three levels, (no addition of Ab0, 5 tons h^{-1} as a second level Ab1, 10 tons h^{-1} as a third level Ab2), and the third factor, a nanofertilizer symbolized as N, applied at four levels, (no addition N0, 4 kg h^{-1} nanozinc N1, 2 kg h^{-1} nanoboron N2, and 1 kg h^{-1} nanoboron + 2 kg h^{-1} nanozinc N3). These factors were tested for their effects on the number of the bacteria *P. aeruginosa* and stimulation of amidase enzyme activity in the first harvest of stevia crop. The statistically analyzed data indicated that the synergistic effect between the three study factors showed significant superiority through increasing the number of *P. aeruginosa* bacteria and the activity of the amidase enzyme during the two periods, Considering that for the two periods in view, it recorded (153.7,137.7) $\times 10^7$ CFU g^{-1} dry soil and (265.33, 163.00) µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$, respectively, while the control treatment recorded the lowest values during two periods, with (44.3,24.7) CFU g^{-1} dry soil and (61.33,21.67) µg $N\text{-}NH_4^+ g^{-1}$ soil $2h^{-1}$, respectively.

Keywords: Amidease enzyme, bio fertilizers, nano fertilizers, organic waste.

Introduction

Enzymes are very important roles in the decomposition of organic matter, this acts as a mirror of the absorption and use of nutrients. Low Enzyme activity in the soil, it can deter the natural physiological processes taking place in plants and also inhibit their growth and development. (Lemanowicz *et al*.,2020). Enzymes are an important factor in the agricultural ecosystem, whether in a long or short period of time, in addition to the possibility of using enzyme activity to indicate the effectiveness of bioreclamation of ecosystems that have been destroyed by successive agricultural operations (Saraptka *et al*.,2002). Enzymatic activity in soil is mostly evaluated based on the effect of five enzymes (Dehydrogenase, Phosphatase, Urease, Invertase, and Protease), the most important of which is the amidase enzyme (Acylamide amidohydrolase, EC3.5.1.4) Amidase, which catalyzes the hydrolysis of a small group of amides and produces carboxylic acid and ammonia (Figure 1) (Valina *et al*.,2004).

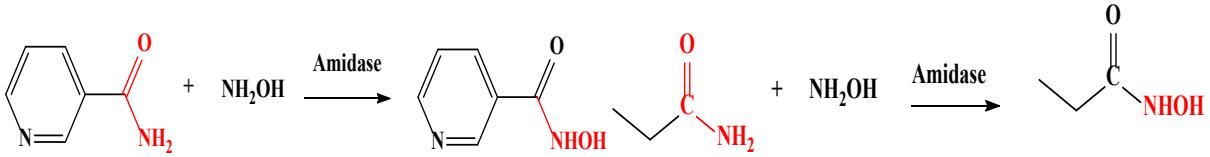

Fig. (1): The basic structure of the amidase shown (Valina *et al.*,2004).

These are common enzymes that have come to be noticed more due to the varied biochemical application opportunities they offer. Studies have found that the use of agrochemical fertilizers lowers their activity; hence, it can be an indicator for the presence and effects of such agrochemical fertilizers, which may harm enzyme activity. (Mills *et al.*,1997). This enzyme is very sensitive to chemical fertilizers and has garnered considerable interest because of the distinctions in the characteristics of its substrate and inhibitor in the covalent catalysis of *P.aeruginosa* amidase. (long *et al.*,2016). Microorganisms with specialized substrates play an important role in the detoxification and removing pesticides from soils containing toxic amides. For example, amidase is mployed to produce acrylic acid from acrylamide and thus convert toxic pollutants into raw materials (nawaz *et al.*,1991). Hence, the efficiency of *P. aeruginosa* in inducing amidase enzyme activity in the soil is a characteristic feature, as proved by (Al-muhamady,2020), in his study on biomass and enzymatic activity in the soil, "Effect of *P. aeruginosa*." The reason Gram-negative *P. aeruginosa* bacteria are superior or have an edge over other species is that they can adapt to live in all kinds of polluted environments, yet they maintain essential decomposition enzymes in their outer membrane., unlike Gram-positive bacteria, release large amounts of their enzymes outside the cell (Hiroshi,2003). These bacteria decompose organic pollutant with its enzymes and waste products resulting from such processes of metabolism usually act as carbon sources and energy. It is obvious that they have different types of enzymes; one of the most significant is the enzyme amidase, it also means that with the help of these enzymes, bacteria can easily utilize most other organic waste and soil waste materials, as a source of carbon and energy. This leads to increased biomass and enzyme activity in soil. (Adeleye *et al.*,2017; Adeleye *et al.*,2018). The most important of these wastes is the waste of the mushroom Agaricus bisporus; it constitutes the remains of the biomass after the mushroom harvest in commercial cultivation (Sendi *et al.*,2013). The biomass evidently increased drastically upon supplementing the soil with organic waste of high carbon content (chernysheva *et al.*,2023). This finding was supported by (fanin *et al.*,2022) in their study, which addressed a cocontrol of effects resulting from waste of consumed mushrooms and mineral fertilizer on the quality of the soil, related to activities of biomass transformation in C, P, and N. White mushroom waste proved a good substitute for organic fertilizer and can be applied on an annual basis, besides without causing toxic effects in the initial stages of microbes and enzymes. As a result, the researcher has contended that with such findings, agricultural waste, including consumed mushroom waste, will be better managed, to ensure increased soil fertility according to the principle of sustainable development. Zinc and boron are essential trace elements that are present in all six enzyme classes (Andernini & Birtini,2012). Zinc and boron are involved in the formation and activation of a number of enzymes, including lactic acid dehydrogenase, glutamic acid dehydrogenase, alcohol

dehydrogenase, proteinases, peptidases, and enolase (Ali *et al*.,2008). (Raliya *et al*.,2008) found to cause enhancement of enzyme activity and biomass at the rhizosphere level of the soil. Some works have proposed that nano-zinc and boron fertilizers can improve the resistance of plants to Soil status by enhancing biomarker due to positive effects on soil biomarker (Trafdar *et al*.,2014; Narendhran *et al*.,2016;Kale & gawada,2016), zinc deficiency may play a role in the decrease in starch content and enzyme activity (Bious *et al*.,2011). Recognizing the importance of enzymes in soil and the increasing urge to maintain a sustainable environment, the current study aimed to evaluate the combined effect of boron, nano zinc, white fungus waste and *P. aeruginosa* bacteria in increasing the number of *P. aeruginosa* bacteria and stimulating the activity of the amidase enzyme in the first and second harvest of stevia crop.

Materials & Methods

Soil Sample Collection Before Planting

Soil samples were collected from a depth of (0-30) cm at an agricultural site affiliated with the Agricultural Research Department/ Diwaniyah Research Station on 15/1/2024. After collection, the soil samples were air-dried and stored until biological, chemical analyses were conducted (Table 1). The experiment was carried out during the winter agricultural season 15/2/2024. Plowing, smoothing and leveling operations were carried out for the selected area to prepare it for the experiment. The field was divided into three sectors, each sector containing 24 panels with dimensions of 2 m × 2 m as an experimental unit, and a distance of 2 m was left between sectors and 1 m between experimental units*Experimental treatments*

Treatments of fertilizers shown in Table (2) were applied in the experiment, and the rate of 3 replications for each treatment was followed, thus rendering the number of experimental units as 72. The experiment was laid out in the design of a complete block with three replications.

The fertilizer recommendation is hereby incorporated in the form of split application granular urea (47.619 kg N h-1), and split urea application atFirst and second harvest of the stevia crop. Phosphate fertilizer (71.428 P kg h-1) shall be applied in one batch at 120 days period Single superphosphate as per scientific recommendations. Phosphate fertilizer was applied in two equal splits at 60 kg P2O5 h-1 with the addition of potassium fertilizer in the form of potassium sulphate at the same rate in one dose. Factors of the study are three. The first factor, which is biofertilizer, represented by P. aeruginosa bacteria marked by symbol B, has two levels, which are the addition of bacteria P. aeruginosa B1 injected 0.92 ml bacterial vaccine with a syringe into the rhizosphere after 14 days of planting of stevia, B0 without the addition of liquid P. aeruginosa vaccine, white fungus waste two levels, as a second level Ab0 without the addition of 5 tons h-1 and the third level Ab1 10 tons h-1 added in one batch upon planting. The nano fertilizer, Coded N, was also added at four levels, which are (without adding N0, 4 kg h-1 nano zinc N1, 2 kg h-1 nano boron N2, 1 kg h-1 nano boron + 2 kg h-1 nano zinc N3) added in one batch simultaneously with the addition of white fungus waste.

Table (1): chemical and biological properties of the study soil before planting.

Parameter	Value	Unit
pH $_{1:1}$	7.23	-
EC $_{1:1}$	4.78	**ds m^{-1}**
CEC	15.18	**Centi mole $^{+}$ kg^{-1}**
Available Nitrogen	23.8	**mg Kg^{-1} soil**
Available phosphorus	4.6	
Available Potasium	258.91	
$CaCO_3$	201.00	**g Kg^{-1} soil**
Amidase Enzyme activity	18.33	**µg N-NH_4^+ g^{-1} soil $2h^{-1}$**

Table (2): Experimental treatments and their symbols

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S	*Treatment*	*Name of the Treatment*
1	$B_0Ab_0N_0$	*Without addition*
2	$B_0Ab_0N_1$	*4 kg h^{-1} of nanozinc*
3	$B_0Ab_0N_2$	*2 kg h^{-1} of nanoboron*
4	$B_0Ab_0N_3$	*1 kg h^{-1} of nanoboron + 2 kg h^{-1} of nanozinc*
5	$B_0Ab_1N_0$	*5 tons h^{-1} of white mushroom waste Level 1*
6	$B_0Ab_1N_1$	*5 tons h^{-1} of white mushroom waste + 4 kg h^{-1} of nanozinc*
7	$B_0Ab_1N_2$	*5 tons h^{-1} of white mushroom waste + 2 kg h^{-1} of nanoboron*
8	$B_0Ab_1N_3$	*5 tons h^{-1} of white mushroom waste + 1 kg h^{-1}1 of nanoboron + 2 kg h^{-1} of nanozinc*
9	$B_0Ab_2N_0$	*10 tons h^{-1} of white mushroom waste*
10	$B_0Ab_2N_1$	*10 tons h^{-1} of white mushroom waste + 4 kg h^{-1} of nanozinc*
11	$B_0Ab_2N_2$	*10 tons h^{-1} of white mushroom waste + 2 kg h^{-1} of nanoboron*
12	$B_0Ab_2N_3$	*10 tons h^{-1} of white mushroom waste + 1 kg h^{-1} of nanoboron + 2 kg h^{-1} of Zinc Nano*
13	$B_1Ab_0N_0$	*Add* P. aeruginosa *vaccine*
14	$B_1Ab_0N_1$	*Add* P. aeruginosa *vaccine + 4 kg h^{-1} of nanozinc*
15	$B_1Ab_0N_2$	*Add* P. aeruginosa *vaccine + 2 kg Nanoboron*
16	$B_1Ab_0N_3$	*Add* P. aeruginosa *vaccine + 1 kg h^{-1} of nanoboron + 2 kg h^{-1} of nanozinc*
17	$B_1Ab_1N_0$	*Add* P. aeruginosa *vaccine + 5 tons h^{-1} of white fungus waste*
18	$B_1Ab_1N_1$	*Add* P. aeruginosa *vaccine + 5 tons h^{-1} of white fungus waste + 4 kg h^{-1} of nanozinc*
19	$B_1Ab_1N_2$	*Add* P. aeruginosa *vaccine + 5 tons h^{-1} of white fungus waste + 2 kg h^{-1} of nanoboron*
20	$B_1Ab_1N_3$	*Add* P. aeruginosa *vaccine + 5 tons h^{-1} of white mushroom waste + 1 kg h^{-1} of nano boron + 2 kg h^{-1} of nano zinc*
21	$B_1Ab_2N_0$	*Add* P. aeruginosa *vaccine + 10 tons h^{-1} of white mushroom waste*
22	$B_1Ab_2N_1$	*Add* P. aeruginosa *vaccine + 10 tons h^{-1} of white mushroom waste + 4 kg h^{-1} of nanozinc*
23	$B_1Ab_2N_2$	*Add* P. aeruginosa *vaccine + 10 tons h^{-1} of white mushroom waste + 2 kg h^{-1} of nano boron*
24	$B_1Ab_2N_3$	*Add* P. aeruginosa *vaccine + 10 tons h^{-1} of white mushroom waste + 1 kg h^{-1} of nano boron + 2 kg h^{-1} of nano zinc*

Population density of *P. aeruginosa*

The culture medium for *P. aeruginosa* (HiFlouro™ *Pseudomonas* Agar Base) was prepared by dissolving 46.75 g in 1000 ml of distilled water containing 10 ml of glycerin, the mixture then was heated to boiling to fully dissolve the medium, after that the steam sterilization process was carried out at a pressure of 15 bars and a temperature of (121 °C) for 15 minutes,

then the medium was cooled to 45-50 °C and mixed well and poured into sterile Petri dishes (King and Raney,1954). Serial dilutions of soil samples were prepared by adding 1 g of the soil sample to 9 ml water, creating 10^{-1}-10^{-7} decimal dilutions 1 ml of soil suspension to be performed in tubes containing 9 ml of sterile distilled water for each soil sample.

The prepared medium was inoculated with the decimal dilutions. The plates were incubated at 28ºC After incubation the plates were taken out for counting the growing colonies using a colony counter (Black,1965).

Amidase activity

Amidase activity was determined by adding 0.2 ml of dye to a 5g soil sample as described above and measuring the release of ammonium nitrogen by the method of Frankenberger and tabatbai(1980) with 9 ml of THAM buffer (0.1 M, pH 8.5) and 1 ml of 0.5 M formamide solution, at 37 0C for 2 hr. To this 35 ml of a solution containing (potassium chloride 2.5 M, uranium acetate 0.005 M and for inhibition of enzyme activity KCl $UO_2(C_2H_3O_2)_{2.H2O}$), by adding the reagents to a volume of 50 ml and completed to the volume with the same solution.

Statistical analysis of the data:

The measured data for the study indicators were statistically analyzed using the Genstat program, and the averages were compared using the least significant difference (LSD) test at a probability level of 5% (Al-Rawi Kalaf Alaah,1980).

Results & Discussion

Numbers of P. aeruginosa bacteria (CFU g^{-1} dry soil) In the first and second harvest of stevia crop.

As shown in (Figure 2) that A and b show the process of comparing the addition of the inoculum to the soil sample treated with white fungus waste compared to the control treatment, as *P. aeruginosa* cells appear clearly in Figure b, while no cells appeared in the dish that not contain the inoculum, as shown in Figure A. Figure c also shows a close-up image of *P. aeruginosa* bacteria, as it appears in a pale color, while Figure d shows the process of pouring the culture medium into the dishes.

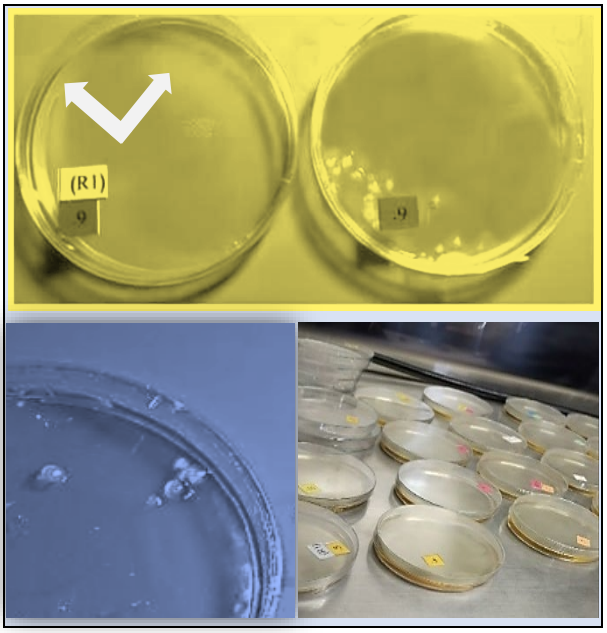

Fig. (2): A and b show the difference between the inoculum of the soil sample treated with white fungus waste compared to the control treatment, Figure c shows a close-up image of *P. aeruginosa* bacteria, Figure d shows the process of pouring the culture medium into the dishes.

Tables (3 and 4) indicate a study of the combined effect of boron, nano zinc, white fungus waste and *P. aeruginosa* bacteria on the number of *P. aeruginosa* bacteria in the rhizosphere soil of the stevia plant after 120 and 240 days. Tables (3 and 4) show that the addition of the B1 bio-vaccine was significantly superior and recorded the highest number of *P. aeruginosa* bacteria during the two periods, reaching (132.0,120.2)×10^7 CFU g^{-1} dry soil, respectively, compared to the control treatment B0, which recorded the lowest number of bacteria during the two periods, reaching (72.7,63.5)×10^7 CFU g^{-1} dry soil, respectively. The reason for the increase in the number of Gram-negative *P. aeruginosa* bacteria is due to their adaptation to living in

different environments while maintaining the vital decomposition enzymes present in their outer membrane, unlike Gram-positive bacteria, which release large amounts of their enzymes outside the cell, thus increasing their numbers in addition to increasing the activity of their enzymes (Hiroshi,2003). The results of the statistical analysis showed the significant superiority of the study factor that includes the addition of white mushroom waste at three levels (Ab0, Ab1 and Ab2) the Ab2 level produced the highest number of *P. aeruginosa* bacteria after 120 and 240 days of addition, reaching (123.5,112.0)×10^7 CFU g^{-1} dry soil, respectively, compared to the control treatment Ab0, which recorded (72.4,66.6)×10^7 CFU g^{-1} dry soil, respectively. The superiority effect of mushroom waste may be due to the fact that it is fresh, application to the soil immediately after the mushroom harvest stage, as it contains in its composition different types of microorganisms, including bacillus bacteria such as *P. aeruginosa*, which is considered one of the dominant bacteria in organic fertilization systems because it contributes to the decomposition of waste due to its tolerance to high temperatures (Sendi et al.,2013). It was found that it is very active in the first stage of cultivation after 120 days, and its number gradually decreased in the second stage after 240 days, i.e. in the second harvest of the stevia plant.

The analysis data showed that the study factor included the addition of nano fertilizer at four levels (N0,N1,N2,N3), as the results shown in Tables (3 and 4) the N3 level was significantly superior during the two periods, as it recorded (109.20,97.7)×10^7 CFU g^{-1} dry soil, respectively, compared to the Control treatment N0, which recorded (94.9, 88.6)×10^7 CFU g^{-1} dry soil, respectively. The superiority of the treatment consisting of half the recommendation of boron with half the recommendation of zinc is due to the joint role of boron and zinc, as boron and zinc are essential components in the formation of many enzymes secreted by microorganisms that work to accelerate metabolic activities, thus contributing to increasing the numbers of microorganisms, including *P. aeruginosa bacteria* (Ali et al.,2008).

The interaction between *P. aeruginosa* bacteria and white fungus waste resulted in a significant superiority in increasing the numbers of *P. aeruginosa*, as the dual treatment B1Ab2 recorded the highest rate of bacterial numbers in the two periods, reaching (142.8,132.3)×10^7 CFU g^{-1} dry soil, respectively, compared to the cocontrol treatment B0Ab0, which gave (30.5,29.3)×10^7 CFU g^{-1} dry soil, respectively. The reason for the superiority of adding *P. aeruginosa* bacteria with white mushroom waste is that these bacteria are able to metabolize organic waste using their enzymes, and the waste that is added with them is often a source of carbon and energy. It is also clear that they secrete a wide range of enzymes, the most important of which is the amidase enzyme, and these enzymes enable them to consume the largest number of white mushroom waste, which leads to an increase in their numbers in the soil (Adeleye et al.,2017). The interaction between *P. aeruginosa* bacteria and nano-fertilizer showed a significant effect, as the dual treatment B1N3 recorded the highest rate of *P. aeruginosa* bacteria numbers at both time periods, reaching (139.9,115.0)×10^7 CFU g^{-1} dry soil, respectively, compared to the cocontrol treatment B0N0, which recorded (65.1,61.9)×10^7 CFU g^{-1} dry soil, respectively. The reason for the greater effectiveness of adding P. aeruginosa bacteria and nano fertilizer is based on the capacity of boron and nano zinc to keep up the performance of the bacteria that are introduced into the soil, from

both living-organism-related and environmental pressures because of its physical and chemical attributes which set it apart from other types of plant nutrients (Panishikal *et al*.,2021). Tables (3 and 4) show that the binary interaction between white mushroom waste and nano fertilizer gave a significant superiority in the rate of *P. aeruginosa* bacteria numbers, as the Ab2N3 treatment outperformed in both periods, recording (126.8,115.0)×10^7 CFU g^{-1} dry soil compared to the cocontrol treatment, which recorded the lowest number in both time periods, reaching (67.0,62.2)×10^7 CFU g^{-1} dry soil, respectively. The superiority of this treatment is attributed to the fact that the mushroom waste contains a variety of microorganisms that accelerate the decomposition process and nutrient availability, as boron and nano zinc also act as a catalyst for the organisms that decompose the added organic waste, thus increasing the activity of microorganisms, including *P. aeruginosa* bacteria (Sendi et al.,2013;upadhayay *et al*.,2023).

Table (3): Effect of *P. aeruginosa*, white fungus waste and nano fertilizer on the number of *P. aeruginosa* bacteria 10^7 (CFU g^{-1} dry soil) In the first harvest of stevia crop.

P. aeruginosa inoculation (B)		B0	B1		
		72.7	132.0		
LSD 0.05		1.0			
White mushroom waste (Tones h^{-1})		Ab0	Ab1	Ab2	
		72.4	111.2	123.5	
LSD 0.05		1.2			
Nano fertilizer (kg h^{-1})		N0	N1	N2	N3
		94.9	102.2	103.2	109.2
LSD 0.05		1.4			
Bilateral interaction between inoculation with P. aeruginosa and white fungus waste					
		Ab0	Ab1	Ab2	
B0		30.5	83.3	104.3	
B1		114.3	139.0	142.8	
LSD 0.05		1.7			
Bilateral interaction between P. aeruginosa inoculation and nanofertilizer					
		N0	N1	N2	N3
B0		65.1	73.4	73.8	78.4
B1		124.7	130.9	132.6	139.9
LSD 0.05		2.0			
The dual interaction between white mushroom waste and nano fertilizer					
		N0	N1	N2	N3
Ab0		67.0	71.5	73.8	77.2
Ab1		99.7	110.3	111.2	123.5
Ab2		118.0	124.7	124.5	126.8
LSD 0.05		2.4			
Triple interaction between study factors					
		N0	N1	N2	N3
B0	Ab0	24.3	30.7	31.7	35.3
	Ab1	69.7	84.7	85.7	93.3
	Ab2	101.3	105.0	104.0	106.7
B1	Ab0	109.7	112.3	116.0	119.0
	Ab1	129.7	136.0	136.7	153.7
	Ab2	134.7	144.3	145.0	147.0
LSD 0.05		3.4			

The statistically analyzed data in Tables (3 and 4) indicate that the triple interacton between the study factors showed a significant superiority in increasing the number of *P. aeruginosa* bacteria during the two periods, as the triple combination B1Ab1N3 outperformed in the two periods, which recorded (153.7,137.7)×10^7 CFU g^{-1} dry soil, respectively, compared to the cocontrol treatment, which recorded the lowest values, reaching (44.3,24.7)×10^7 CFU g^{-1} dry soil, respectively. The reason for the superiority of the combination of *P. aeruginosa* bacteria, white fungus waste, boron and nano zinc is that the combination of bio-, organic and nano-fertilizers represents a sustainable approach to eliminate the negative effects of environmental stresses in the soil, as boron is characterized by Nanozinc has slow decomposition, which increases its effectiveness in improving biochemical processes in the soil, in addition to the role of white fungus waste, which in turn is decomposed by microorganisms to give nucleophosphate and proteins, in addition to trace and essential elements and amino acids, which encourages the growth of bacterial strains more quickly, including *P. aeruginosa* bacteria (Adeleye *et al*.,2017;Raliya *et al*.,2018;Panishikal *et al*.,2021

Table (4). Effect of *P. aeruginosa* bacteria, white fungus waste and nano fertilizer on the number of *P. aeruginosa* bacteria10^7 (CFU g^{-1} dry soil) In the second harvest of stevia crop.

P. aeruginosa inoculation (B)	B0			B1	
	63.5			120.2	
LSD 0.05			1.3		
White mushroom waste (Tones h^{-1})	Ab0	Ab1		Ab2	
	66.6	97.1		112.0	
LSD 0.05			1.5		
Nano fertilizer (kg h^{-1})	N0	N1	N2	N3	
	88.6	89.9	91.1	97.7	
LSD 0.05			1.8		
Bilateral interaction between inoculation with P. aeruginosa and white fungus waste					
	Ab0	Ab1		Ab2	
B0	29.3	69.7		91.6	
B1	103.8	124.5		132.3	
LSD 0.05			2.2		
Bilateral interaction between P. aeruginosa inoculation and nanofertilizer					
	N0	N1	N2	N3	
B0	61.9	61.5	62.6	68.2	
B1	115.3	118.3	119.7	127.5	
LSD 0.05			2.5		
The dual interaction between white mushroom waste and nano fertilizer					
	N0	N1	N2	N3	
Ab0	62.2	64.5	67.0	72.5	
Ab1	95.5	93.8	93.0	106.0	
Ab2	108.2	111.3	113.3	115.0	
LSD 0.05			3.1		
Triple interaction between study factors					
	N0	N1	N2	N3	
B0 Ab0	24.7	27.7	29.7	35.3	
B0 Ab1	73.0	67.3	64.0	74.3	
B0 Ab2	88.0	89.3	94.0	95.0	
B1 Ab0	99.7	101.3	104.3	109.7	
B1 Ab1	118.0	120.3	122.0	137.7	
B1 Ab2	128.3	133.3	132.7	135.0	
LSD 0.05			4.3		

Activity of amidase enzyme in the soil after 120 and 240 days of addition.

Tables (5 and 6) present the combined effect of boron, nano zinc, white fungus waste and *P. aeruginosa* bacteria on the activity of the amidase enzyme in the rhizosphere soil of stevia plant after 120 and 240 days. Tables (5 and 6) show that the addition of the bio-vaccine B1 was significantly superior and recorded the highest average amidase activity during both periods, reaching 177.58 and 93.81 µg $N\text{-}NH_4^+ g^{-1}$ soil $2h^{-1}$, respectively, compared to the cocontrol treatment B0, which recorded the lowest amidase activity during both periods, reaching 91.36 and 42.31 µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$, respectively. The reason for the superiority of *P. aeruginosa* is due to its superiority in the number of bacteria (Tables 3 and4), as it plays a major role in controlling the activity of the amidase enzyme, as it activates the amidase enzyme by the division proteins present in its cell membrane, which are used to reduce the self-inhibition of the amidase enzyme. These proteins work to regulate the activity of amidase (Uhara *et al*.,2010; Peters *et al*.,2013; Yakhnina *et al*.,2015; Al-Maamouri *et al*.,2024).

The results of the statistical analysis showed the significant superiority of the study factor that includes the addition of white mushroom waste at three levels (Ab0, Ab1 and Ab2), The level Ab2 was superior by giving the highest activity of amidase enzyme at the periods of 120 and 240 days of addition, reaching 172.67 and 94.71 µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$, Respectively, as compared to treatment Ab0, which were 91.29 and 39.83 µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$, respectively (Tables 5 and6). The reason for the superiority of the waste in increasing the activity of the amidase enzyme in the soil during both periods is due to the fact that it was superior in increasing the numbers of *P. aeruginosa* bacteria as in (Tables 3 and4), which has a close association with the enzyme. The addition of white mushroom waste to the soil also plays an important role as it works to increase the microorganisms that are considered biological control elements and regulators of the ecosystem as they produce different types of enzymes, including amidase enzyme, which is one of the hydrolysis enzymes that works to decompose the organic matter in it and convert it into biomass, organic acids and carbon dioxide, and decompose the components of the soil at the same time to maintain the balance of nutrients (Sivojiene *et al*.,2021;Rabago *et al*.,2024).

The analysis data showed that the study factor included the addition of nano fertilizer at four levels (N0,N1,N2,N3), as the results shown in Tables (5and6) show that the N3 level was significantly superior during both periods, as it recorded 158.11 and 85.17 µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$, respectively, compared to the cocontrol treatment N0, which recorded 119.56 and 57.94 µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$, Respectively; the reason may be due to the superiority of the same treatment in increasing the number of *P. aeruginosa* bacteria that stimulate the secretion of the amidase enzyme as shown in Table (3and6) Additonally, the role of boron and zinc

nanoparticles in biological stimulation, which improved the levels of functional categories of soil bacteria in the rhizosphere region, which led to a significant improvement in their biosphere membranes, which helped some types of bacteria to overcome environmental pressures, which led to improving the process of carbohydrate metabolism and enhanced the rate of carbon decomposition by bacteria, thus increasing the activities of enzymes in general, including the amidase enzyme (Fei *et al*.,2020;lu *et al*.,2020;Al-Saadawi & Al-Taweel,2024a;Al-kafaji *et al*.,2024a).

The synergistic effect between *P. aeruginosa* bacteria and white fungus waste led to a marked superiority in the amidase activity. The dual treatment B1Ab2 recorded the maximum rate of amidase activity during both periods, which was 229.58 and 131.50 µg $N\text{-}NH_4^+$ g^{-1} soil 2 h^{-1} respectively, in cocontrol to B0Ab0 treatment, which was 69.00 and 27.75 µg $N\text{-}NH_4^+$ g^{-1} soil 2 h^{-1} respectively. The superiority of the treatment with *P. aeruginosa* bacteria and white fungus waste comes as a result of the former's superiority in *P*. aeruginosa richness as illustrated in Tables (3and4). which stimulates the secretion of the amidase enzyme in the soil, in addition to the fact that the fungus waste contains a high percentage of organic matter and many enzymes, but it is somewhat slow to decompose, so this problem can be solved when added in conjunction with the bio-inoculum, which helped in increasing the enzymatic activity in the soil, since microorganisms are known for their rapid response to biological decomposition and thus adapt quickly to various changes as they work to convert and decompose white fungus waste to produce elements that improve the biological properties of the soil (Das *et al*.,2010;Sarkar *et al*.,2022;Masa *et al*.,2024).

The interaction between *P. aeruginosa* bacteria and nano-fertilizer showed a significant effect, as the dual treatment B1N3 recorded the highest rate of amidase enzyme activity during both periods, reaching 215.67 and 123.22 µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$, respectively, compared to the cocontrol treatment B0N0, which recorded 83.78 and 37.89 µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$, respectively. The addition of *P. aeruginosa* bacteria plays a vital role in enhancing soil fertility by fixing atmospheric nitrogen, dissolving phosphorus and making other nutrients available that are often unavailable, leading to an increase in the number of enzymes secreted. However, the sensitivity of bioinoculation to environmental conditions, including pH and temperature, is one of the setbacks which was later minimized by using a combination with nanoparticles. This creates more efficient biosystems that enhance the activity of the enzymes in the soil; therefore, amidase enzyme activity is enhanced (Al-Saadawi and Al-Taweel,2024b; Verma *et al*.,2024; Al-kafaji *et al*.,2024b). These superiorities were really evident at the amidase enzyme activity (tables 6 and 7), wherein the interaction between white mushroom waste and nano-fertilizer manifested the trend; Ab2N3 treatment significantly out-yielded at the first harvest of stevia crop with a value of 187.83 µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$ compare to

Ab1N3 treatment, which showed significance at the second harvest of stevia crop of 106.33 µg N-NH_4^+ g^{-1} soil $2h^{-1}$ The control treatment, showed the lowest values at these periods with values of 77.83 and 35.17 µg N-NH_4^+ g^{-1} soil $2h^{-1}$ respectively.

Table (5): Effect of *P. aeruginosa*, white fungus waste and nanofertilizer on amidase activity (µg N-NH_4^+ g^{-1} soil $2h^{-1}$) In the first harvest of stevia crop.

P. aeruginosa *inoculation (B)*		B0			B1
		91.36			177.58
LSD 0.05		**1.72**			
White mushroom waste (Tones h^{-1})		**Ab0**	**Ab1**		**Ab2**
		91.29	139.46		172.67
LSD 0.05		**2.11**			
Nano fertilizer (kg h^{-1})		**N0**	**N1**	**N2**	**N3**
		119.56	127.89	132.33	158.11
LSD 0.05		**2.44**			
Bilateral interaction between inoculation with P. aeruginosa *and white fungus waste*					
		Ab0	**Ab1**		**Ab2**
B0		69.00	89.33		115.75
B1		113.58	189.58		229.58
LSD 0.05		**2.98**			
Bilateral interaction between P. aeruginosa *inoculation and nanofertilizer*					
		N0	**N1**	**N2**	**N3**
B0		83.78	89.00	92.11	100.56
B1		155.33	166.78	172.56	215.67
LSD 0.05		**3.45**			
The dual interaction between white mushroom waste and nano fertilizer					
		N0	**N1**	**N2**	**N3**
Ab0		77.83	90.67	93.50	103.17
Ab1		117.83	125.00	131.67	183.33
Ab2		163.00	168.00	171.83	187.83
LSD 0.05		**2.22**			
Triple interaction between study factors					
		N0	**N1**	**N2**	**N3**
B0	**Ab0**	61.33	70.00	70.33	74.33
	Ab1	81.33	84.67	90.00	101.33
	Ab2	108.67	112.33	116.00	126.00
B1	**Ab0**	94.33	111.33	116.67	132.00
	Ab1	154.33	165.33	173.33	265.33
	Ab2	217.33	223.67	227.67	249.67
LSD 0.05		**5.97**			

It may attribute the reasons behind the superiority of the binary treatment of white mushroom waste, boron, and nano zinc to overproduce the same to be superior in the populations of *P. aeruginosa*, as shown in Tables (4 and 5), which stimulates the secretion of amidase enzyme in the soil. In addition to the joint and direct effect of boron and nano zinc elements to the soil, This resulted in that it provoked the microorganism in the white-rot fungi waste decomposition, in return increases the rate of vitality in the soil for this plant which will consequently lead to more number of microorganisms in the soil that take part in producing and boosting the activity of many enzymes among them including amidase enzyme. (Bahir *et al*.,2020; Al-Taweel & Al-budairy,2024; Al-Jubouri & Al-Taweel,2024; Al-Hasnawi &Jarallah,2024).

Table (6): Effect of *P. aeruginosa*, white fungus waste and nanofertilizer on amidase activity (µg N-NH_4^+ g^{-1} soil $2h^{-1}$) In the second harvest of stevia crop.

P. aeruginosa inoculation (B)		B0			B1
		42.31			93.81
LSD 0.05		**1.63**			
White mushroom waste (Tones h^{-1})		**Ab0**	**Ab1**		**Ab2**
		39.83	69.62		94.71
LSD 0.05		**2.00**			
Nano fertilizer (kg h^{-1})		**N0**	**N1**	**N2**	**N3**
		57.94	62.89	66.22	85.17
LSD 0.05		**2.31**			
Bilateral interaction between inoculation with P. aeruginosa *and white fungus waste*					
		Ab0	**Ab1**		**Ab2**
B0		27.75	41.25		57.92
B1		51.92	98.00		131.50
LSD 0.05		**2.82**			
Bilateral interaction between P. aeruginosa *inoculation and nanofertilizer*					
		N0	**N1**	**N2**	**N3**
B0		37.89	40.78	43.44	47.11
B1		78.00	85.00	89.00	123.22
LSD 0.05		**3.26**			
The dual interaction between white mushroom waste and nano fertilizer					
		N0	**N1**	**N2**	**N3**
Ab0		35.17	39.17	41.00	44.00
Ab1		51.83	57.33	63.00	106.33
Ab2		86.83	92.17	94.67	105.17
LSD 0.05		**3.99**			
Triple interaction between study factors					
		N0	**N1**	**N2**	**N3**
B0	**Ab0**	21.67	27.00	29.67	32.67
	Ab1	35.67	37.67	42.00	49.67
	Ab2	56.33	57.67	58.67	59.00
B1	**Ab0**	48.67	51.33	52.33	55.33
	Ab1	68.00	77.00	84.00	163.00
	Ab2	117.33	126.67	130.67	151.33

The data in Tables (5 and 6) show that, the triple interaction among the studied factors significantly superior in enhancing amidase activity during both phases. For instance, the triple combination B1Ab1N3 was best in both phases with as high as 265.33 and 163.00 µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$ while the lowest values recorded during the two periods were 61.33 and 21.67 µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$ for their control treatment as illustrated in (Table 6 and 7). The superior performance of the bacteria combination of with, white fungus waste, boron and nano zinc may be due to the superiority of the treatment itself in the numbers of *P. aeruginosa* as shown in Tables (4 and 5) that stimulate the secretion of the amidase enzyme in the soil, Additonally, the importance of using these bacteria along with white fungus waste to be the main element in its decomposition, It could be attributed to the secretion of some hormones , acids , antibiotics amidase enzyme by the bacteria with works added effect of increasing effectiveness towards hydrolysis. Furthermore, the positive effect of boron or nano-zinc that acts as bio-stimulating elements for enhancing the effectiveness of enzymes in the soil (Panishikal *et al*.,2021; Upadayay *et al*.,2023; Al-Kalidi R. & Al-Taweel,2024; Al-Kalidi A. & Al-Taweel,2024; Abdul karim & H).

Conclusion

The interactive effect of boron, nano zinc, white fungus waste and bacteria *P. aeruginosa* revealed superiority in stimulating the amidase enzyme throughout both periods. The triple combination B1Ab1N3 which was the highest activity levels, recording 265.33 and 163.00 µg $N\text{-}NH_4^+$ g^{-1} soil $2h^{-1}$ respectively compared to the control treatment which recorded the lowest values (61.33 and 21.67 µg $N\text{-}NH_4^+g^{-1}$ soil $2h^{-1}$ respectively).

Acknowledgments

Based on the results of this study, we recommend to adopt the combination of nano fertilizer, waste of white mushrooms and *P. aeruginosa* as a bio-inculum. We recommend further research into other species of bacteria in the rhizosphere of Iraq's diversified environmental sites, isolating the effective ones, and using them as a bio-inculum. We also recommend an analysis at the other extreme, namely of the enzymes generated in the rhizosphere by bacteria, particularly *P. aeruginosa*, which represents a very sensitive pointer of soil vitality reflecting the activity of different groups of organisms toward their production in addition to the impact of the non-biotic environment on the soil itself; this factor indicates any reduction in the microbial activity. Even with an active role within the element cycle of the soil, some have proven sensitive to variation in the composition of microbes as well as the soil formation, especially a source rich in organic material like the residue from the pleurotus ostreatus mushroom, where the activity of the enzyme amidase was significantly enhanced in the soil with the inoculation of *P. aeruginosa* bacteria along with said residue of the white mushroom thus biological and organic management.

Contributions of authors

Z.J.K. : The research is extracted from the doctoral thesis of researcher Zahraa Jassim Kazim Al-budairy.
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Conflicts of interest

The authors declare no conflicts of interest.

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التأثير المشترك للبورون والزنك النانوي واللقاح الحيوي ومخلفات الفطر الأبيض في فعالية انزيم الأميديز في التربة

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المستخلص: أجريت تجربة في موقع زراعي تابع لدائرة البحوث الزراعية في العراق / محطة أبحاث الديوانية وبتاريخ 2024/1/15 بهدف معرفة تأثير عوامل الدراسة الثلاثة والمتضمنة العامل الأول وهو السماد الحيوي المتمثل ببكتريا *P. aeruginosa* والمرمز له بالرمز B بمستويين (عدم اضافة لقاح من بكتريا *P. aeruginosa* B_0 ، إضافة بكتريا *P. aeruginosa* (B_1) ، والعامل الثاني مخلفات الفطر الابيض والمرمز له Ab أضيف بثلاثة مستويات هي (دون إضافة Ab_0 ، 5 طن هـ$^{-1}$ كمستوى ثاني Ab_1، 10 طن هـ$^{-1}$ كمستوى ثالث Ab_2) ، اما العامل الثالث وهو السماد النانوي المرمز له N بأربعة مستويات هي (دون اضافة N_0 ، 4 كغم هـ$^{-1}$ زنك نانوي N_1، 2 كغم هـ$^{-1}$ بورون نانوي N_2 ،1 كغم هـ$^{-1}$ بورون نانوي + 2 كغم هـ$^{-1}$ زنك نانوي N_3) في زيادة أعداد بكتريا *P. aeruginosa* وتحفيز نشاط انزيم الاميديز بعد 120 و240 يوم من الاضافة بتربة مزروعة بنبات سكر الستيفيا .تشير البيانات المحللة احصائياً أن التأثير التآزري بين عوامل الدراسة الثلاثة أظهر تفوقاً معنوياً من خلال زيادة أعداد بكتريا *P. aeruginosa* وفعالية انزيم الاميديز خلال الفترتين، إذ تفوقت التوليفة الثلاثية B1Ab1N3 في الفترتين والتي سجلت (153.7و137.7) ×10^7 CFU g^{-1} dry soil و(265.33 و163.00) µg N-NH_4^+ g^{-1} soil $2h^{-1}$ بالتتابع قياساً بمعاملة المقارنة التي سجلت أدنى القيم في الفترتين بلغت (44.3 و24.7)×10^7 CFU g^{-1} dry soil و (61.33و 21.67 µg N-NH_4^+ g^{-1} soil $2h^{-1}$) بالتتابع.

الكلمات المفتاحية: انزيم الأميديز، الأسمدة النانوية، المخلفات العضوية، اللقاح الحيوي.