TRIPLE CONTROL AGAINST FUSARIUM WILT DISEASE OF FABA BEAN AND ITS IMPACT ON PRODUCTIVITY

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Fusarium wilt, a fungal disease, significantly threatens faba bean yield in Egypt. This current study investigated the potency of a combined application of endophytic bacteria, decomposed olive leaves extract (OLE), and iron nanoparticles (FeNPs) for its biocontrol and growth promotion in faba bean. Two endophytic bacterial strains isolated from helthy faba bean plants and identified using 16S rRNA nucleotide sequence as *Bacillus subtilis* and *Pseudoroseomonas wenyumeiae* were studied for their antagonistic and plant growth promoting traits activities, FeNPs was biosynthesized via *Pseudoroseomonas wenyumeiae*. OLE was biodegraded using a fungus *Bosea thiooxidans* to enhance its antifungal potential. A research experiment was designed based on natural field infection. Significant differences in disease severity index (DSI) and disease incidence (DI) were noticed. While untreated plants exhibited the highest disease burden (DI = 72.5%, DSI = 0.275),the combined application of bioagents, decomposed OLE, and FeNPs as both soil drench and foliar spray (FeNPs) showed the lowest disease severity (DI = 34.2%, DSI = 0.089). Treated plants demonstrated improved growth parameters, including increased height, wet and dry weight, number of pods, weight of 100 seeds, macro- and microelement content. This study suggested that the synergistic combination of endophytic bacteria, OLE, and FeNPs offered a promising and sustainable biocontrol strategy against *Fusarium* wilt in faba bean, promoting plant growth and reducing reliance on chemical pesticides.

Keywords: *Fusarium oxysporum*, bacterial endophyte, olive leaves extract, synthesized iron nanoparticles

INTRODUCTION

Wilt disease caused by *Fusarium oxysporum* is a dead world widely vascular syndrome in several economically important crops as tomato and

faba bean (Sampaio et al., 2020), results in its prolonged survival of the fungus in soil (Jamil et al., 2021), and generation of resistant species to commercially labile fungicides (Florencio-Anastasio et al., 2022). Fungal spores were found to survive for many years in soil (Mbasa et al., 2021), the fungus affects the water and the flow of nutrients in the plant after infecting (Joshi, 2018), causing yellowing, wilting and plant death (Soleha et al., 2022).

Endophytic bacteria live within tissues of plant without causing harm and often benefit the plant parts (Compant et al., 2021). Their beneficial effects include promoting plant growth, improving health, and enhancing the efficiency of phytoremediation in the rhizosphere (the area around plant roots) (Li et al., 2023). They apply mechanisms such as: competition for nutrients, antibiosis, ecological niches and induced systemic resistance to supersede the phytopathogen (Prakash, 2023). The efficient of endophytes depends on several factors such as: host specificity, colonization pattern, population dynamics, ability to move within tissues of host and induced systemic resistance (Wippel, 2023). Endophytic bacteria put down pathogens that cause economically important diseases in several crops (Sulaiman and Bello, 2023), they can produce antibiotics and enzymes such as glucanases, chitinases, lipases and proteases, which cause cellular lysis (Ajuna et al., 2023). Leaves of the olive tree (*Oleaeur opaea* L.) are the first botanical mentioned in the Bible (Kazmi et al., 2023 and Jasprica et al., 2023) and have been applied for medicinal purposes ancient times ago. The researches have concluded that olive leaves extract (OLE) have antimicrobial properties (Yangui et al., 2021), include compounds with potent antimicrobial activities against fungi, mycoplasma and bacteria (Silvestrini et al., 2023), and have high content of phenols and flavonoids (luteolin, oleuropein and their derivatives). These compounds are demarcated as secondary metabolites and used as antioxidant, antimicrobial activities and anti-inflammatory. Ibrahim et al. (2021) described that olive leaves inhibit fungi as well as gram negative and positive bacteria. Zemheri-Navruz et al. (2019) and Gholamhosseini et al. (2020) reported that OLE can increase the immune response against viruses by stimulating phagocytosis. Nanotechnology implementation in agriculture has considered a revolution in agrotechnology in recent years (Chanu and Singh 2022), that completely replace the current farming practices. Therefore, disease management in plants applying nanotools could be beneficial in providing protection to plants against pathogens or management of disease through highly regulated and battered disposition of active components at require places (Zhera et al., 2021).

The green synthesized of iron nanoparticles (FeNPs) technique is a better alternative to chemical and physical methods for the generation of nanoparticles (Afolalu et al., 2020 and Al-Radadi, 2022). It is not only

cheap, but it is also less complex and consuming time, safe, eco-friendly, and non-toxic. Moreover, it contains far less requirement for energy, more practical control of chemicals and reagents and less wastage of inputs (Nadeem et al., 2021). FeNPs have promising antimicrobial properties, they can fight bacteria and fungi through various mechanisms, including generation of reactive oxygen species, membrane disruption, and interference with cellular processes (Godoy-Gallardo et al. 2021). Overall, iron nanoparticles hold immense promise for revolutionizing disease management. Their unique properties and versatility offer exciting possibilities for developing novel diagnostic tools, targeted therapies, and effective antimicrobial agents (Patra et al., 2018). Therefore, the purpose of this study was the triple control against *Fusarium* wilt disease of faba bean caused by *F. oxysporum* using two effective endophyte bacterial strains, fermented OLE and FeNPs.

MATERIALS AND METHODS

1. Isolation of Causal Agent *Fusarium oxysporum*

F. oxysporum isolates were isolated from the diseased roots of faba bean (*Vicia faba* L.) plants taken from Toshka station, Aswan governorate, Egypt. Pieces of root were cut down and placed aseptically on potato dextrose agar (PDA) medium. Incubated at 22ºC under 12 h photoperiod. *Fusarium* isolates identification was made depending on their morphological characters. One *F. oxysporum* monoconidial isolate was taken and maintained on PDA medium at 22ºC.

2. Biocontrol Agents Preparation

2.1. Preparation of endophytic bacteria

2.1.1. Isolation of bacterial bioagent

According to Mathiyazhagan et al. (2004), endophytic bacterial were isolated with some modifications. Healthy faba bean plants were gently collected and transported to research laboratory. A sterile scalpel was used to cut the roots into pieces 2–3 cm long, sample roots were removed, and they were surface sterilized by immersing them in 70% alcohol for one minute, 2.5% sodium hypochlorite for two minutes, and 0.1% $HgCl_2$ for one minute. They were then cleaned in four variations of sterile phosphate buffered saline (PBS). The sterility of aliquots from the last buffer wash was examined. Using a sterile pestle and mortar, the chosen samples were triturated in 10 ml of PBS.

2.1.2. *In vitro* antagonism against pathogenic fungi

On PDA medium, the effectiveness of each endophyte bacterial isolate against *F. oxysporum* was examined by plate assay (Ishikawa et al., 2000). The selected isolates were inoculated in vitro on the superficial of agar plate, 2 cm away from fungal disc. Abacterial culture loop was applied to the surface of PDA and King B plate and after 96 hours of incubation at

28±1°C, Every Petri dish was injected with a loopful of *F. oxysporum* mycelia. After ten days of incubation at 25°C, fungal growth was detected. The length of the cleared zones of antagonistic growth that is, the distances between the growth of bacteria and fungi were measured. Each test was repeated four times with mean values and standard error deviation. The formula 100 × C − T/C (T, treatment; C, control) was used to calculate the value of inhibition. As a control, a Petri plate without bacterial isolate was used. Two bacterial bioagents with high antagonistic activities were selected.

2.1.3. Properties of endophytic bacterial isolates that promote the growth of plants

Phosphate Solubilization

The selected bacterial bioagents were added to Pikovaskya (PKV) agar plates to qualitatively assess the phosphate solubilization. After a 5-day incubation period at 28±1ºC, a clear zone formed surrounding the bacterial growth, signifying its ability to solubilize phosphate were recorded, as reported by Gaur (1990).

Indole acetic acid (IAA) production

Using a spectrophotometer, produced indole acetic acid (IAA) was identified in accordance with Ehmann (1977).

Production of NH3

The ability of bacterial isolates to produce ammonia in peptone water was examined. After inoculating freshly formed cultures with 10 ml peptone water in each tube, the cultures were cultured for 48–72 hours at 28±2ºC. Each tube received 0.5 ml of Nessler's reagent and a positive test result for ammonia production was the development of a brown to yellow colour (Cappuccino and Sherman, 1992).

2.2. Prepatation of decomposed olive leaves extract (OLE)

2.2.1. Decomposed olive leaves extract (OLE) preparation

The cellulose decomposing activity of the fungus *Bosea thiooxidans* (provided from microbiology lab, Desert Research Center) was confirmed by detecting the colonies on CMC agar and then incubating them for 48 hours at 28°C. The plates were submerged with 0.1% Congo red for 20 minutes following incubation, then for a further 20 minutes with 1 M NaCl (Teather and Wood, 1982), alternatively, they were submerged for five minutes in Gram's iodine (2 g potassium iodide and 1 g iodine in 300 ml of distilled H_2O) (Kasana et al., 2008). According to a modified method of (Ishikawa et al., 2000), colonies exhibiting discolouration of the Congo-Red or Gram's iodine were deemed positive cellulose decomposers and were selected for identification using universal 16S rRNA primers.

2.2.2. Solid-state fermentation of olive leaves

Fresh olive leaves samples were collected, washed, cut into small pieces, and employed, as substrates for the solid-state fermentation process

that produces antifungal agents (Pan et al., 2012). Fresh olive leaves weighing around 200 g each were cleaned, chopped into tiny bits, placed inside glass jars, and autoclaved for 15 minutes at 121°C. Nutrient agar medium containing 5% yeast extract, 1% glucose, 0.5% peptone, and 2% agar was used to cultivate *Bosea thiooxidans* for a full day at 30°C. After incubation, 2×10^6 fungal colony / ml of sterile dist. water was used to form a bacterial suspension. Each glass jar holding 200 g of sterile olive leaf had about 100 cm of the bacterial suspension injected aseptically. The jars were then incubated at 30°C for 21 days. The jars were shaken every 2 days. After incubation, a clear supernatant was obtained by filtering the fermented olive leaves through cotton wool and centrifuging the mixture for 10 minutes at 6000 rpm. For assessing OLE antifungal potency, the acquired stock supernatant was maintained as 100%.

2.2.3. Evaluation of the antimycotic activity of OLE *in vitro*

Antagonistic activity of OLE was tested according to the technique of Babu Joseph and Kumar (2008). Sterile PDA medium was combined with varying quantities of crude extract (3, 5, 7, 10, and 15%), and the mixture was then transferred into sterile Petri dishes. After a week of growth, 0.5 cm diameter pieces from the fungal colony were placed onto PDA plates and incubated at 25±2°C. The growth diameters of the colonies were measured once the fungal growth in the control treatment reached the plate edge. The following formula was used to get the inhibition percentage:

Inhibition percentage = fungal growth average at control - growth average at treatment) / (growth average at control × 100.

2.2.4. Chemical screening of olive leaves extract (OLE)

For OLE, chemical assay of total saponins, terpenoids, alkaloids and tannins were carried out using high-performance liquid chromatography (HPLC) following standard methods (Biswas et al., 2013).

2.3. Iron oxide nanoparticle (Fe_2O_3NPs) biosynthesis

FeNPs synthesis was tested by the two selected endophytic bacteria isolates (isolates 1 and 2) which controlling the wilt disease caused by *F. oxosporium*. Only bacterial isolate no. 2 can synthesize iron oxide nanoparticle (Fe_2O_3NPs). The synthesis of FeNPs was done by selected isolate of endophytic bacteria which controlling *Fusarium* Wilt disease that is caused by *F. oxosporium*.

The fresh endophyte bacterial isolate was maintained on nutrient agar medium at 37°C for 24 hours. Further, the culture was subcultured into nutrient broth medium at continuous orbital shaking at 150 rpm (LM-570RD, Yihder, Thiwan), 37°C for 24 hours. The supernatant was collected after centrifugation at 5000 rpm for 5 min in overnight bacterial culture and it is used for synthesis of Fe_2O_3NPs. The cell free supernatant (100 ml) and 100 ml of ferric chloride solution (0.01 M) was taken in a 500 ml beaker. The beaker containing mixture solution was placed on a magnetic stirrer at room temperature for 24 hours and it was dried at 120°C. This dried sample

was annealed at 700°C for 5 h because the energy from the heat can enhance the vibration and diffusion of the lattice atoms for crystallization (Fig. 1).

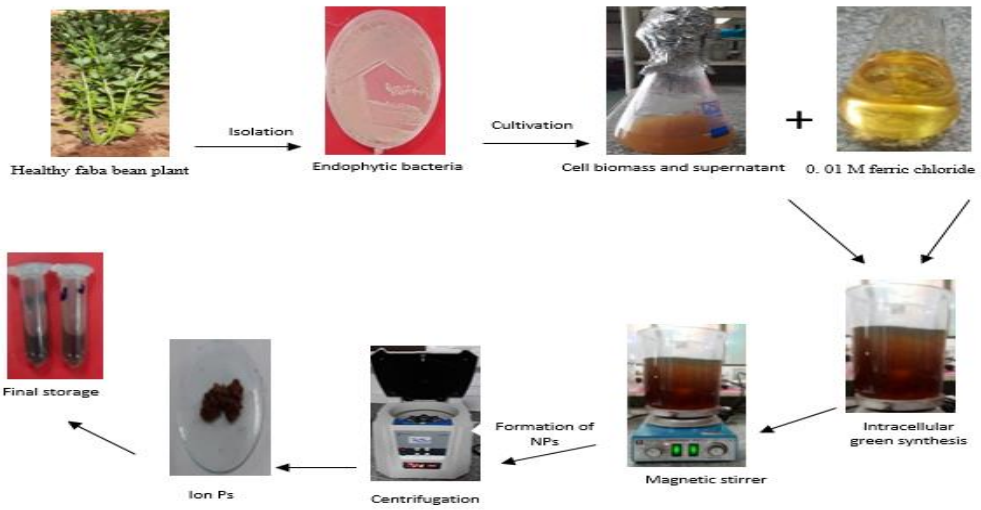

Fig. (1). Schematic diagram of Fe-NPs biosynthesis.

2.3.1. Iron oxide nanoparticle (Fe_2O_3NPs) characteristics

Spectrum analysis in UV-Vis

UV-Visible absorption spectroscopy (UV340-111503), one of the crucial methods to confirm the development of metal nanoparticles given surface plasmon resonance occurs for the metal, was used to monitor the formation of nanometal (Basavaraja et al., 2008). The ability of coloured materials to selectively absorb light within the visible portion of the electromagnetic spectrum and scan the spectra between 200 and 600 nm at a resolution of 1 nm is what gives materials their colour. UV-Vis's spectrum analysis performed at Desert Research Center, Cairo, Egypt.

Particle size analyzer

Using dynamic light scattering, the particle size of newly synthesized FeNPs was determined (DLS). An UV-Vis spectrophotometer was used to find the FeNPs absorption peak.

Utilizing energy dispersive X-ray (EDX) analysis with scanning electron microscopy (SEM)

Using SEM analysis, EDX (JEOL, JSM-6360LA, Japan) examined the qualitative and quantitative chemical makeup of the bacterially produced FeNPs (Hamza et al., 2022).

An infrared spectrophotometer with a Fourier transform (FTIR)

FTIR To ascertain the nature of related functional groups and structural elements of green produced nanoparticles, spectroscopy was used. Using the KBr pellet approach, 100 mg of KBr pellet were used to encapsulate 10 mg of nanoparticle powder, which was then analyzed in a FTIR spectroscope with a scan range of 400 to 4000 cm^{-1} and a resolution of 4 cm^{-1} (Ashokkumar and Ramaswamy, 2014).

2.3.2. Antifungal assay of nano iron

The antibacterial effectiveness of the produced FeNPs was evaluated against *F. oxosporium*. To achieve this, various quantities of Fe_2O_3NPs (0.01, 0.03, 0.05, 0.07, and 0.1%) were added to PDA medium. The antifungal activity of a chemical fungicide that is sold commercially (Metalaxyl + Mancozeb) was also evaluated for improved assessment. This chemical fungicide was used as a negative control and was diluted with PDA at the same concentrations.

3. *In Vivo* Efficacy Test

3.1. Field experiment

The study was conducted during the 2023 winter growing season at Toshka station, Desert Research Center, Aswan governorate, Egypt. The soil texture was sandy with 59.5% nitrogen, 0.39% organic matter, pH 7.95 and electrical conductivity of 1.82 $DS.m^{-1}$. The fertilizer used was calcium super phosphate (15.5% P_2O_5) and potassium sulphate (48.5 K_2O) in the rate of 200 and 50 kg/fed and were broadcasting before sowing. Nitrogen fertilizer in the form of ammonium sulphate (20.5% N) in the rate of 15 kg N/fed to enhancing saymputic bacteria and face the nutrients needs in the early stage of faba bean plants in this new cultivated area. Maryout-2 cultivar of faba bean was acquired from the Genetic Resources Department, Desert Research Center, Cairo, Egypt. While two endophytic bacteria bioagents (10^8 cfu/ml) were added into the soil at 20 ml/plant during sowing. Crude extract of decomposed olive leaf at 15% dilution and Fe_2O_3NPs (1000 ppm) were added after 30 and 60 days of sowing which called triple control. The experiment was statistically designed as a completely randomized design with three replicates. The following treatments were conducted: T1: control (without treatment), T2: soil application of OLE and FeNPs, T3: foliar spraying of OLE and FeNPs and T4: soil application + foliar spray.

3.2. Growth traits, yield and its components

Plant height (cm), number of branches, fresh and dry weights (g), and the number of pods/plants (g) and 100 seeds weight (g) at harvest were measured. For the chemical examination of faba bean plants, nitrogen was assessed using the micro-Kjeldahl method (Bremner and Mulvaney, 1982). Spectrophotometers and flame photometers were used to quantify potassium and phosphorus, respectively (Page et al., 1982). In contrast, the Ionic

Coupled Plasma determined the values of Ca, Fe, Zn, and Mg (Alloway, 1995).

3.3. Disease incidence

The disease incidence percentage (DI%) was determined using the following equation: Percentage of disease incidence = number of infected plants / total number of plants observed x100

3.4. Disease Severity (%)

Data of disease severity were recorded using the nine-category disease rating scale (Sahile et al., 2008). The disease severity (%) was calculated using the following equation:

$$DS\% = (\sum n)/9N \times 100$$

$\sum n$= the sum of all scale ratings, 9 is the highest category on the rating scale and N= sample size.

4. Analytical statistics

The results were statistically analyzed using Statistics version 9 software. Differences across treatment options were deemed significant when they exceeded the least significant differences (LSD) at the 5% mark (Analytical Software, 2008).

RESULTS AND DISCUSSION

1. Isolation and Identification of *Fusarium oxysporum*

To conduct an antagonistic investigation, *F. oxysporum* monoconidial isolate was isolated and kept on PDA medium at 22°C. *Fusarium* species are the most prevalent types of fungi that hinder faba bean growth and consequently resulting in notable yield reductions (Xiao et al., 2021).

1.1. Selection and identification of endophytic bacteria

Investigation of the antagonism against *F. oxysporum* revealed that only two bacterial species–out of 14 had antagonistic activities against *F. oxysporum* (Table 1). However, these two isolates had antagonism against *F. oxysporum*, the range of the inhibition zone was 6-9 ml. The higher ability to compete for nutrients or the products released in the medium, which slow down or eliminate *F. oxysporum* pathogen, are thought to be the causes of inhabitation (Gajera et al., 2013). These findings are consistent with the study of Zheng et al. (2011). For bacterial identification, through partial sequencing of 16S rRNA, the molecular characteristics of bacterial isolate 1 was ascertained. It was belonging to *Bacillus subtilis* with blast identity of 100% while, isolate 2 was closely related to *Pseudoroseomonas wenyumeiae* with 95% explosive persona. The two bacterial strains' phylogenetic tree and the strains that were closely connected are shown in Fig. (2).

Table (1). Morphology and enzyme production by the two endophytic bacterial isolates.

Characteristic	Colony colour	Shape	Gram stain	Motility	Spore forming	Production of enzymes	Alkaline phosphatase	Lipase	Catalase	Oxidase	Cellulase	Protease	Pectinase
Isolate (1)	Slightly yellow	Rod	+	+	+		+	+	+	+	+	+	+
Isolate (2)	White	Coccus	-	-	-		+	+	+	+	+	+	+

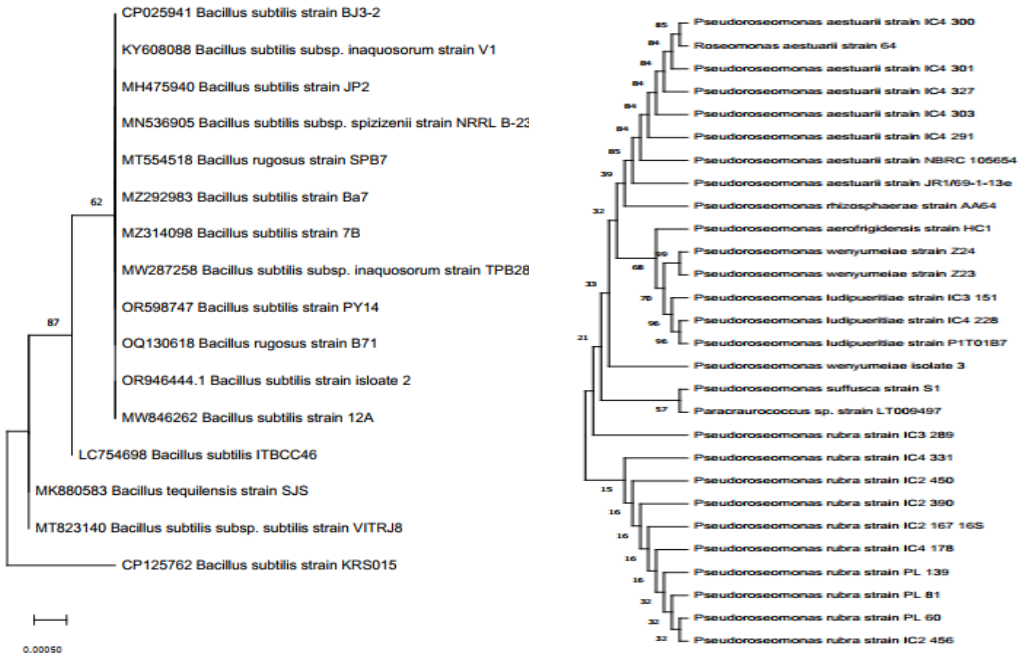

Fig. (2). Phylogenetic tree of partial 16S rRNA sequence for *Basillus subtilis* and (on the left) and *Pseudoroseomonas wenyumeiae* isolates (on the right).

1.2. Properties of plant growth promoting traits of endophytic bacterial isolates

The results of an investigation into the chosen strains' plant growth-promoting characteristics are given in Table (2).

1.3. Solubilization of phosphate

On PKV agar plates, inorganic phosphate was qualitatively dissolved. Both isolates under investigation were capable of effectively solubilizing inorganic phosphate. *B. subtilis* was more active (10 ml) than *Ps. wenyumeiae* (Fig. 3). It is thought that the solubilization of insoluble phosphate from rock phosphate was caused by the excretion of microbial metabolites such organic acids. Phosphate is frequently the limiting nutrient for microbial and plant growth in soil (Rodriguez et al., 2004). According to reports, the plant's yield grew because of microorganisms that solubilize phosphate in various crops (Shi et al., 2014).

Table (2). Characteristics of plant growth promoting traits of two endophytic bacterial strains.

Characteristics	Phosphate activity	Indole acetic acid test	Ammonia production
Basillus subtilis	++	++	+
Pseudoroseomonas wenyumeiae	+	+	+

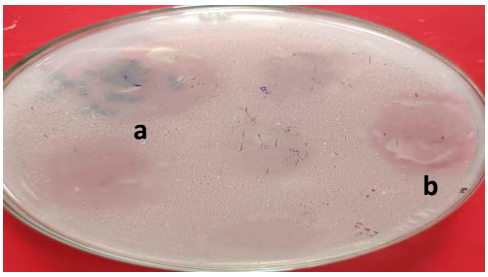

Fig. (3). The ability of **a.** *Basillus subtilis* and **b.** *Pseudoroseomonas wenyumeiae* to solubilize inorganic phosphate performed on Pikovaskya (PKV) agar plates qualitatively.

1.4. Indole acetic acid (IAA) production

Indole acetic acid is a phytohormone that directly promotes plant development. The amount of IAA produced varied from isolate to isolate, with relatively high amounts (61 µg/ml) in *B. subtilis*, followed by 42 µg/ml in *Ps. Wenyumeiae* (Fig. 4). These findings closely matched those of several investigations on the rhizobacteria's synthesis of IAA, which demonstrated a notable increase in IAA by the addition of tryptophan to the growth medium (Patten and Glick, 2002 and Sauvêtre and Schröder, 2015). Through root initiation, cell division, enlargement, higher growth rate, and apical dominance, auxin may promote plant growth (Frankenberger et al., 1995).

1.5. Production of NH3

Both *B. subtilis* and *Ps. wenyumeiae* showed a positive production of ammonia as shown in Fig. (4). It has been proposed that generated ammonia increases the amount of nitrogen that is accessible in the soil and may benefit plant growth. According to these results, Ahmed et al. (2008) analyzed 52 isolates of rhizobacteria that promote plant growth and discovered that every isolate of *Ps. wenyumeiae* that was evaluated was capable of producing ammonia. Similar results to those achieved by Anitha and Kumudini (2014) were reported.

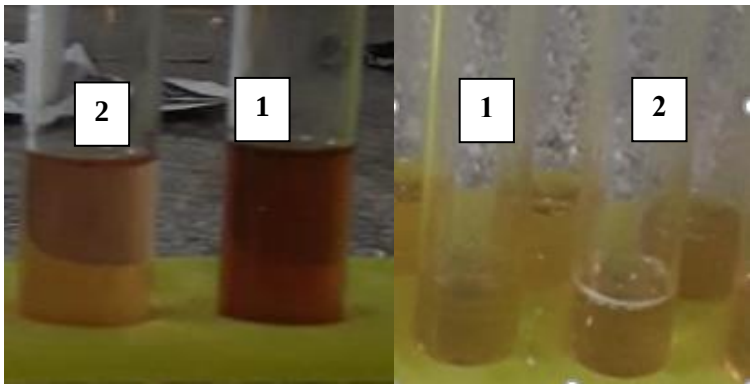

Fig. (4). Activity of (1) *Basillus subtilis* and (2) *Pseudoroseomonas wenyumeiae* to **a.** IAA test and **b.** ammonia production.

2. Chemical Screening of Olive Leaves Extract (OLE)

Screening of phenolic compounds in decomposed OLE using HPLC revealed the presence of tannins, terpenoids, alkaloids and flavonoids, while saponins were not detected in decomposed OLE. Through comparison of standard retention periods and UV spectra under HPLC settings, many chemical classes of phenolic components were found in the decomposed OLE (Table 3). There was a higher concentration of hydroxybenzoic acids and hydroxycinnamic acids in the decomposed OLE. Thirteen of the nineteen unique phenolic compounds that were examined and measured in the extract were found. Pyrocatechol was the main phenolic compound in decomposed OLE (22.85 µg/ml), followed by chlorogenic acid (10.63 µg/ml) and gallic acid (9.59 µg/ml). Other phenolic chemicals were found in the crude extract include methyl gallate (1.85 µg/ml), naprenin (1.26 µg/ml), catechin (1.07 µg/ml), daidzein (0.57 µg/ml), and caffeic acid (0.51 µg/ml). Minor amounts of ellagic acid, gallic acid, syringic acid, p-hydroxybenzoic acid and apigenin were also observed.

Antifungal activity of decomposed olive leaves extracts (OLE)

Different concentrations of decomposed OLE (3-15%) were tested for their inhibitory effect of *F. oxysporum* growth *in vitro*. The data presented in Fig. (5) demonstrate that the mycelial development of *F. oxysporum* was inhibited in a concentration-dependent manner by all tested concentrations of OLE. The concentration of 15% provided the highest growth inhibitions (71%) whereas the lowest growth inhibition (12.5%) was observed at 3%. Most studies confirmed the relationship between OLE and its biological activity. Additionally, antifungal effects of OLE have been already described in many studies. Subsequently, oleuropein was reported to be the major component of OLE that exhibited good antifungal activity (Zorićet al., 2016 and Hallouma et al., 2023).

Table (3). Phenolic compounds in decomposed olive leaves extract as determined by HPLC analysis.

No.	Compounds	Chemical classes	Conc. (µg/ml)
1	Gallic acid	Hydroxybenzoic acids	9.59
2	Chlorogenic acid	Hydroxycinnamic acids	10.63
3	Catechin	Flavan-3-ols	1.07
4	Methyl gallate	Galloyl esters	1.85
5	Coffeic acid	Hydroxycinnamic acids	0.51
6	Syringic acid	Dimethoxybenzene	0.28
7	Pyrocatechol	Hydroxylated phenols	22.85
8	Rutin	Flavonoids	0.00
9	Ellagic acid	Hydroxybenzoic acids	0.00
10	Coumaric acid	Hydroxycinnamic acids	0.09
11	Vanillin	Phenolic aldehyde	0.16
12	Ferulic acid	Hydroxycinnamic acids	0.35
13	Naringenin	Flavonoids	1.26
14	Daidzein	Isoflavonoids	0.57
15	Querectin	Flavonoids	0.00
16	Cinnamic acid	Carboxylic acid	0.20
17	Apigenin	Flavanon-glycoside	0.00
18	Kaempferol	Flavonoids	0.00
19	Hesperetin	Flavanon-glycoside	0.00

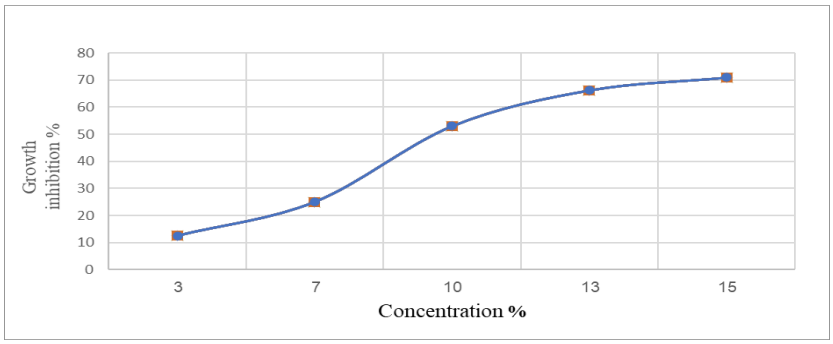

Fig. (5). Effect of decomposed olive leaves extract concentrations on the growth inhibition percentage of *Fusarium oxysporum.*

3. Iron Nanoparticles Synthesized Using Biosynthesis

By exposing a precursor aqueous salt $FeCl_3$ solution (0.01M $FeCl_3$ of final concentration) to bacterial cell-free filtrate of *Ps. wenyumeiae* in an aqueous solution, FeNPs was synthesized. The reaction was run for 72 hours at 35°C, in the dark, and with 150 rpm of shaking. The distinctive dark yellowish-brown changing colour of the mixture indicated the synthesis of FeNPs. *Ps. wenyumeiae* bacterial cell-free filtrate were reduced aqueous iron ions to FeNPs, and the colour reaction was caused by the metal nanoparticles' surface Plasmon vibration being excited (Shahverdi et al., 2007).

3.1. Iron nanoparticle characterization

3.1.1. Spectrum analysis in UV-Vis

As previously reported, absorption peaks were detected at 200-700 nm ranges due to the stimulation of surface plasmon vibrations in FeNPs (Tran et al., 2010). According to Fig. (6), nanoparticles were seen at 212 and 250 nm, which was because of charge transfer spectra. By monitoring UV/Vis spectra, the bio reduction of Fe ions in aqueous solutions was observed. The absorption spectra of green produced FeNPs were studied using UV-Vis. The resulting NPs had a particle size of 84.8 nm (Fig. 7). Considering the results of other researchers, FeNPs produced by *Ps. wenyumeiae*, the UV-Vis spectrophotometer displayed approximately peaks at wavelengths of 226 and 276 nm (Mazumdar and Haloi, 2011). However, two peaks were seen in the FeNPs study employing the fungus *Alternaria alternate*, one at 238 nm and the other at 265 nm (Mohamed et al., 2015). According to Saranya et al. (2017), Fe_2O_3NPs exhibited a distinctive absorption peak at 310 nm in UV-Vis absorption. However, two absorption peaks of FeNPs produced by *Sargassum muticum* aqueous extract were added at wavelengths of 402 and 415 nm, according to Mahdavi et al. (2013).

3.1.2. SEM and EDX studies

SEM images of the prepared FeNPs at different magnifications displayed its hexagonal shape. EDX graphs show that iron oxide was formed. Results in Fig. (8), display the spot-profile mode registered EDX spectrum. The EDX analysis clearly shows that the weight percentage of iron in the FeNPs generated by *Ps. wenyumeiae* was 25.8% (Fig. 9).

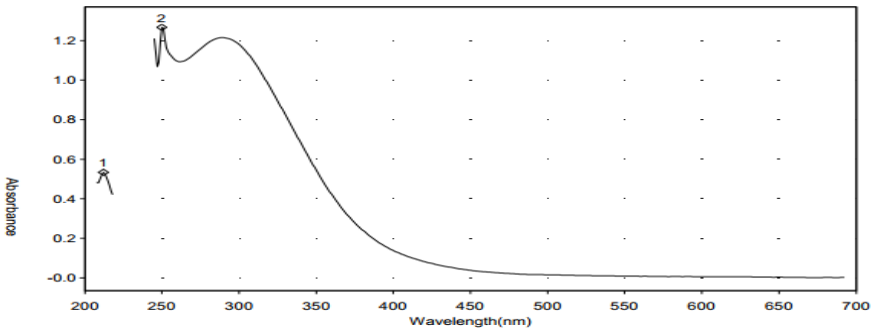

Fig. (6). The detected iron nanoparticles using UV-visible absorption spectroscopy at 212–250 nm.

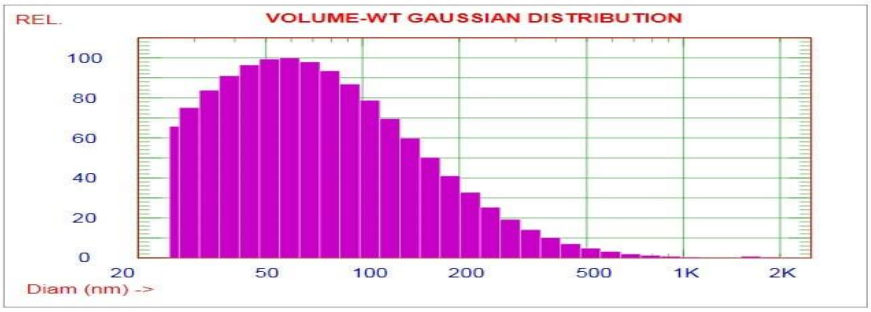

Fig. (7). Particle size of iron nanoparticles synthesized by *Pseudoroseomonas wenyumeiae*.

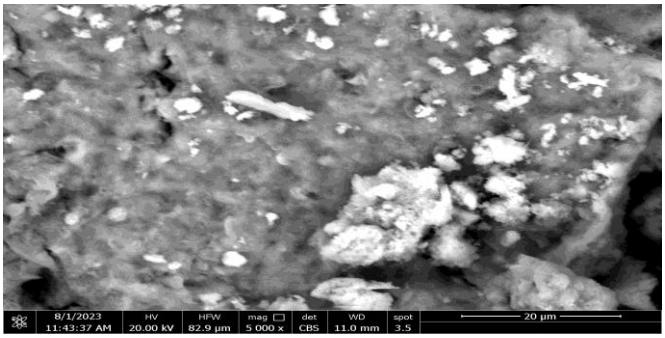

Fig. (8). SEM image of iron nanoparticle.

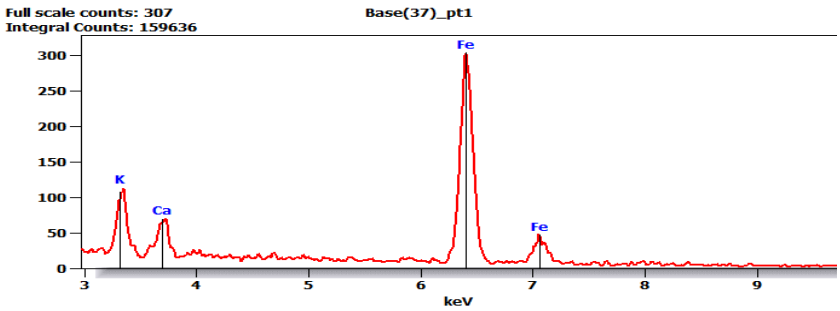

Fig. (9). Iron nanoparticles EDX spectrum. Iron made up 25.8% of the weight, and Ca was 15%.

3.1.3. FeNPs analysis using Fourier transform infrared spectrophotometry (FTIR)

FTIR spectrum of nano green synthesized FeNPs displayed stretching vibrations as illustrated in Fig. (10), the amide linkages connecting amino acid residues in proteins provide the well-known signatures in the infrared region of the electromagnetic spectrum. The band observed at 3202 cm^{-1} was assigned to the stretching vibrations of primary and secondary amines. for alkyne group, 1604.83 cm^{-1} for C=C, 1035.25 cm^{-1}. This result supports the presence of protein and other bioactive compounds on the surface of biosynthesized FeNPs, confirming that metabolically produced bioactive compounds act as capping agents during production and prevent the reduced iron particles agglomeration. In addition, O–H absorption peak of 439.18 cm^{-1} refer to Fe-O stretches of Fe_3O_4 and Fe_2O_3, confirming the formation of biosynthesized FeNPs. Manivasagan and Kim (2015) and Ahmad et al. (2017) have reported that, bonds functional groups such as –C-O-C-, -C-O-, -C=C- are derived from heterocyclic compounds like proteins, which may be the capping ligands of AgNPs.

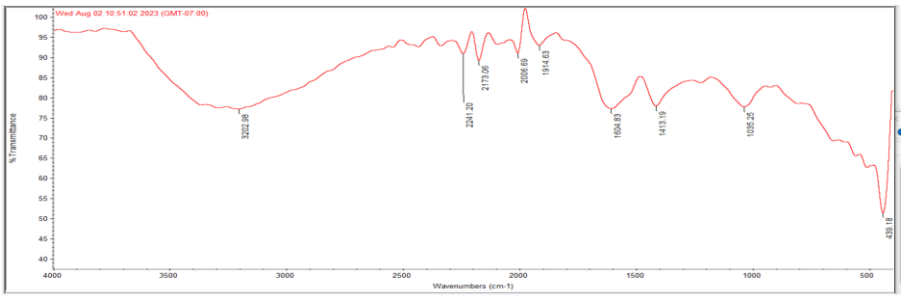

Fig. (10). FTIR spectra demonstrating that the surface of biosynthesized iron nanoparticles containing proteins and bioactive substances.

3.1.5. Assay for antifungals

Various concentrations of chemical fungicides (Metalaxyl + Mancozeb) and Fe_2O_3NPs showed varying levels of growth inhibition (Fig. 11). Yet, 0.1% concentration produced the best and most comparable results in both situations. Chemical fungicides and Fe_2O_3NPs demonstrated 81.3% and 81% growth suppression at this dose, respectively. The growth inhibition of nanoparticles was superior to chemical fungicides at lower concentrations. The findings also showed that *F. oxysporum's* mycelial growth can be significantly inhibited by nanoparticle concentrations as low as 0.01%. These results suggest that nanoparticles may play a part in the management of disease. To protect crops from a variety of plant diseases, different concentrations of these nanoparticles dramatically suppressed the growth of *F. oxysoprum*. This makes them an effective substitute for synthetic chemicals.

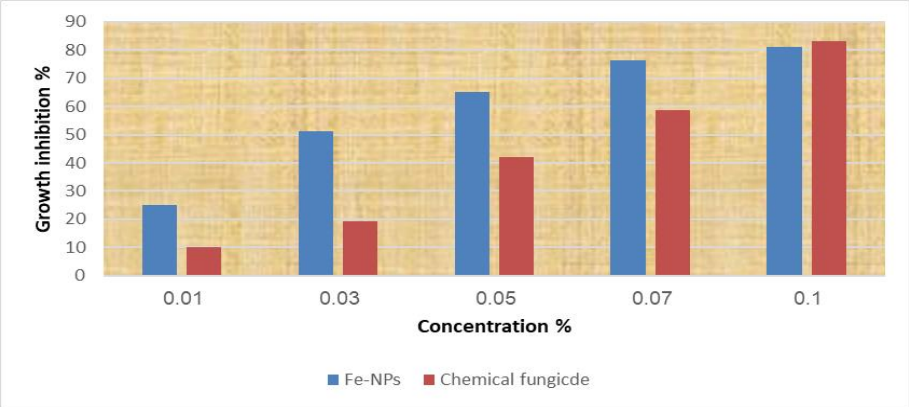

Fig. (11). Growth inhibition of *Fusarium oxysporum* at different concentrations of synthesized nanoparticles and chemical fungicide.

Numerous studies have reported on the antifungal properties of metal oxide nanoparticles mediated by plants. Fe_2O_3NPs have been shown to exhibit high growth suppression (84%) against *Alternaria mali*, *Diplodia seriata*, and *Botryosphaeria dothidea* in addition to other metals (Ahmad et al., 2016). Because of their small size and durability, Fe_2O_3NPs exhibit increased antifungal activity. Small particles usually induce cell harm by interfering with intracellular Ca^{2+} absorption, increasing intracellular matrix dispersion and penetration, and increasing cell damage (Machado et al., 2015). When nanoparticles are reduced, metal ions and the microbial cell membrane begin to interact electrostatically, damaging the cell membrane and internal organelles (Srihasam et al., 2020). By interacting with the

electron transport chain, damaging DNA by breaking phosphate and hydrogen bonds, denaturing protein by changing tertiary structure, and destroying the mitochondria through oxidative stress, the penetration of nanoparticles into microbial cells further increases microbial inhibition (Jesudoss et al., 2016). Reactive oxygen species (ROS) are created because of interactions between inorganic metal and metal oxide nanoparticles, which harm cells (Basak et al., 2014).

4. Plant Growth, Productivity and Yield as Affected by Endophytic Bacteria, Decomposed Olive Leaves Extract (OLE) and Iron Nanoparticles (Fe_2O_3NPs) Treatment

The data reported in Table (4) demonstrate how the potency triple control can be used as ground treatment, foliar spraying, or both to improve faba bean plants infected with *F. oxysporum*. The treatment T4 of soil application + foliar spray increased significantly the height of plant, fresh, dry weight, branches, number of pods per plant, and weight of hundred seeds compared to control. A significantly high value was noted for bean plant height of T3 (125.66) followed by T2 (124) and the lowest was the control (104.33). Plants treated with bioagents in combination with fermented OLE and FeNPs produced the highest fresh and dry weights. In contrast, plant branches were not significantly influenced by treatments. The number of pods per plant were significantly increased by T4 application, T3 and T2 compared with the control. The weight of 100 seeds of faba bean was reduced due to *F. oxysporum* infection since infected plants gave 75 g while treated plants gave 104 g. In the case of T4, the response was the strongest, followed by T3, T2, and T1, respectively.

The collected data align with previous reports indicating that endophyte bacterial strains can effectively increase lentil plant height, dry weight, fresh weight, and pod count when *F. oxysporum* is present and producing wilt disease (Santhosh et al., 2023). Iron is a trace element that is crucial for photosynthetic activities and plant growth, as is widely known. Numerous researchers have noted how iron significantly affects many plants' growth, yield, and other biochemical characteristics (Liaqat et al., 2023). Ibrahim et al. (2021) examined the effects of varying crystal sizes of Fe_2O_3NPs on wheat. They found that foliar treatment with tiny particle sizes promote the contents of total phenolic compounds and photosynthetic pigments. The results are in line with Elizabeth et al. (2023), who demonstrated how Fe_2O_3NPs stimulated tomato seeds and affected their growth. Studies have employed Fe_2O_3NPs as a nanofertilizer and plant growth promoter in a variety of plants, including spinach (Jeyasubramanian et al., 2016), peanuts (Rui et al., 2016), *Nicotiana benthamiana* (Cai et al., 2020) and chickpea (Irum et al., 2020). The use of nano fertilizers present a special opportunity to create plant nutrients with a high rate of absorption, increased usage efficacy, and minimal losses that promote the uptake of

nutrients by the plants (Fatima et al., 2021). Based on the previous statements, it seems that combined application of a bacterial bioagent, decomposed OLE, and FeNPs has a positive impact on faba bean plant growth and yield. This is great news for agricultural sustainability and potentially increased bean production. Disease incidence (DI) and severity index (DSI) significantly varied among different treatments represented in Table (4) and Fig. (12). Untreated plants suffered the highest disease burden, with a DI of 72.5% and DSI of 0.275.

Table (4). Morphological features and degree of wilt disease in faba bean plants after treatment with triple control against *Fusarium* wilt disease.

Treatments	The parameters							
	Plant height (cm)	**Fresh wt. (g)**	**Dry wt. (g)**	**No. of branches/ plant**	**No. of pods/ plant**	**Hundred seed weight (g)**	**Disease incidence (%)**	**Disease severity index**
Control	104.33^{d}	3.00^{d}	0.9500^{d}	8.1 ^{a}	30.0^{d}	75^{c}	72.5^{a}	0.275^{a}
Soil application	124.00 ^{c}	3.46^{c}	1.1245^{c}	8.5^{a}	68.0^{c}	92^{b}	70.5^{b}	0.200^{b}
Foliar spraying	125.66^{b}	4.24^{b}	1.2200^{b}	8.5^{a}	71.5^{b}	95^{b}	54.8^{c}	0.155^{c}
Soil application + foliar spray	126.50^{a}	4.83^{a}	1.5300^{a}	8.5^{a}	81.0^{a}	104^{a}	34.2^{d}	0.089^{d}

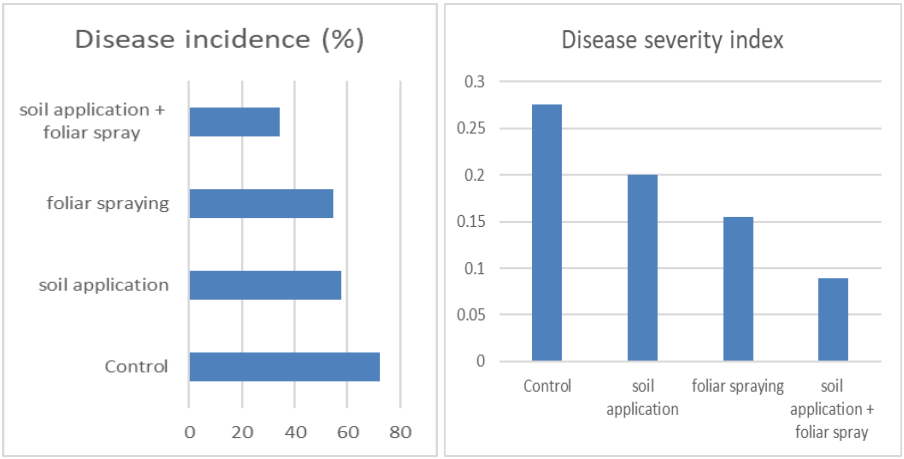

Fig. (12). Effect of triple control on the incidence and severity of *Fusarium oxysporum.*

The "triple control" combination of bioagents, decomposed OLE, and FeNPs in both soil and spray forms yielded the lowest disease severity, with a DI of 34.2% and DSI of 0.089. Spray application alone and soil application alone also offered significant improvements compared to the control further validating the effectiveness of the chosen approach. The synergistic effect of all three components (endophytes, decomposed OLE, and FeNPs) seems to be driving the superior disease control. Endophyte bacteria colonize plant tissues, promoting growth and offering direct antagonism against harmful pathogens like *F. oxysporum* (Gupta et al., 2022). OLE possesses antifungal properties due to its bioactive compounds, further suppressing fungal growth (Alowaiesh et al., 2023). FeNPs might enhance the antifungal activity of both bacteria and OLE, potentially through mechanisms like nutrient competition or disruption of fungal cell membranes (Garg et al., 2023). This suggests that each element contributes to disease suppression to some extent, and their combined action amplifies the overall effect. This study highlights the potential of eco-friendly approaches like employing beneficial microbes and natural extracts for managing plant diseases. Integrating these strategies with traditional methods could pave the way for sustainable disease control practices in agriculture.

Data in Table (5) demonstrate that the various applied treatments (T2: soil application, T3: foliar spraying, and T4: soil application + foliar spray) considerably ($p \leq 0.05$) enhanced the concentration of macro-elements (N, P, K, and Ca) in the leaves of faba bean plants. The plants treated with treatment T4 showed the highest values of macronutrient content (N, P, K, and Ca) when compared to the control treatment, followed by treatment T3 and treatment T2. The micro-element content (Fe, Zn, and Mg) in faba bean plants showed similar significance ($p < 0.05$). Concentration of Fe, Zn, and Mg in plants increased by the various applied treatments more than by the control treatment (T1) compared to control. Endophyte bacteria can fix nitrogen, solubilize phosphorus, and produce hormones, all of which contribute to enhanced nutrient uptake and growth (Rana et al., 2020; Eid et al., 2021 and Di et al., 2023). Decomposed OLE contains various bioactive compounds like phenolic acids and oleocanthal, which stimulate antioxidant defense systems and improve nutrient utilization (Baccouri et al., 2022 and Cuffaro et al., 2023). FeNPs offer a higher surface area for efficient iron absorption, improving chlorophyll synthesis and photosynthesis (Feng et al., 2022 and Singh et al., 2023). Applying these factors together could lead to a synergistic interaction, where the combined effect is greater than the sum of individual effects. For example, OLE might enhance the efficacy of endophytes, while FeNPs could boost the impact of both (Garg et al., 2023).

Table (5). Effect of triple control on both macro and micronutrients, found in the leaves of faba bean.

Treatments	Leaves macro and micronutrients						
	N %	P %	K %	Ca %	Fe %	Zn %	Mg %
Control	1.63^{b}	0.13^{d}	0.60^{c}	1.08^{c}	0.05^{c}	0.01^{b}	0.26^{c}
Soil application	2.22^{a}	0.17^{c}	1.07^{ab}	2.81^{b}	0.12^{b}	0.03^{ab}	0.41^{b}
Foliar spraying	2.32^{a}	0.21^{b}	1.08^{ab}	2.82^{b}	0.12^{b}	0.03^{ab}	0.42^{b}
Soil application + foliar spray	2.40^{a}	0.28^{a}	1.09^{a}	3.49^{a}	0.16^{a}	0.04^{a}	0.47^{a}

CONCLUSION

Farmers often rely on chemical fungicides to combat *Fusarium* wilt, a soil-borne fungal disease. However, these chemicals are significant dangers to the environment and human health, and their effectiveness can be limited due to fungal resistance. A promising alternative is a triple-pronged approach combining (1) beneficial microbes like *B. subtilis* and *Ps. wenyumeiae*, (2) decomposed OLE, and (3) FeNPs. This strategy leverages the natural defense mechanisms of plants and the synergistic effects of these elements. The interaction between FeNPs, OLE and the beneficial microbes can trigger systemic defense responses in plant organs beyond the site of application. This enhanced immunity translates to improve plant growth and productivity, ultimately offering a more sustainable and effective solution for controlling *Fusarium* wilt.

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مكافحة ثلاثية ضد مرض الذبول الفيوزاريومي لنبات الفول وتأثيره على الإنتاجية

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الذبول الفيوزاريومي، وهو مرض فطري، يهدد بشكل كبير إنتاج الفول البلدي في مصر. بحثت هذه الدراسة في إمكانية التطبيق المشترك للبكتيريا الداخلية ومستخلص أوراق الزيتون (OLE) وجزيئات الحديد النانوية (FeNPs) في المكافحة الحيوية وتعزيز النمو في الفول البلدي. تم عزل سلالتين من البكتيريا الداخلية (*Bacillus subtilis* و*Pseudoroseomonas wenyumeiae*) من نباتات الفول الصحية وتم تشخيصهما باستخدام 16S rRNA nucleotide sequence ودراسة آثارهما المضادة والصفات المعززة لنمو النبات (القادره على التخليق الحيوي لـ FeNPs). تم تحلل OLE بيولوجيًا باستخدام *Bosea thiooxidans* لتعزيز إمكاناته المضادة للفطريات. صممت تجربة بحثية تعتمد على العدوى الحقلية الطبيعية. وقد لوحظت اختلافات كبيرة في حدوث المرض (DI) ومؤشر شدة المرض (DSI). أظهرت النباتات غير المعاملة أعلى شدة للمرض (DI=٧٢.٥٪، DSI=٠.٢٧٥). أظهر التطبيق المشترك للعوامل الحيوية وOLE المحلل وFeNPs كغمر التربة والرش الورقي أقل خطورة للمرض (DI=٣٤.٢٪، DSI= ٠.٠٨٩). أظهرت النباتات المعاملة تحسنًا في مؤشرات النمو، بما في ذلك زيادة الطول، والوزن الرطب والجاف، وعدد القرون، ووزن ١٠٠ بذرة، ومحتوى العناصر الكبرى والصغرى في النبات. تشير هذه الدراسة إلى أن المزيج التآزري من البكتيريا الداخلية وOLE وFeNPs يقدم استراتيجية واعدة ومستدامة للمقاومة الحيوية ضد الذبول الفيوزاريومي في الفول البلدي، مما يعزز نمو النبات ويقلل الاعتماد على المبيدات الحشرية الكيميائية.