Scientific Journal of King Faisal University:
Basic and Applied Sciences

Novel Cell-Free Suspensions of Symbiotic Bacteria for Biocontrol of Phytopathogenic Bacteria

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LINK	RECEIVED	ACCEPTED	PUBLISHED ONLINE	ASSIGNED TO AN ISSUE
https://doi.org/10.37575/b/sci/250009	10/03/2025	26/05/2025	26/05/2025	01/06/2025
NO. OF WORDS	**NO. OF PAGES**	**YEAR**	**VOLUME**	**ISSUE**
6289	7	2025	26	1

ABSTRACT

Biofertilizers utilize plant growth-promoting bacteria (PGPB), which are symbiotic bacteria found in the root nodules of leguminous plants that fix atmospheric nitrogen. This study utilized cell-free bacterial suspensions from several leguminous plants, including Vicia faba, Trifolium repens, Lens culinaris, Trigonella foenum-graecum, Lens culinaris subsp. orientalis and Medicago sativa. Six isolates (MA1–MA6) were tested as biocontrol agents against Agrobacterium tumefaciens RB04, Lelliottia amnigena MN1 and Xanthomonas campestris. After establishing that these bacteria are pathogenic, we evaluated their antagonistic activity using the cross method. This identified MA2 as the most effective isolate, with MA5 following closely. Among the tested isolates, MA2 was the most successful in inhibiting the growth of pathogenic bacteria, followed by MA5. The other isolates only impacted the growth of two bacterial species: L. amnigena strain MN1 and X campestris. The agar well diffusion method showed bacterial sensitivity to cell-free supernatants (CFSs) at 67%, 34% and 17% for A. tumefaciens, X. campestris and L. amnigena, respectively. CFS MA6, the most effective supernatant, was analyzed via gas chrom atography-mass spectrometry, revealing four active compounds: C7H13NO2, C11H18N2O2, C28H53NO3 and C21H39NO3, with molecular weights of 143, 210, 451 and 353, respectively.

KEYWORDS
Agrobacterium tumefaciens, antagonistic, biofertilizers, GC-MS, legume plants, PGPB

CITATION
Nayef, M.A. and Al-Barhawee, N.I.K. (2025). Novel cell-free suspensions of symbiotic bacteria for biocontrol of phytopathogenic bacteria. *Scientific Journal of King Faisal University: Basic and Applied Sciences*, **26**(1), 57–63. DOI: 10.37575/b/sci/250009

1. Introduction

In the rhizosphere, plants coexist with a diverse range of soil bacteria known as plant growth-promoting bacteria (PGPB). These bacteria have a positive influence on plant growth, development and resistance to both biotic and abiotic stresses, while also producing secondary metabolites, which explains their prevalence (Pantigoso *et al.*, 2022). Research highlights phytohormones (Egamberdieva *et al.*, 2017), organic acids and various secondary metabolites, including volatile chemicals and exopolysaccharides (Naseem *et al.*, 2018), as significant contributors. Additionally, they enhance plants' resilience against biotic threats, such as bacterial and fungal infections and abiotic stressors, including drought and salinity (Pellegrini *et al.*, 2020). Over recent decades, PGPBs have garnered substantial attention due to their sustainability and other beneficial traits. However, maintaining their activity in the root zone has proven difficult due to a decline in most PGPB species' populations and suboptimal bacterial biomass production from inoculating soil with bacterial suspensions lacking suitable carriers or formulations. Moreover, these bacteria face competition from soil microbes that have adapted to prey on them and compete with naturally occurring established microbial communities (Mokrani *et al.*, 2020). The low survival rates of PGPB bacteria in agricultural settings likely contribute to the limited mention of PGPB strains in scientific literature. To address this issue, various researchers have proposed using a cell-free suspension (CFS) as a viable alternative. A CFS can be produced through a specific protocol that includes centrifugation and microfiltration, followed by purification and an examination of the biocontrol properties of the substances it contains (Morcillo *et al.*, 2022). Although research on this topic remains limited, CFS shows promise in both the medical and food sectors, with most studies focusing on its use as a biocontrol agent in laboratory settings to combat bacterial and fungal plant diseases. Among the various genera researched for their potential biostimulant and biocontrol capabilities, considerable emphasis has been placed on Bacillus (Pellegrini *et al.*, 2020).

Having acquired this insight, we set out to utilize the conjugate filtrate system of symbiotic bacteria sourced from the root nodules of six legume plants (e.g., *Trigonella foenum-graecum, Lens culinaris subsp. orientalis* and *Medicago sativa*) as a method for biological control. Our objective was to inhibit the proliferation of three soil-borne bacterial species, *Agrobacterium tumefaciens* strain RB04, *Lelliottia amnigena MN1* and *Xanthomonas campestris*, that are harmful to various plant species. We then confirmed their pathogenicity on our target plants in vitro and identified their bioactive compounds through gas chromatography-mass spectrometry (GC-MS) analysis.

2. Materials and Methods

2.1. Symbiotic Bacteria:

Six bacterial isolates from root nodules of leguminous plants, isolated in a previous study, were used, namely: *Vicia faba, Trifolium repens, Lens culinaris, T. foenum-graecum, L. culinaris* subsp. *orientalis* and *M. sativa* (MA1, MA2, MA3, MA4, MA5 and MA6).

2.2. Pathogenic Bacteria:

Three different types of bacteria that colonize the soil and are pathogenic to plants were used in this study.

2.2.1. Agrobacterium *tumefacien* strain RB04

PhD student Rayan Salim from the University of Mosul's Department of Plant Protection, in the College of Agriculture and Forestry, conducted this research. After modifying the method outlined by Trigui *et al.* (2013), one colony of *A. tumefaciens* was transferred to Luria-Bertani (LB) broth and incubated at 30°C for 24 hours. The population was measured at 107 CFU/ml, with an absorbance of 0.96 ± 0.02 at 600 nm. Carrots (*Daucus carota L.*) were thoroughly cleaned by rinsing under running water with a brush. Subsequently, discs measuring 5 mm × 8 mm were created and sterilized in 2% sodium hypochlorite (NaClO) for 30 minutes. After three 15-minute rinses in

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sterile water, the discs were removed and dried on sterile paper towels. They were then positioned on sterile water agar medium in Petri plates. Following this, 50 µL of bacteria was applied to the discs using a small syringe. The discs were then carefully placed onto the water agar medium using sterile forceps. The plates were incubated at 25°C for 16 hours in light, followed by 8 hours in darkness. Regular monitoring of crown gall development was conducted.

2.2.2. Lelliottia amnigena (MN1)

This bacterium was obtained from the Soil Science and Water Resources Department, College of Agriculture/Mosul University. Two methods were employed to confirm its diagnosis.

The first involved assessing pathogenicity using the modified methodology of Osai *et al.* (2022), which employed mushroom plants instead of potato tubers. Healthy mushroom samples were carefully chosen, washed with tap water and subjected to surface sterilization using 75% ethanol. To eliminate any remaining alcohol, samples were rinsed three times with sterile distilled water and dried on sterile filter paper. Small incisions were made on the surface using a sterile scalpel and approximately 0.3 ml of *L. amnigena* suspension (3.5×10^1 CFU/ml) was applied. Sterile distilled water served as a negative control. The samples were then placed in a sterile plastic dish and incubated at 25°C, with daily observations of their characteristics made over 25 days.

The second method involved the molecular identification of these bacteria at the genus and species level, according to the following steps:

- A - DNA extraction: This process utilized Chelex 100 from Bio-Rad, USA. A small volume of purified colonies was collected using a loop and placed in a 1-ml tube containing 200 µl of Chelex 100 and 100 µl of TE buffer. The sample was then incubated in a water bath at 95°C. After 10 minutes, the sample was carefully lifted and centrifuged at 13,000 rpm for another 10 minutes. The resulting upper aqueous layer, which contained the DNA, was carefully removed and transferred to a 0.5-ml tube, then stored at −4°C in a refrigerator until needed.
- B-*16S rRNA* gene sequencing: As described by Rudolf *et al.* (2009), the sequencing process starts with PCR amplification of the target areas within the *16S rRNA* gene using specified primers: forward (F: GTGTAGCGGTGAAATGCG) and reverse (R: ACGGGCGGTGTGTACAA), in a total reaction volume of 25 µl. This mixture consists of 12.5 µl of master mix, 0.5 µl of forward primer, 0.5 µl of reverse primer, 6.5 µl of distilled water and 5 µl of genomic DNA. The PCR reaction involves denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 1 minute and extension at 72°C for 30 seconds, concluding with an extension at 72°C for 5 minutes. Following this, PCR products are resolved on a 2% agarose gel with ethidium bromide and visualized using UV light. The sequence is then determined by sending the sample to Korea, followed by analyzing the results in the National Center for Biotechnology Information online database.

2.2.3. Xanthomonas campestris

PhD student Rayan Salim from the University of Mosul's Department of Plant Protection and College of Agriculture and Forestry obtained this bacterium. The diagnosis was validated by introducing this bacterium into cabbage (*Brassica oleracea*) plant leaves and monitoring its pathogenicity according to the methodology described by Popović *et al.* (2013). The bacteria were cultivated on a solid yeast extract-dextrose-CaCO3 medium for 48 hours at 28°C, then centrifuged and diluted in sterile distilled water to a concentration of 10^8 cfu/ml. Later, the petioles and leaves of the cabbage were injected with the inoculum to conduct the pathogenicity test. The leaves were incubated in a low-humidity environment at 25°C with a 16/8-h light cycle for a total of 24 hours. Once removed from the humid environment, the samples were kept in an incubator maintaining the same light and temperature conditions. Symptoms were visible 1-week post-inoculation.

2.2.4. Antagonistic activity test

We employed the cross-linking technique outlined by Skowronek *et al.* (2020) to evaluate whether six bacterial species isolated from legume root nodules (MA1, MA2, MA3, MA4, MA5 and MA6) can inhibit three naturally occurring soil-pathogenic bacteria (*A. tumefaciens RB04, L. amnigena MN1* and *X. campestris*). LB agar plates were prepared and inoculated with bacteria sourced from root nodules by drawing a single line down the center of each Petri dish. Following a 2-day incubation at 28°C, the plates were inoculated with phytopathogenic bacteria by creating a single line at a 90° angle to the previous line. We examined microbial interactions by measuring the size of the inhibition zone.

2.3. Testing Antibacterial Efficacy of The Cell-free Suspension:

2.3.1. Cell-free supernatant preparation

For the preparation of CFS, the procedure of El-Mokhtar *et al.* (2020) was modified slightly by adjusting the incubation conditions. The six isolated bacteria (MA1–MA6) were grown in 100 mL of LB broth for 18 hours at 28°C, then centrifuged to obtain the supernatant at 6,000 × g for 15 minutes at 4°C. The centrifuged supernatant was filtered using a sterile filter (Sigma, Germany) with a 0.22-µm pore size.

2.3.2. Agar well diffusion method

For 24 hours, each type of harmful bacteria (*A. tumefaciens RB04, L. amnigena MN1* and *X. campestris*) was cultivated in LB broth and maintained at 28°C. After 100 µL of sterile swab was used to disperse each suspension over the surface of the solid LB medium, corresponding to McFarland's standard of 0.5, the mixture was left to dry at room temperature (25°C). Then, using a sterile cork borer, holes 6 mm in diameter were punched into the agar. For each bacterial species, the holes were filled with approximately 100 µL of CFS and the plates were incubated at 28°C for 24 hours, as described by Gopal and Thirupathi (2020). A transparent graduated ruler was used to measure the inhibition area surrounding each hole in millimeters.

2.3.3. Testing pathogens' susceptibility to antibiotics

The same method described earlier was utilized to inoculate the medium with pathogenic bacteria, substituting the holes with antibiotic discs. A total of 10 different antibiotics were employed: Trimethoprim (TMP), Ceftazidime (CAZ), Erythromycin (E), Vancomycin (VA), Cefotaxime (CTX), Ampicillin (AM), Rifampin (RA), Streptomycin (S), Chloramphenicol (C), Penicillin (P), Neomycin (N) and Gentamicin (CN), all produced by Oxoid™, United Kingdom. Following incubation under identical conditions, the zone of inhibition around each disc was measured in millimeters.

2.4. Gas Chromatography-mass Spectrometry Analysis of Components of Cell-free Supernatant:

Test results indicated that the CFS from bacterial isolates of plant root nodules (AM6) was the most effective against phytopathogenic bacteria. Using a gas chromatography-mass spectrometry (GC-MS) QP210 ULTRA gas chromatograph (Shimadzu, Japan) at Basrah University, we identified chemical compounds with antibacterial properties by examining their retention times in the mass spectrometer's capillary column and then comparing them with the mass spectra from the NSTA08 library database and GC-MS Solution software (Al-Barhawee and Al-Rubyee, 2024).

2.5. Statistical Analysis:

The results were statistically analyzed according to Goad (2020) to identify significant differences between means in terms of standard deviation values at a significance level of $p = 0.05$.

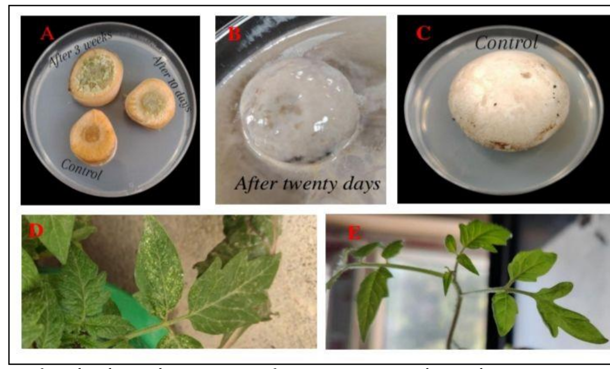
3. Results and Discussion

3.1. Pathogenicity:

The pathogenicity of the bacteria from the three species in this study was confirmed through their effects on a specialized plant (Figure 1). Carrot discs, a type of dicotyledonous plant, were infected with *A. tumefaciens* strain RB04, allowing us to observe the progression of crown gall disease over 10 days and 3 weeks post-infection. *A. tumefaciens* strains collected from six distinct dicotyledonous plants on the campus of Rajshahi University in Rajshahi, Bangladesh, were identified as *A. tumefaciens*, capable of inducing crown gall disease on potato slices, when compared to the standard *A. tumefaciens* strain ATCC 23308T. These strains were assigned registration numbers based on their host plants (Islam *et al.*, 2010). According to Soriful *et al.* (2010), the number of crown galls formed on carrot discs varied with the strain, resulting in 12–16 tumors per disc, exceeding the 12 tumors per disc observed with the control *A. tumefaciens* ATCC 23308T. It is important to note that crown gall formation occurs when T-DNA from the tumor-inducing plasmid (Ti) integrates into the host plant genome (Hooykaas, 2023).

Regarding the bacterium *L. amnigena*, diagnosis was confirmed due to the infected mushroom plant exhibiting symptoms of soft mold disease. This condition is characterized by the infected plant's attachment area being submerged in a viscous solution that emits a foul, soft, mold odor, ultimately leading to the plant's demise. Molecular diagnosis was conducted using the *16S rRNA* gene primer, which revealed 95% identity with the isolate *L. amnigena* strain BW102, as documented in the gene bank with accession number PQ803913. The less than perfect match can be attributed to deletions or substitutions among some of its nitrogenous bases: C and G were deleted at two sites; G was substituted by A at five sites; A was changed to G at five sites; C was converted to both T and A at two different locations; A switched to G at four places; and T changed to A at one site (Figure 2). Consequently, it has been registered in GenBank as *L. amnigena* strain MN2, with accession number PV030017. Genome evolution occurs when there is a shift in the sequence of nitrogenous bases in a short genomic segment. This alteration is termed a mutation, caused by gene deletion or reduction (Lynch, 2006). Laboratory evolutionary studies indicate that bacterial genomes are susceptible to deletion events over brief evolutionary time frames (Nilsson *et al.*, 2005). In this study, we identified 21 independent deletion and substitution mutations occurring at varying rates, often referred to as biased mutations since they occur more frequently than others (Payne *et al.*, 2019). These mutations tend to arise at specific genomic sites, particularly those more prone to change, such as regions distant from the origin of replication (Long *et al.*, 2015). Furthermore, these mutations may occur infrequently and within a single gene (Horton and Taylor, 2023), causing damage to single-stranded sequences during replication or when used as templates for gene expression. This is frequently associated with bacterial exposure to toxic agents, such as free radicals and radiation, or variations in nutrient medium components or growth conditions (Selveshwari *et al.*, 2021). Mutations can also result from the interactions between the replication complex and mononucleotide repeats, such as CCCCC, leading to polymerase enzyme slippage that causes insertion or deletion processes (Hefetz *et al.*, 2023). Notably, this phenomenon was not observed in our study, as shown in Figure 2. However, we found a marked preference for substitutions over deletions, a tendency often associated with their simpler formation mechanisms, more efficient repair processes and generally less specific evolutionary implications (Horton and Taylor, 2023). In *Pseudomonas* sp., Danneels *et al.* (2018) reported 857 insertion and deletion mutations, with the mutations predominantly occurring in the form of deletions rather than insertions. Remarkably, this is the first record of this isolate in Iraq and other Arab countries, as per gene bank documentation. Globally, two strains have been recorded in China, the first derived from a downy mildew disease affecting potato tubers (Osei *et al.*, 2022) and the second isolated from downy mildew impacting numerous fields of purple mustard, with infection rates reaching around 20–30% across a 5-hectare agricultural area, leading to significant economic losses (Li *et al.*, 2025). Recent developments have classified *L. amnigena* as a soil-endemic bacterium and an opportunistic pathogen responsible for downy mildew in plants (Wu *et al.*, 2023). The third isolate analyzed in this study was *X. campestris*, due to its capacity to infect cabbage leaves and cause soft rot disease. Iglesias-Bernabé *et al.* (2019) reported that *B. oleracea* crops infected with *X. campestris pv. campestris* have led to significant economic damages worldwide.

Figure 1: Pathogenicity of pathogenic bacteria on carrots, mushroom and cabbage leaves



A: infected with *Agrobacterium tumefaciens* strain RB04 and control
B: infected with *L. amnigena* MN1,
C: control
D: infected with *X. campestris,*
E: control

Figure 2: The sequence of *L. amnigena* MN1 *16S rRNA* gene

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Query: H241104-013_O20_C2_CF.abl 503 Query ID: lcl|Query_8057121 Length: 429
>Lelliottia amnigena strain BW102 16S ribosomal RNA gene, partial sequence
Sequence ID: PQ803913.1 Length: 1442
Range 1: 7 to 425
Score:654 bits(354), Expect:0.0,
Identities:399/421(95%),  Gaps:2/421(0%), Strand: Plus/Plus
Query  9    GGCGGCAGCCTACACATGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGA  68
            ||||||||O|||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7    GGCGGCAG-CTACACATGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGA  65
Query  69   GCGGCGGACGGGTGAGTAATGTCTGGGAGACTGCCTGATGGAGGGGGATAACTACTGGAA  128
            ||||||||||||||||||||||||||||!|||||||||||||||||||||||||||||||
Sbjct  66   GCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAA  125
Query  129  ACGGTAGCTAATACCGCATAACGTCGCAGGACCGAAGAGGGGGACCTTCGGGCCTCTTGC  188
            ||||||||||||||||||||||||||||!||||!||||||||||||||||||||||||||
Sbjct  126  ACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGC  185
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Query  369  GCGTGTATGAAGAAAGCCTTCCGCTTGTAAACCACTTTCAACCCGGGAGGAAAGCTTTGT  428
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Sbjct  366  GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGA-GGAGGAAGGCATTGT  424
Query  429  G  429
            |
Sbjct  425  G  425
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O: Delete of C, ©: Delete of G, !: Substitute G with A, *: Substitute A with G
Θ: Substitute C with G, θ: Substitute T with G, +: Substitute C with A, ■: Substitute C with T, ®: Substitute T with A

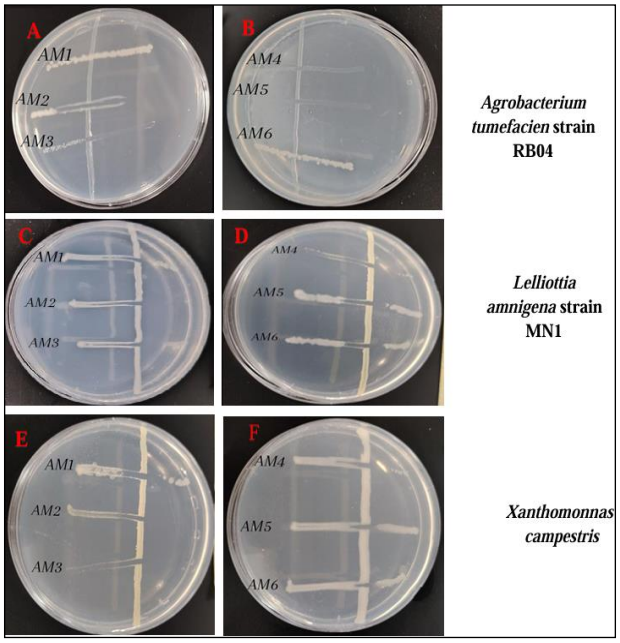
3.2. Evaluation of The Antagonistic Activity:

3.2.1. Symbiotic bacteria by cross-streak assay

The potential antagonistic properties of symbiotic isolates were assessed using cross-line assays against three phytopathogenic bacteria: *A. tumefaciens* strain RB04, *L. amnigena* strain MN1 and *X. campestris* (Figure 3). Six isolates, labeled MA1 through MA6, successfully inhibited the growth of these bacterial species (Table 1). Among them, the isolate MA2 exhibited the strongest inhibitory effect on pathogenic bacteria, followed by MA5. In contrast, the

remaining isolates (MA1, MA3, MA4 and MA6) only affected the growth of two species: *L. amnigena strain* MN1 and *X. campestris*. This variation can be attributed to the release of secondary metabolites during the biocontrol process, which are often toxic to other microorganisms yet not critical for the cell functions of the producing organisms. The ability to generate such metabolites can vary significantly across different strains and isolates (Tariq *et al.*, 2020). The antagonistic effects of these aerobic nitrogen-fixing bacteria might stem from competition for space and nutrients rather than antimicrobial substances (Esteban-Herrero *et al.*, 2023). Alternatively, these bacteria may produce biocontrol enzymes, such as protease, lipase, amylase and DNase, or generate stable antimicrobial substances that permeate the medium and remain active throughout the analysis (Riseh *et al.*, 2024). Additionally, resistance may arise from the production of virulence factors by pathogenic bacteria, especially Gram-negative types, including efflux pumps that are crucial for expelling diverse compounds, thus lowering their concentration and effectiveness in the cell (Gaurav *et al.*, 2023).

Figure 3: Cross-tests between the six isolates (MA1–MA6)



Plant symbionts are represented by the horizontal lines and the vertical lines represent the three plant pathogenic isolates.

Table 1: Determination of the antagonistic activity of the six plant symbiotic isolates against the pathogenic isolates using cross-validation tests.

Symbiotic bacteria	Size of Inhibition Zones (mm)		
	Pathogenic bacteria		
	A. tumefacien strain RB04	*L. amnigena* strain MN1	*X. campestris*
MA1	R (0) c	25 ± 1.00 c	17 ± 1.528 d
MA2	40 ± 2.000* A	45 ± 1.000 a	40 ± 1.732 a
MA3	R (0) c	30 ± 2.517 b	27 ± 2.082 b
MA4	R (0) c	26 ± 3.000 bc	23 ±1.732 c
MA5	20 ± 2.517 B	20 ± 3.464 d	12 ± 2.000 e
MA6	R (0) c	15 ± 2,646 e	10 ± 2.646 e
Resistance (%)	67	0.0	0.0
Sensitivity (%)	33	100	100

R: Resistance, *: value of standard deviation, p-value: < 0001. Data is the average of three replicates. Similar letters indicate no significant differences, while different letters indicate significant differences.

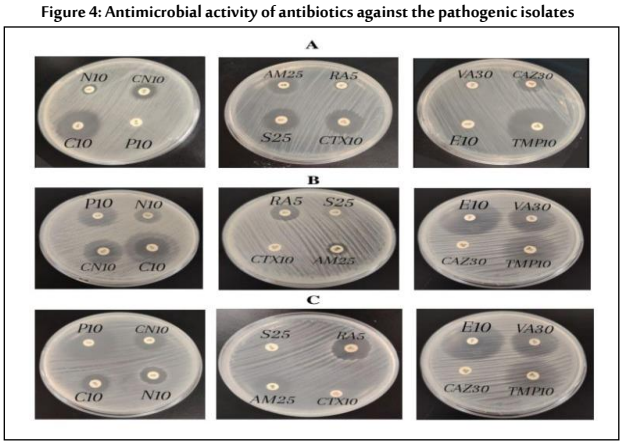
3.2.2. Symbiotic bacterial cell-free supernatants

When assessing the inhibitory effects of six CFSs on plant pathogens known for their drug resistance, 16% for *A. tumefaciens* RB04 and *L. amnigena* MN1 and 25% for *X. campestris* (see Figure 4 and Table 2), most tested pathogens generally exhibited varying resistance levels to all types of CFS, with complete resistance noted for CFS from MA3 and MA5. Conversely, the CFS from AM6 demonstrated the greatest inhibitory effect against plant pathogenic bacteria, showing inhibition zones of 20 mm for *A. tumefaciens* RB04, 17 mm for *X. campestris* and 16 mm for *L. amnigena* MN1, compared to the other CFS types (refer to Figure 5 and Table 3). Statistical analysis revealed significant differences among the variables, as indicated by a probability level of <0.05.

Regarding the resistance of Gram-negative bacteria, the studied plant pathogens are resistant to the following antibiotics: *X. campestris* (CAZ), *L. amnigena* MN1 and *X. campestris* (CTX) and *A. tumefaciens* RB04 (P). This resistance likely arises from their production of the beta-lactamase enzyme, which effectively cleaves the beta-lactam ring in antibiotic structures (Vivekanandan *et al.*, 2025). *A. tumefaciens* RB04's resistance to the antibiotic E is presumably due to modifications in the 23S ribosomal RNA within the 50S ribosomal subunit, which obstructs E's binding to the ribosome and allows continued protein synthesis (Liang and Han, 2013). Meanwhile, the resistance of *L. amnigena* MN1 and *X. campestris* to S could result from alterations in the genes encoding the 16S ribosomal RNA (Spagnolo *et al.*, 2016). Regarding the sensitivity of these three types of pathogenic bacteria to trimethoprim, this may stem from its inhibition of the reduction of dihydrofolate to tetrahydrofolate, the active form of folic acid, as suggested by Gleckman *et al.* (1981). On the other hand, the sensitivity to chloramphenicol may be attributed to its ability to inhibit protein synthesis by binding to the 50S subunit of the bacterial 70S ribosome (Yu and Zeng, 2024).

Furthermore, Jumaah *et al.* (2022) observed that CFS from bacteria isolated from the root nodules of leguminous plants inhibited the growth of certain plant pathogenic fungi, suggesting their potential as biological control agents. This antimicrobial action from these bacteria potentially suppresses the growth of plant pathogens by degrading compounds secreted by them and inhibiting the germination of pathogenic fungal spores (Al-Ani *et al.*, 2012). In a recent study, the cell-free supernatant of *P. aeruginosa*, isolated from soil, was found to play a role in preventing biofilm formation by *A. tumefaciens*, a plant pathogenic bacterium that attaches to plant wounds and forms crown galls (Al-Barhawee and Al-Rubyee, 2024).

Figure 4: Antimicrobial activity of antibiotics against the pathogenic isolates



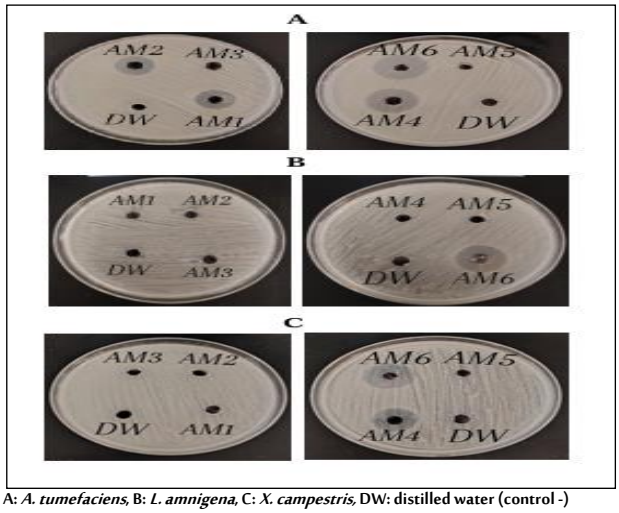
A: A. tumefaciens, B: L. amnigena, C: X. campestris.

Table 2: Effect of antibiotics on pathogenic bacteria by the diameter of the inhibition zone (mm)

Antibiotics (µg/disk)	Size of Inhibition Zones (mm)		
	Pathogenic bacteria		
	A. tumefacien strain RB04	*L. amnigena* strain MN1	*X. campestris*
TMP (10)	31 ± 2.082* a**	27 ± 3.055 bc	22 ± 2.082 b
CAZ (30)	16 ± 1.732 b	34 ± 2.517 a	R (0) e
E (10)	R (0) e	26 ± 2.309 bc	23 ± 3.055 b
VA (30)	6 ± 0.000 c	24 ± 2.000 cd	30 ± 2.082 a
CTX (10)	19.33 ± 1.528 b	R (0) e	R (0) e
AM (25)	17 ± 2.887 b	11 ± 2.000 f	16 ± 1.000 cd
RA (5)	8 ± 1.155 c	15 ± 0.000 e	19 ± 2.646 c
S (25)	18 ±1.732 b	R (0) e	R (0) e
C (10)	30 ± 4.619 a	28 ± 4.041 b	15 ± 1.155 d
P (10)	R (0) e	24 ± 1.732 cd	29 ± 1.000 a
N (10)	7 ± 1.732 c	16 ± 1.732 e	14 ± 0.000 d
CN (10)	17 ± 2.646 b	20 ± 3.215 d	17 ± 3.512 cd
Resistance (%)	16	16	25
Sensitivity (%)	84	84	75

R: Resistance, *: value of standard deviation, p-value: < 0001. Data are an average of three replicates. **: Similar letters indicate no significant differences between the variables and different letters indicate significant differences between the variables.

Figure 5: Antagonistic activity of six CFSs against three pathogenic bacteria



A: *A. tumefaciens*, B: *L. amnigena*, C: *X. campestris*, DW: distilled water (control -)

Table 3: Effect of CFS of Symbiotic bacteria on pathogenic bacteria by the diameter of the inhibition zone (mm).

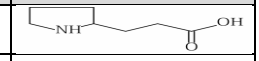
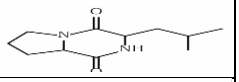
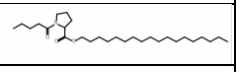
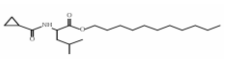
CFS of Symbiotic bacteria	Size of Inhibition Zones (mm)		
	Pathogenic bacteria		
	A. tumefacien strain RB04	*L. amnigena* strain MN1	*X. campestris*
AM1	16 ± 1.732* bc**	R (0) e	R (0) e
AM2	13 ± 1.155 c	R (0) e	R (0) e
AM3	R (0) e	R (0) e	R (0) e
AM4	18 ± 3.464 ab	R (0,0,0)	16 (14, 17,17)
AM5	R (0) e	R (0) e	R (0) e
AM6	20 ± 3.215 a	16 ± 1.732 a	17 ± 2.517 a
Resistance (%)	33	83	66
Sensitivity (%)	67	17	34

R: Resistance, *: value of standard deviation, p-value: < 0001. Data are an average of three replicates. **: Similar letters indicate no significant differences between the variables and different letters indicate significant differences between the variables.

3.2.3. Gas chromatography-mass spectrometry analysis

Isolate MA6 CFS was chosen for GC-MS analysis due to its strong effectiveness in inhibiting pathogenic bacteria growth, as indicated by the size of the inhibition zone (mm). Table 4 shows the presence of four chemical compounds: (3-Pyrrolidin-2-yl-propionic acid), (Pyrrolo[1,2-a] pyrazine-1,4-dione hexahydro), (L-Proline, N-valeryl-, octadecyl ester) and (L-Leucine, N-cyclopropylcarbonyl-, undecyl ester). Several studies have identified these chemical compounds in various bacterial sources. For example, GC-MS analysis of an ethyl acetate extract from *Streptomyces sp.* revealed several compounds, including 3-pyrrolidin-2-yl-propionic acid, which demonstrated a notable antibacterial effect against a range of pathogenic bacteria (Veilumuthu, 2022). According to Kiran *et al.* (2018), an antibiotic called pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro was found to be effective against methicillin-resistant *Staphylococcus aureus* after being isolated from the marine bacterium *Bacillus tequilensis* MSI45. Additionally, Ser *et al.* (2015) reported its antioxidant properties when extracted from *Streptomyces* bacteria sourced from soil. Conversely, laboratory tests demonstrated that *Aspergillus oryzae* YRA3 could completely inhibit the growth of *Rhizoctonia solani*. The chemical composition of the secondary metabolites produced by this fungus, as analyzed by GC-MS, revealed the presence of 32 distinct compounds. L-Proline, N-valeryl and octadecyl esters were found to be the most effective, while no antagonistic effects were observed for Leucine, N-cyclopropylcarbonyl- and undecyl esters throughout the experiment (Rashad *et al.*, 2023).

Table 4: The four chemical compounds and their structure from the GC-MS analysis of AM6 CFS

Compound Name	Formula	Molecular Weight(g/mol)	Structures
3-Pyrrolidin-2-yl-propionic acid	$C_7H_{13}NO_2$	143	
Pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro	$C_{11}H_{18}N_2O_2$	210	
L-Proline, N-valeryl-, octadecyl ester	$C_{28}H_{53}NO_3$	451	
L-Leucine, N-cyclopropylcarbonyl-, undecyl ester	$C_{21}H_{39}NO_3$	353	

4. Conclusions

Symbiotic bacteria associated with legume plants, particularly those isolated from the root nodules of beans, can inhibit the development of bacterial plant diseases by producing bioactive compounds. Therefore, these bacteria show promise as biological agents to control bacterial infections in economically valuable crops and future research should promptly evaluate them in real-world settings as novel, environmentally safe antibacterial agents. Investigating this topic could lead to the development of new and more effective antibacterial agents based on the active compounds and diverse secondary metabolites produced by these environmentally friendly bacteria, thereby illuminating the complex relationships among different microbes coexisting in the soil. Additionally, the data stored in the gene bank indicate that *L. amnigena* has been isolated in only two previous studies conducted in China and was recently isolated for the third time in this study by the Laboratory of Molecular Genetics, Department of Biology, College of Pure Sciences, University of Mosul, Iraq. This marks the first registration in Iraq and the first time this bacterium has been used for in vitro biocontrol. We anticipate that future field applications will support sustainable agriculture efforts in producing healthy crops free from microbial diseases.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

Acknowledgments

We extend our sincere appreciation to the University of Mosul for providing us with the opportunity to conduct this research and for publishing the results in your esteemed publication.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflicts of Interest

No conflicts of interest exist.

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