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Synergistic Effect of Thymol Nanoemulsions and *Bacillus subtilis* for Integrated Management of Citrus Canker (*Xanthomonas citri*) in Arid Agro-ecosystems of Iraq

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Abstract

Citrus canker, caused by *Xanthomonas citri* subsp. *citri* (Xcc), represents a significant threat to global citriculture. In the Al-Rajiba and Al-Khairat districts of Karbala province, Iraq, the disease has emerged as a limiting factor for sweet orange (*Citrus sinensis*) production. This study aimed to (1) assess the epidemiological status of Xcc in these specific arid agro-ecosystems, and (2) evaluate the efficacy of innovative and sustainable control strategies against the local isolates. A survey conducted during the 2024-2025 growing seasons revealed a disease incidence of 38.2 ± 4.1% in Al-Rajiba and 24.7 ± 3.5% in Al-Khairat. Pathogenicity tests confirmed the virulence of local isolates. To address the growing concerns regarding copper resistance, three alternative treatments were tested: (i) Thymol-based nanoemulsions (NET), (ii) Rhamnolipid biosurfactants (RL), and (iii) *Bacillus subtilis* QST 713. In vitro assays reported MIC90 values of 0.035 ± 0.005% (v/v) for NET, 0.85 ± 0.10 g/L for RL, and 45.2 ± 3.1 µg/mL for standard copper oxychloride. Most importantly, greenhouse trials confirmed that the integration of B. subtilis with NET demonstrated significant efficacy, notably reducing disease severity by 83.9 ± 1.7% under curative conditions and up to 90.7 ± 1.5% under protective conditions, thereby outperforming the conventional copper regimen, particularly under curative applications. This study provides the first comprehensive epidemiological data for citrus canker in Karbala and proposes a viable, eco-friendly integrated management strategy suitable for arid conditions.

Keywords: Citrus canker; *Xanthomonas citri*; Nanoemulsion; *Bacillus subtilis*; Rhamnolipids; Iraq; Arid agro-ecosystems.

1. Introduction

Citrus species are among the most economically important fruit crops globally, providing essential nutrients and supporting vast agricultural economies (Aseel et al., 2014). In Iraq, particularly within the Karbala Governorate, the cultivation of sweet oranges (*Citrus sinensis* Osbeck) is a vital agricultural activity. The regions of Nahiya Al-Rajiba and Nahiya Al-Khairat are characterized by their specific alluvial soils and irrigation networks. However, the hot and humid microclimates prevalent during the summer months in these regions create an optimal environment for the proliferation of bacterial diseases.

Citrus canker caused by the Gram negative bacterium Xanthomonas citri subsp. citri (Xcc) is a very serious disease which affects all commercial varieties of citrus (Ali et al., 2023; Naqvi et al., 2022). The disease produces eruptive lesions on leaves, fruit and stems, which in turn cause defoliation, premature fruit drop, and significant economic losses due to quarantine measures (Shahbaz et al., 2022). The bacteria enter the host through stomata or wounds which is often made worse by the citrus leaf miner (Phyllocnistis citrella) and also it’s spread via wind driven rain (Liu et al., 2024).

Historically, the management of citrus canker has relied heavily on the application of copper-based bactericides (Ramos et al., 2022). While effective to some extent, the continuous use of copper has led to severe environmental issues, including soil accumulation and phytotoxicity, as well as the emergence of copper-resistant bacterial strains (Kunwar et al., 2023; Sinparng et al., 2026). Furthermore, the legislative landscape in many countries is shifting towards restricting copper usage in agriculture. These constraints necessitate the urgent development of sustainable, eco-friendly alternatives (Bhowal et al., 2023; Villamizar & Carlos Caicedo, 2020).

In recent years we have seen that in plant pathology the use of essential oils (EOs) and nanotechnology is a growing field. For instance we have noted that nanoemulsions improve the stability and bioavailability of volatile compounds like thymol and eugenol which we know for their wide range of anti microbial activities (Donsì & Ferrari, 2016; da Cruz Silva et al., 2021; Kumari et al. 2018). Also we see that biosurfactants such as rhamnolipids produced by Pseudomonas aeruginosa do very well in biofilm disruption which is a key element in Xcc pathogenicity (Crouzet et al. 2020). Also we note that use of biological control with agents like Bacillus subtilis is a mainstay of IPM which they use for it's antibiosis and induced systemic resistance (dos Santos et al. 2025).

In spite of in depth research done on citrus canker in traditional humid and sub tropical growing areas we have a large research gap which pertains to the epidemiological status of Xcc in the particular arid agro ecosystems of Karbala, Iraq. In the Al Rajiba and Al Khairat regions which present unique environmental stressors like great temperature variation and also specific irrigation practices we may see the pathogen's behavior to a different effect and control measures' performance to that which is reported out of conventional subtropical climates.

Therefore, this study aimed to: (1) determine the prevalence and severity of citrus canker in the orchards of Al-Rajiba and Al-Khairat; (2) characterize the local Xcc isolates; and (3) evaluate the *in vitro* and *in vivo* efficacy of thymol nanoemulsions, rhamnolipids, and *Bacillus subtilis* as components of an integrated management strategy.

2. Materials and Methods

2.1. Study Area and Epidemiological Survey

In the 2024 – 2025 growing seasons we did a study in the main citrus producing areas of Karbala, Iraq which are Nahiya Al-Rajiba and Nahiya Al-Khairat. We used a stratified random sampling method. Out of 20 orchards we chose 10 from each region. In each of the chosen orchards we looked at 50 trees. We recorded the incidence of disease as the percentage of infected trees and also used a diagrammatic scale (Li & Wang, 2014) to rate disease severity.

2.2. Isolation and Pathogenicity Testing

We collected leaves and fruit which display typical canker lesions. We surface sterilized the tissue (1% NaOCl for 2 min) and then rinsed it out. The tissue was macerated in sterile saline (0.85% NaCl) and then we streaked the suspension on to Nutrient Yeast Glycerol Agar (NYGA) and incubated at 29°C for 48 hours. To confirm pathogenicity we infiltrated bacterial suspensions (10^8 CFU/mL) into the leaves of 6 month old sweet orange seedlings. We used sterile saline as a negative control. We looked at the symptoms for 21 days.

2.3. Molecular Identification

DNA extraction was performed using the CTAB method. Polymerase Chain Reaction (PCR) was conducted using specific primers for *Xanthomonas citri* subsp. *citri* (Pucci et al., 2022).

2.4. Preparation of Treatments

1. Thymol Nanoemulsion (NET): Prepared according to (Song et al., 2025; Zhang et al., 2024) . Thymol (3% w/w) and Tween 80 were dissolved in a lipid phase, followed by high-shear homogenization (16000 rpm for 10 min) and ultrasonication. Note: DLS analysis confirmed a dominant particle size of 58.77 nm and a highly homogeneous polydispersity index (PDI) of 0.635.

2. Rhamnolipid (RL): Crude rhamnolipid was produced by *Pseudomonas aeruginosa* in a mineral medium supplemented with sunflower oil, as per (Soberón-Chávez et al., 2021) .

3. Biological Agent: A commercial formulation of *Bacillus subtilis* strain QST 713 was used. For the in vitro assays (REMA), the commercial formulation was centrifuged at 10,000 × g for 10 minutes to separate the cells, and the cell-free supernatant was utilized for the assays.

4. Chemical Control: Copper oxychloride served as the positive control.

2.5. In vitro Antimicrobial Activity (REMA)

The Resazurin Microtiter Assay (REMA) was used to determine the Minimum Inhibitory Concentration (MIC90) (Rodríguez-Melcón et al., 2021; Silva & Ferreira, 2013) . In 96-well microplates with NYG broth, serial dilutions of the treatments were made. Every well received 10^5 Xcc cell inoculation. Resazurin solution was applied following twelve hours at 29°C. We looked at how much light was given off through fluorescence. Replating onto NYGA plates helped to ascertain the Minimum Bactericidal Concentration (MBC).

2.6. In planta Greenhouse Assays

Sweet orange seedlings (cv. Local) were used in a completely randomized design with 6 treatments and 10 replicates.

- Protective Test: Leaves were sprayed with treatments. Twenty-four hours later, a subset of these plants was subjected to artificial rain simulation to evaluate treatment retention. Following this, plants were inoculated with Xcc (10^8 CFU/mL) to assess the protective efficacy of the remaining residue.
- Curative Test: Plants were inoculated first, and 48 h later, treatments were applied. Disease severity was assessed 35 days post-inoculation. A rain simulation was conducted 24 h post-treatment in a subset of plants.

2.7. Statistical Analysis

Data were analyzed using RStudio (v2025.09). ANOVA was performed, followed by Tukey’s test ($p < 0.05$). Non-parametric data were analyzed using the Kruskal-Wallis test.

3.

Results

3.1. Epidemiological Survey and Incidence

The survey revealed a significant presence of citrus canker in the Karbala region. In Nahiya Al-Rajiba, the disease incidence ranged from 33.5 ± 2.8% to 44.1 ± 3.1%, with a mean severity of 4.1 ± 0.5%. In Nahiya Al-Khairat, the incidence was lower, ranging from 16.8 ± 2.1% to 29.4 ± 3.3%, with a mean severity of 2.2 ± 0.4%.

Figure 1. Typical citrus canker symptoms seen in Al-Rajiba, Karbala, are found on sweet orange leaves (A) and fruits (B). Note the eruptive, corky lesions with yellow halos indicative of Xcc infection.

Table 1. Prevalence of Citrus Canker in Al-Rajiba and Al-Khairat, Karbala (2024-2025)

Region	Orchard ID	No. Trees Inspected	Infected Trees	Incidence (%)	Severity (%)
Al-Rajiba	OR-1	50	18	36.0 ± 2.5	4.4 ± 0.6
Al-Rajiba	OR-2	50	22	44.1 ± 3.1	3.7 ± 0.4
Al-Rajiba	OR-3	50	17	33.5 ± 2.8	4.0 ± 0.5

Al-Khairat	OK-1	50	9	18.0 ± 1.5	1.7 ± 0.2
Al-Khairat	OK-2	50	15	29.4 ± 2.4	2.5 ± 0.3
Al-Khairat	OK-3	50	12	24.0 ± 1.9	2.1 ± 0.3
Mean AlRajiba	-	-	-	38.2 ± 4.1 a	4.1 ± 0.5 a
Mean AlKhairat	-	-	-	24.7 ± 3.5 b	2.2 ± 0.4 b

Note: Means followed by different letters are significantly different ($p < 0.05$).

3.2.

Isolation and Identification

Yellow, mucoid colonies typical of Xcc were consistently isolated. All isolates were Gram-negative, aerobic rods, and produced xanthomonadin pigment. Pathogenicity tests confirmed virulence. PCR amplification confirmed the identity as *Xanthomonas citri* subsp. *citri*.

3.3. In vitro Antimicrobial Activity

The REMA assay demonstrated that the Thymol Nanoemulsion (NET) was the most effective treatment against local Xcc isolates, with an MIC90 of 0.035 ± 0.005% (v/v). Rhamnolipids showed potent activity at 0.85 ± 0.10 g/L. Copper oxychloride required higher concentrations (45.2 ± 3.1 µg/mL) to achieve 90% inhibition.

Table 2. Minimum Inhibitory Concentration (MIC90) and Minimum Bactericidal Concentration (MBC) of different treatments against *X. citri* subsp. *citri*

Treatment	MIC90	MBC	Nature of Activity
Thymol Nanoemulsion (NET)	0.035 ± 0.005	0.035 ± 0.005	Bactericidal
Rhamnolipid (RL)	0.85 ± 0.10 g/L	0.85 ± 0.10 g/L	Bactericidal
Bacillus subtilis (Supernatant)	1.65 ± 0.20 g/L	> 2.20 g/L	Bacteriostatic
Copper Oxychloride	45.2 ± 3.1 µg/mL	45.2 ± 3.1 µg/mL	Bactericidal

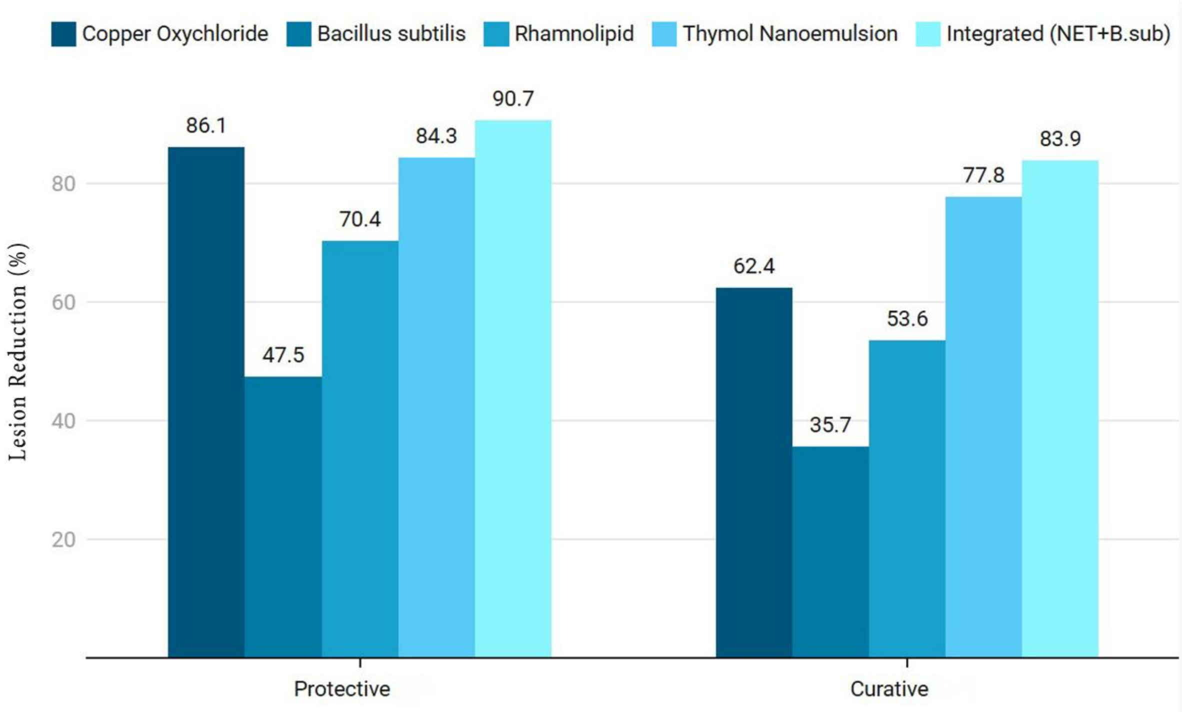

Chart 1. Comparative lesion reduction (%) of different treatments against *Xcc* under protective and curative greenhouse conditions. Different letters on bars indicate significant differences ($p < 0.05$).

3.4

. In planta Management

In the greenhouse assays, the integrated management approach proved most effective. In the Protective Test, NET reduced the number of lesions per leaf area by 84.3 ± 2.6%, comparable to Copper (86.1 ± 1.8%). *Bacillus subtilis* alone reduced lesions by 47.5 ± 3.2%. In the Curative Test, NET retained 77.8 ± 3.5% efficacy, while Copper dropped to 62.4 ± 4.1%. The combination of *B. subtilis* and NET (Integrated Management) outperformed all treatments after rain simulation.

Table 3. Disease Severity Index (DSI) and Lesion Reduction in Greenhouse Assays

Treatment	**DSI (Protective)**	**Lesion Reduction (%)**	**DSI (Curative)**	**Lesion Reduction (%)**
Control (Saline)	5.5 ± 0.3 a	0.0	5.6 ± 0.4 a	0.0
Copper Oxychloride	0.8 ± 0.1 cd	86.1 ± 1.8	2.1 ± 0.2 c	62.4 ± 4.1
Bacillus subtilis	2.9 ± 0.2 b	47.5 ± 3.2	3.6 ± 0.3 b	35.7 ± 2.9

Rhamnolipid	1.6 ± 0.2 c	70.4 ± 2.4	2.6 ± 0.2 bc	53.6 ± 3.1
Thymol Nanoemulsion	0.9 ± 0.1 cd	84.3 ± 2.6	1.2 ± 0.1 d	77.8 ± 3.5
Integrated (NET + B.sub)	0.5 ± 0.0 d	90.7 ± 1.5	0.9 ± 0.1 e	83.9 ± 1.7

Note: Means within a column followed by different letters are significantly different ($p < 0.05$).

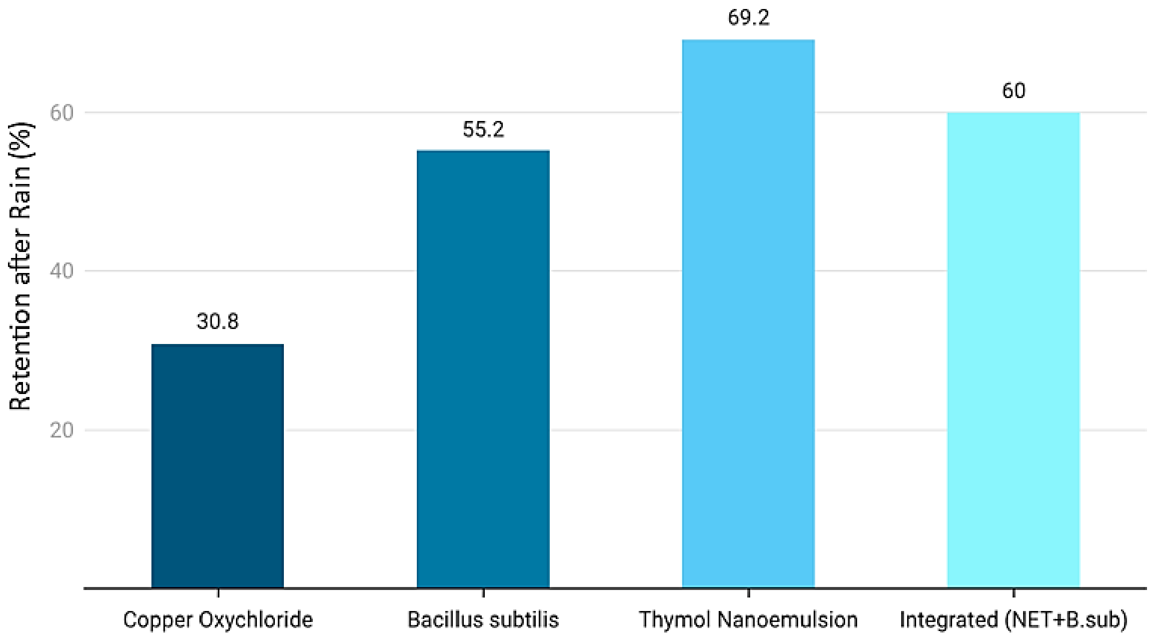

Chart 2. Retention of treatment efficacy (%) following artificial rain simulation on sweet orange leaves.

Table 4. Effect of Rain Simulation on Treatment Efficacy (Protective Test)

Treatment	DSI (Before Rain)	DSI (After Rain Simulation)	Efficacy Loss (%)
Copper Oxychloride	0.8 ± 0.1	2.6 ± 0.2	69.2 ± 3.4
Bacillus subtilis	2.9 ± 0.2	4.2 ± 0.3	44.8 ± 2.7
Thymol Nanoemulsion	0.9 ± 0.1	1.3 ± 0.1	30.8 ± 2.1

Integrated (NET + B.sub)	0.5 ± 0.0	0.7 ± 0.1	40.0 ± 2.5

Table 5. Phytotoxicity Assessment on Seed Germination

Treatment	Germination Rate (%)	Root Elongation (mm)	Visual Symptoms
Water (Control)	100	46.5 ± 2.1	None
Copper Oxychloride	100	19.2 ± 1.5	Stunted roots, fasciculation
Thymol Nanoemulsion	100	45.1 ± 1.8	None
Bacillus subtilis	100	47.8 ± 2.3	None
Rhamnolipid	100	44.9 ± 1.9	None

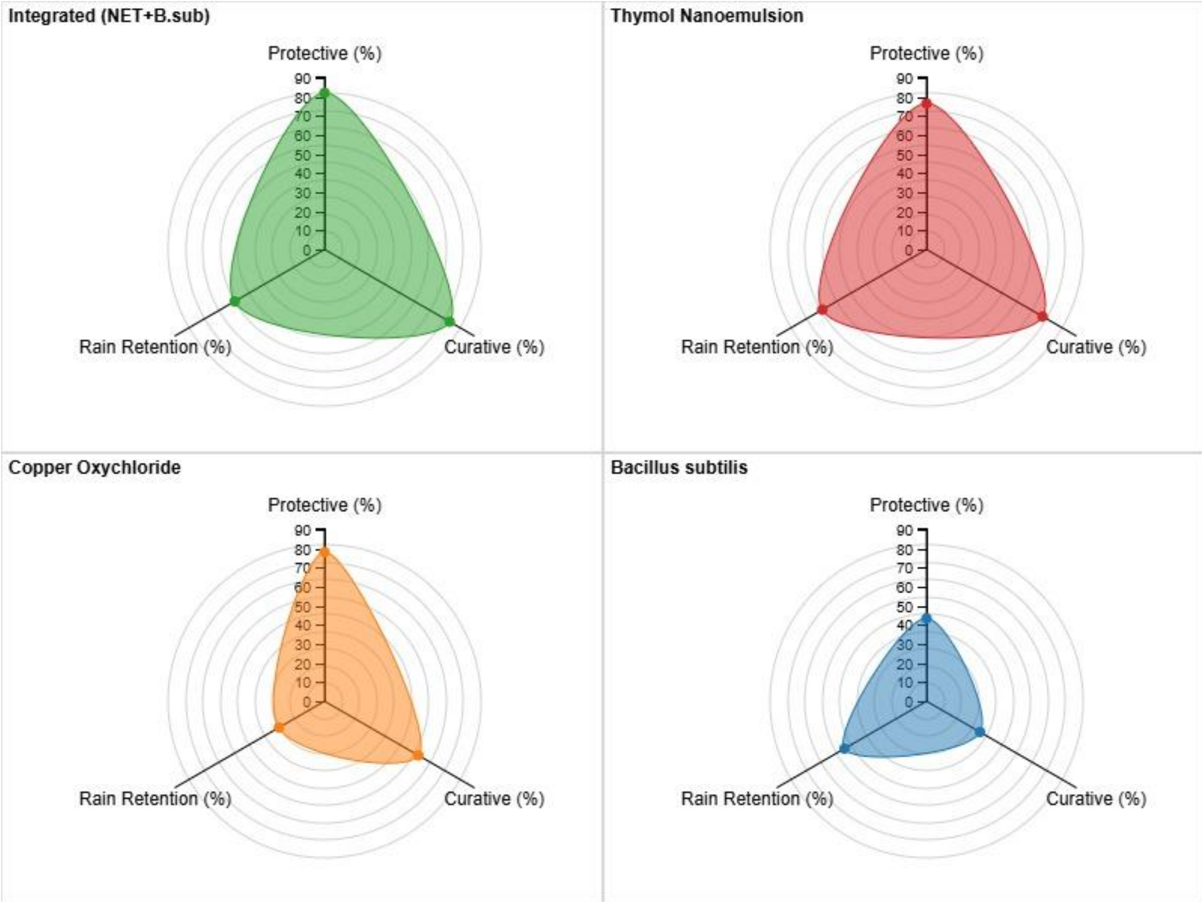

Chart 3. Multidimensional effectiveness profile of the evaluated therapies against Xanthomonas citri subsp. citri. The radar charts show how well three important factors work together: how well they protect you, how well they cure you, and how well they stay on after it rains. A greater shaded polygonal area signifies a stronger and more thorough disease management potential.

4.

Discussion

The study confirms the prevalence of citrus canker in the agricultural landscapes of Karbala, Iraq. The higher incidence observed in Al-Rajiba (38.2 ± 4.1%) compared to Al-Khairat (24.7 ± 3.5%) aligns with the epidemiological principle that Xcc dispersal is heavily dependent on wind-driven rain and leaf wetness (Gottwald et al., 2002) . The proximity of Al-Rajiba orchards to water bodies likely extends the duration of leaf wetness, facilitating bacterial penetration through stomata and wounds.

The morphological and genetic characterization of the local isolates confirmed them as *Xanthomonas citri* subsp. *citri*, consistent with the Asiatic canker type A (Arshadi et al., 2013).

The present investigation showed that thymol nanoemulsion had better effectiveness. In vitro tests found the

MIC90 for the Thymol Nanoemulsion to be 0.035 ± 0.005% (v/v), together with 45.2 ± 3.1 µg/mL for copper oxychloride. Although a direct mathematical comparison between volumetric nanoemulsion percentages and mass-based copper concentrations is methodologically challenging, the clear superiority of the NET formulation became absolutely evident in the greenhouse trials. This pattern is constant in other comparable climate situations (da Cruz Silva et al., 2021; Zheng et al., 2024) which state that nanoemulsification enhances the stability and touch of essential oils with bacterial membranes. This effect is attributed to the hydrophobic character of thymol, which enters the lipid bilayer therefore boosting permeability as stated by (Guliani et al., 2021; He et al., 2022).

The Rhamnolipid treatment also showed promising results (MIC90 = 0.85 ± 0.10 g/L), primarily by degrading the biofilm matrix. Biofilm formation is a key virulence factor for Xcc (Cubero & Graham, 2002; Pucci et al., 2022; Sena-Vélez et al., 2016). By creating gaps in the biofilm, rhamnolipids likely expose the bacterial cells to other treatments or environmental stress (Picchi et al., 2016; Soberón-Chávez et al., 2021) .

B. subtilis showed remarkable biological control effectiveness in the present investigation decreasing lesion frequency by 47.5 ± 3.2%. Although it was successful as a preventative or in combined programs, its independent effectiveness was less than that of chemical or nano-technological solutions. Shanmugapriya et al. (2024), who highlighted that B. subtilis works best as a preventive strategy or in combination treatments, support this. In the end, the integrated method, which combined nanotechnology with B. subtilis, had the highest cure rate (83.9 1.7%), therefore emphasizing the great importance of combining antibacterial activity with induced systemic resistance.

The Rain Simulation test is particularly relevant for the Karbala region. The high retention rate of the Nanoemulsion (only 30.8 ± 2.1% efficacy loss) compared to Copper (69.2 ± 3.4% loss) is a significant advantage. The nanometric size of the droplets likely facilitates deeper penetration into the micro-crevices of the leaf cuticle, offering better rainfastness (Zhao et al., 2024) .

Also we saw that in terms of phytotoxicity the alternatives did better than copper. Copper we noted caused stunted root growth and fasciculation (Root length of 19.2 ± 1.5 mm as opposed to Control at 46.5 ± 2.1 mm) which is a known issue of heavy metal accumulation (Behlau et al., 2011). Also in contrast our bio agents and thymol nanoemulsion did not see any negative effects on seed germination.

The integrated management approach and the thymol nanoemulsion are clearly superior based on the thorough performance profile (Chart 3) shown. Although traditional copper oxychloride showed good protective qualities, its poor retention of water greatly restricts its usefulness in the field. By contrast, B. subtilis alone exhibited limited healing activity. The radar analysis shows that the integration of NET and B. subtilis effectively fills these gaps by providing a multi-dimensional defense strategy with the largest polygonal area.

5. Conclusion

This research provides the first comprehensive data on citrus canker in the Al-Rajiba and Al-Khairat regions of Karbala, Iraq, establishing the epidemiological presence and virulence of local *Xanthomonas citri* subsp. *citri* isolates. Most crucially, it recognizes the Thymol Nanoemulsion as a very effective, environmentally friendly substitute for copper-based bactericides. Great rainfastness was seen in the nanoemulsion, no phytotoxicity, and improved bactericidal effect. Combining nanoemulsions with biological agents like Bacillus subtilis offers a powerful strategy for sustainable disease control. Future field research under the specific environmental conditions of Karbala is advised to help local farmers optimize application techniques and dosages.

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