Evaluation of certain chemical and biological pesticides for the control of crown gall disease in rose plants (*Rosa* sp.)

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Abstract:
Crown Gall is a soil-borne bacterial disease caused by *Agrobacterium tumefaciens*, leading to significant economic losses across numerous plant species. This study aimed to investigate and evaluate the efficacy of certain biological agents and chemical pesticides in controlling this pathogen. In vitro results demonstrated that the biological agents *Trichoderma harzianum* and *Bacillus thuringiensis* exhibited high antagonistic activity against *A. tumefaciens*. Specifically, *T. harzianum* outperformed other treatments with an inhibition rate of 86.41%, showing no significant statistical difference compared to *B. thuringiensis*. Furthermore, the chemical pesticides Nordox 75 WG (at 1.25 g/L) and Beltanol (at 1 ml/L) proved highly effective, achieving a 100% growth inhibition rate of the bacteria. The findings of this study suggest that integrating biological and chemical products can effectively suppress Crown and Root Gall disease in rose plants, thereby enhancing productivity and minimizing economic losses.
Keywords: *Agrobacterium tumefaciens*, Rose plants ,*Trichoderma harzianum* , *Bacillus thuringiensis* .

Introduction

Roses (*Rosa* sp.), belonging to the family Rosaceae, are among the most widely cultivated ornamental plants globally due to their aesthetic floral appeal [1]. They are ranked among the top five ornamental plants in the international market [2]. However, rose production faces significant challenges from various phytopathogens [3]. *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*) is a soil-borne pathogen that poses a severe threat to both the yield and quality of roses [4]. This bacterium can persist in the soil, the rhizosphere of numerous plant species, and various other natural environments [5]. Even under non-agricultural conditions, Crown Gall disease has caused a 60% reduction in global cut rose production [6]. The pathogen can remain asymptomatic within rose plants, serving as a primary source for the spread of the disease and leading to substantial economic losses [7].

This pathogen has been extensively studied due to its critical role in compromising rose health, leading to poor root formation, reduced bud count, and a decline in cut flower productivity, which negatively impacts commercial production [8]. Pathogenic *A. tumefaciens* strains harbor large plasmids known as "Ti" (Tumor-inducing) plasmids

[9]. The bacterium transfers a specific segment of the Ti plasmid (T-DNA) into plant cells, where it is integrated into the host genome. The transfer and integration of T-DNA are regulated by the virulence (*vir*) region [10].

The transferred DNA (T-DNA) contains genes responsible for opine synthesis and the regulation of phytohormone biosynthesis within the host cells. The expression of these T-DNA genes triggers uncontrolled cell division [11], resulting in the formation of galls (tumors) on the stems, crowns, and roots [12]. The pathogen is typically identified using conventional diagnostic methods, such as isolation on selective or general media and pathogenicity tests on indicator plants like carrots or tomatoes [13]. Additionally, molecular techniques, including the use of primers targeting *vir* genes or T-DNA sequences, are employed to detect and identify the pathogen from bacterial cultures, soil, and plant tissues [14]. Previous research indicates that *A. tumefaciens* can be detected in plant or soil samples using qPCR targeting the *cheV* gene. This protein, encoded by the *chvE* gene, is responsible for enhancing the expression of *vir* genes induced by sugars [15].

A previous study demonstrated that sterilizing pruning tools reduced gall formation caused by *A. tumefaciens* in *Datura stramonium* [16]. Historically, several chemical agents, such as methoxyethylmercury chloride, copper oxychloride, and aqueous cuprammonium solution, were tested as pre-planting dips for apple seedlings. Results showed that copper oxychloride and aqueous cuprammonium solution offer significant potential for controlling Crown Gall in rose seedlings [17].

Studies have indicated that the most effective and sustainable approach for managing plant diseases involves an Integrated Disease Management (IDM) system. This system includes the use of host plant resistance, less susceptible cultivars, and intervention through chemical and/or biological controls, alongside sanitary and cultural practices aimed at reducing the primary inoculum. Utilizing pathogen-free soil and planting material, as well as sterilized pruning tools, are critical steps in the prevention and control of Crown Gall disease [18].

Materials and Methods

Isolation of the Causal Agent

A bacterial isolate of *Agrobacterium tumefaciens* was obtained from the Plant Pathology Laboratory for Postgraduate Studies, Department of Plant Protection, College of Agriculture, University of Karbala. The isolate had been previously identified based on morphological and molecular characteristics and was preserved at 4°C for subsequent experiments (19).

Preparation of Bacterial Inoculum

Nutrient Agar (NA) medium was prepared and sterilized in an autoclave at 121°C under 15 psi pressure for 20 minutes. After solidification, plates were inoculated with bacterial isolates and incubated at 30 ± 1°C for 24 hours.

The bacterial growth was harvested by washing the colony surface with 5 ml of sterile distilled water. The Plate Count Technique was employed to prepare the bacterial

inoculum. A serial dilution ranging from 10^{-1} to 10^{-8} was prepared using sterile normal saline solution (0.85% NaCl).

One milliliter from each of the last three dilutions was transferred aseptically into sterile Petri dishes (three replicates per dilution), followed by the addition of 20 ml of NA medium at 45°C. Plates were incubated at 30 ± 1°C, and a bacterial suspension with a concentration of 5×10^6 CFU/ml was selected for subsequent use.

Biochemical Tests of the Studied Bacteria

Growth in Nutrient Broth (NB)

Nutrient Broth (NB) medium (Difco, pH 7.2) was dispensed into test tubes and sterilized. Tubes were inoculated with the bacterial isolates and incubated at 30 ± 2°C for 72 hours. Bacterial growth was recorded qualitatively.

Catalase Test

Several drops of 3% hydrogen peroxide were added to fresh (24-hour-old) bacterial colonies grown on NA medium. The formation of gas bubbles within a few seconds indicated a positive reaction, whereas the absence of bubbles indicated a negative result (20; 21; 22).

Growth at 35°C

Pure bacterial cultures were streaked onto NA plates and incubated at 35°C for 48 hours to differentiate *Agrobacterium* species (19).

Growth on Medium Containing 2% NaCl

NA medium supplemented with 2% sodium chloride was prepared. The tested isolates were inoculated and incubated at 28 ± 2°C for 24 hours to determine their ability to grow under saline conditions (19).

Control of *A. tumefaciens* Causing Crown Gall Disease

Source of Biological Control Agents

The biocontrol fungi *Trichoderma harzianum* and *Trichoderma atroviride* were obtained from the Biological Control Laboratory, Department of Plant Protection, and activated on Potato Dextrose Agar (PDA). The bacterial biocontrol agent *Bacillus thuringiensis* was prepared from the commercial bio-insecticide **Antario KAB** (RUSSELL IPM, Russia), supplied by Al-Dabbana Company for Modern Agriculture (Iraq). It was cultured on NA medium at 30 ± 2°C for 24 hours. All microbial cultures were maintained on slants (NA for bacteria and PDA for fungi) and stored at 5°C for further use.

Evaluation of Antagonistic Efficiency of Biocontrol Agents Against *A. tumefaciens*

The antagonistic activity of *T. harzianum*, *T. atroviride*, and *Bacillus thuringiensis* against *A. tumefaciens* was assessed using the Dual Culture Technique (DCT). NA plates were divided into two equal halves. One half was inoculated with the pathogenic bacterium *A. tumefaciens* by streaking, while the other half was inoculated either with a 5 mm fungal disc taken from the edge of an actively growing colony or with the bacterial antagonist using streaking. Plates were incubated at 25 ± 2°C for 24 hours. Control treatments included plates inoculated only with the pathogen. Each treatment

consisted of four replicates. The percentage of growth inhibition (PGI) was calculated according to (23). using the following equation:

$$\textbf{PGI (\%)} = [(KR - R1) / KR] \times 100$$

Where:

- **PGI (%)** = Percentage Growth Inhibition
- **KR** = Radial growth of the pathogen in control plates (mm)
- **R1** = Radial growth of the pathogen in treated plates (mm)

The inhibition zone diameter was measured, and the data were statistically analyzed. Means were compared using the Least Significant Difference (LSD) test at a probability level of 0.05.

Evaluation of Chemical Pesticides (Nordox 75 WG and Beltanol) Against *A. tumefaciens* In Vitro

In this experiment, several chemical compounds were evaluated for their effectiveness against *A. tumefaciens*, the causal agent of crown gall disease in rose seedlings. These included:

- Nordox 75 WG at 1.25 g/L
- Beltanol at 1 ml/L

Additionally, zinc oxide (ZnO) in both nano and non-nano forms was tested at concentrations of 1.25, 2.5, and 5 g/L.

Each compound was individually mixed with sterilized NA medium prior to solidification and poured into sterile 9 cm Petri dishes. Plates were inoculated with 1 ml of bacterial suspension grown in NB medium at a concentration of 5×10^6 CFU/ml, then gently rotated to ensure uniform distribution.

Plates were incubated at 30 ± 2°C for 72 hours. The experiment was conducted using a Completely Randomized Design (CRD) with four replicates per treatment, including a control treatment without any control agents.

Results were recorded by counting bacterial colonies, and the percentage inhibition was calculated using the following formula:

$$\text{Percentage of growth inhibition}(\%) = \frac{\text{Number of colonies in the comparison} - \text{Number of colonies in the treatment}}{\text{Number of colonies erformed to assess significant}} \times 100$$

Results and Discussion

Isolation of the Causal Pathogen

The isolation results revealed the development of bacterial growth of the causal pathogen on Nutrient Agar (NA) medium after 24 hours of incubation at 30 ± 2 °C. After 48 hours of incubation, individual colonies were distinguishable, exhibiting a white to creamy coloration. The colonies were smooth, convex, and circular with entire margins. As the colonies aged, their color gradually darkened and they developed a viscous, mucoid appearance.

These findings are consistent with previous reports describing the cultural characteristics of Agrobacterium tumefaciens colonies (24,19).

Figure (1): Colonies of Crown Gall Bacterium on Nutrient Agar (NA) medium

Biochemical Characterization of the Bacteria Under Study

The results of the biochemical tests conducted on the bacterial isolates under investigation were positive for all assays, as presented in Table (1).

Table (1): Biochemical Tests of the Bacteria Under Study

No.	Biochemical Test	Result
1	Growth in Nutrient Broth (NB)	+
2	Catalase Test	+
3	Growth at 35 °C	+
4	Growth in medium supplemented with 2% sodium chloride (NaCl)	+

(+) Positive result
(−) Negative result

Management of *Agrobacterium tumefaciens*, the Causal Agent of Crown Gall Disease

In vitro Biological Control of Agrobacterium tumefaciens

The results of this experiment demonstrated that the biological control agents used, namely *Trichoderma harzianum* and *Bacillus thuringiensis*, exhibited high efficiency in suppressing the growth of the pathogenic bacterium *Agrobacterium tumefaciens* under in vitro conditions on Nutrient Agar (NA) medium, compared with the control treatment.

As shown in Table (2), the highest inhibition was recorded with *Trichoderma harzianum,* reaching 86.41%, which did not differ significantly from *Bacillus thuringiensis*, with inhibition percentages of 85.00% and 83.82%, respectively.

Table (2): Antagonistic Potential of *Trichoderma harzianum* and *Bacillus thuringiensis* in Inhibiting the Growth of *Agrobacterium tumefaciens* on Nutrient Agar (NA) medium under in vitro Conditions

Treatment	Growth Inhibition (%)
Control	0.00
Trichoderma harzianum	86.41
Bacillus thuringiensis	83.82

Least Significant Difference (LSD) at P = 0.05: 5.99

The efficiency of the biocontrol fungus *Trichoderma harzianum* is attributed to its ability to produce a range of cell wall–degrading enzymes of phytopathogens, such as proteases, esterases, phosphamidases, and others (25). These enzymes contribute to the degradation of pathogen cell walls, thereby facilitating penetration, colonization, and mycoparasitism of the target pathogen (26).

Furthermore, the antagonistic activity of *T. harzianum* may also be associated with its production of effective proteolytic enzymes, including serine proteases, which inhibit key enzymatic activities in the pathogen, leading to disruption of its developmental processes at early stages (27). These findings are consistent with those reported by (28,29).

Regarding the role of the bacterial biocontrol agent *Bacillus thuringiensis* in the suppression of phytopathogens, members of the genus *Bacillus* are known for their strong ability to inhibit a wide range of plant pathogenic microorganisms. This activity is mainly due to the production of multiple antimicrobial compounds, such as the antibiotic Zwittermicin A, in addition to the enzyme acyl-homoserine lactone lactonase, which plays an important role in disrupting quorum sensing and limiting the growth of competing pathogens (30).

Moreover, the biocontrol efficacy of these bacteria is also associated with the production of insecticidal crystal proteins (Cry proteins) during sporulation, which are largely responsible for their toxic and inhibitory effects (31).

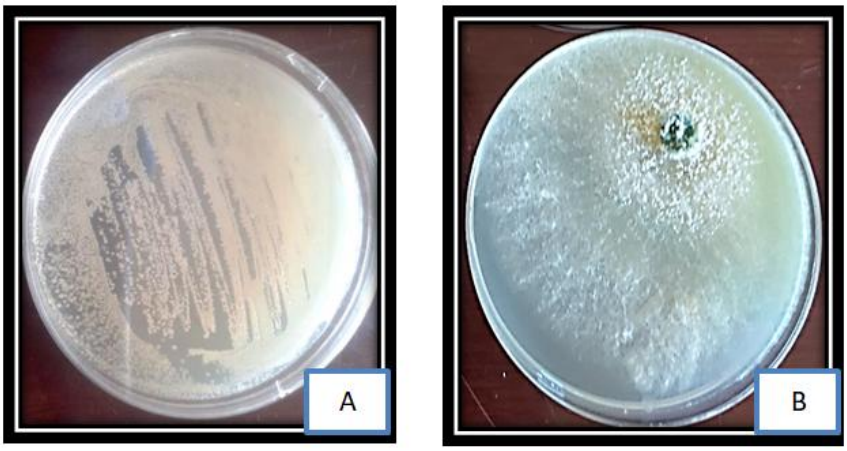

Figure (2): Antagonistic activity of biological control agents: (A) control treatment; (B) antagonism between the pathogenic bacterium and *Trichoderma harzianum*

Effect of the Chemical Pesticides Nordox 75 WG and Beltanol on *Agrobacterium tumefaciens* In Vitro

Both the fungicide Nordox 75 WG at a concentration of 1.25g L^{-1} and the pesticide Beltanol at 1 mL L^{-1} were able to inhibit the growth of *Agrobacterium tumefaciens* on nutrient agar (NA) using the poisoned food technique at the recommended concentrations, as illustrated in Figure (3).

The inhibitory effect of Nordox 75 WG on bacterial growth may be attributed to its content of copper oxide, which suppresses bacterial proliferation and, in many cases, leads to the death of large bacterial populations due to the toxic effects of divalent copper ions (Cu^{2+}) (32). Copper-based compounds have demonstrated considerable efficacy in controlling various plant pathogenic bacteria (33).

Regarding Beltanol, its effect is mainly attributed to the active ingredient hydroxyquinoline sulfate (50% w/v, 500 g L^{-1}), which plays a significant role in inhibiting the growth of the pathogenic bacterium. This finding is consistent with previous studies reporting complete inhibition of pathogen growth by this compound (34).

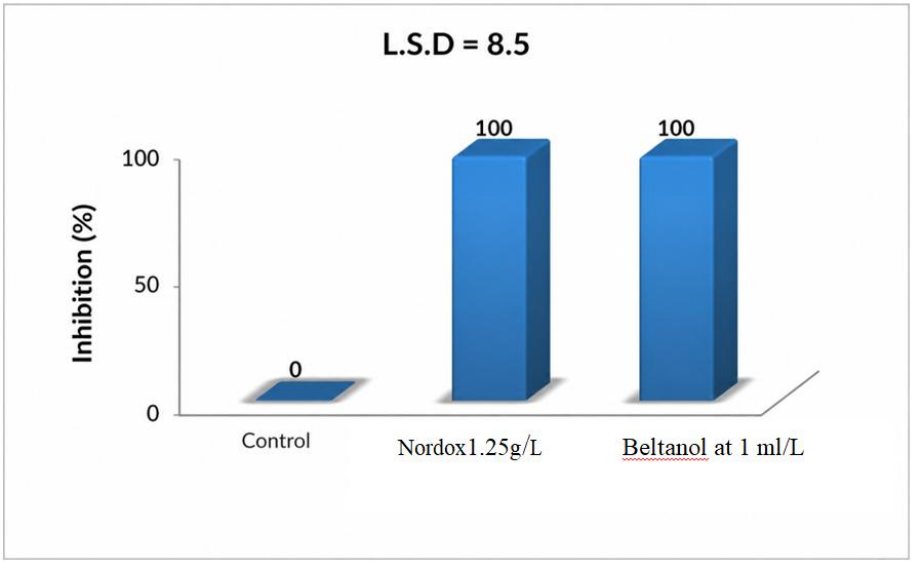

Figure (3): Inhibitory effect of the chemical pesticides Nordox 75 WG and Beltanol on the growth of A. tumefaciens in vitro.

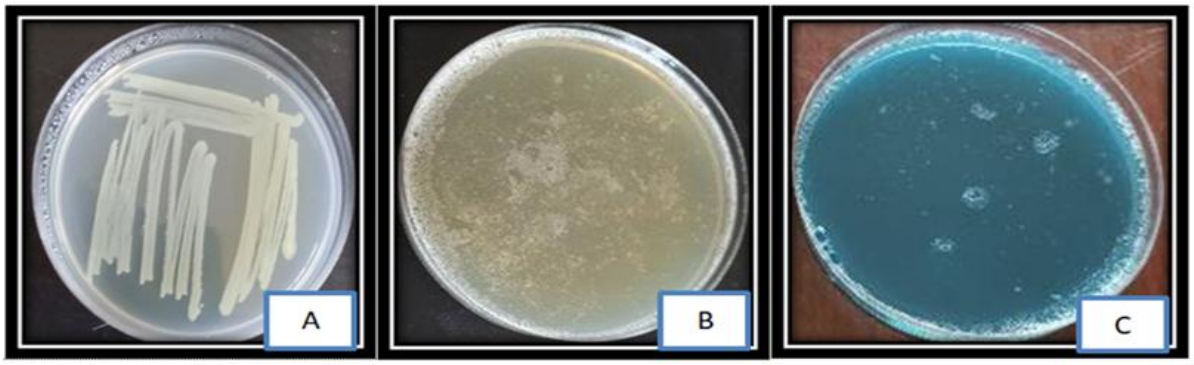

Figure (4): Efficiacy of pesticides against the pathogenic bacteria : (A) Control, (B) Beltanol, (C)Nordox

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