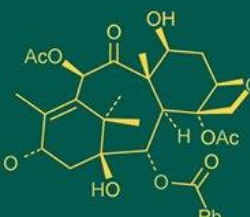
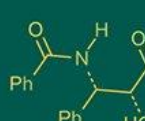


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Screening of cultivable maize rhizosphere bacteria from fall armyworm-infested plants for antibiotic susceptibility and hydrolytic enzyme activities

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Abstract

The fall armyworm (*Spodoptera frugiperda*) is a major insect pest of maize, and the identification of functional microorganisms associated with maize may contribute to the development of sustainable pest management strategies. The present study was undertaken to isolate and characterize cultivable bacterial populations from the rhizosphere of fall armyworm-infested maize and to evaluate their antibiotic susceptibility and selected enzymatic activities. A total of 26 bacterial isolates were obtained from maize rhizosphere soil and screened against six antibiotics, namely erythromycin, colistin, polymyxin, nadifloxacin, methicillin and rifampicin, using the disc diffusion method. Considerable variation was observed in the antibiotic susceptibility profiles of the isolates. Nadifloxacin showed the highest proportion of susceptible isolates (92.3%), followed by colistin and polymyxin, each with 73.1% susceptibility. Erythromycin and rifampicin showed 46.2% and 34.6% susceptibility, respectively, whereas methicillin showed the lowest susceptibility (11.5%). Enzymatic screening revealed proteolytic activity in seven isolates, with MRB-10 exhibiting the highest hydrolysis zone (20.00 ± 0.36 mm), followed by MRB-5 (18.33 ± 0.15 mm) and MRB-9 (16.33 ± 0.21 mm). Urease activity was detected in four isolates, namely MRB-1, MRB-10, MRB-17 and MRB-20. Notably, MRB-10 and MRB-20 exhibited both protease and urease activities, with MRB-10 showing the highest proteolytic activity among all isolates. Overall, the study demonstrates that the rhizosphere of fall armyworm-infested maize harbours cultivable bacteria with diverse antibiotic susceptibility patterns and functional enzymatic traits. The selected isolates, particularly those exhibiting hydrolytic enzyme activities, warrant further molecular identification, evaluation of plant growth-promoting and insecticidal traits, and direct bioassays against *S. frugiperda* to determine their potential for sustainable microbial-based pest management.

Keywords: *Spodoptera frugiperda*, maize rhizosphere, rhizobacteria, antibiotic susceptibility, protease, urease, microbial biocontrol

1. Introduction

Maize (*Zea mays* L.) is an important cereal crop cultivated worldwide for food, feed and industrial purposes. Its production is increasingly threatened by the fall armyworm (FAW), *Spodoptera frugiperda*, which is a highly polyphagous and destructive pest of maize. The rapid spread of FAW and its ability to develop resistance to insecticides have increased the need for alternative and environmentally sustainable management strategies. In this context, microorganisms associated with maize are gaining attention because of their potential roles in plant growth promotion, nutrient cycling and insect suppression (Ranjith *et al.*, 2022; Ranjith and Kalaiselvi, 2024)^[5].

The maize rhizosphere represents a nutrient-rich and biologically active environment that supports diverse bacterial populations influenced by root exudates, soil properties and plant health. Some rhizosphere bacteria produce extracellular enzymes such as proteases and chitinases, which may contribute to nutrient mobilization and antagonistic or insecticidal activities. Winssy *et al.* (2024)^[9] reported that enzyme-producing bacteria associated with FAW showed potential to affect larval survival, indicating that such functional traits can be useful during the preliminary screening of bacterial biocontrol candidates. Similarly, maize-associated *Bacillus amyloliquefaciens* has been reported to influence FAW feeding and enhance plant defence responses (Ranjith and Kalaiselvi, 2024).

The interaction between maize, its associated microorganisms and FAW is complex. Some microorganisms can enhance plant defence and reduce insect performance, whereas others may benefit the insect by contributing to detoxification of plant defensive compounds. For example, Qi *et al.* (2024) [4] demonstrated that a maize-associated *Pantoea dispersa* strain could assist FAW in detoxifying maize benzoxazinoids and promote larval growth. Therefore, characterization of microbial communities associated with FAW-infested maize is important for identifying bacteria with potentially beneficial, neutral or insect-associated functions.

In addition to functional enzyme production, antibiotic susceptibility can provide information about the physiological characteristics and ecological adaptation of bacterial isolates. Screening for antibiotic response together with enzymatic traits can provide a broader assessment of the diversity and functional potential of rhizosphere bacteria. Hence, the present study was undertaken to isolate cultivable bacteria from the rhizosphere of FAW-infested maize and evaluate their antibiotic susceptibility and selected functional traits, particularly protease and urease activities. The study may provide potential bacterial candidates for further characterization and evaluation in sustainable FAW management.

2. Materials and Methods

2.1 Collection of maize rhizosphere soil

Rhizosphere soil samples were collected from maize (*Zea mays* L.) plants naturally infested with fall armyworm (*Spodoptera frugiperda*). Plants showing characteristic symptoms of FAW infestation, such as leaf scraping, windowing and irregular feeding damage, were selected for sampling. The plants were carefully uprooted, and loosely adhering soil was removed without disturbing the soil closely associated with the roots. Soil adhering to the root surface was collected aseptically into sterile polyethylene bags, labelled appropriately and transported to the laboratory under cool conditions for microbiological analysis.

2.2 Isolation of rhizosphere bacteria

Approximately 1 g of rhizosphere soil was transferred aseptically into 9 mL of sterile distilled water or sterile physiological saline and thoroughly mixed using a vortex mixer to obtain a homogeneous soil suspension. Serial ten-fold dilutions were prepared up to an appropriate dilution level. Aliquots of 0.1 mL from selected dilutions were spread uniformly on sterile nutrient agar (NA) plates. The plates were incubated at approximately 28 ± 2 °C for 24-48 h. Following incubation, morphologically distinct bacterial colonies differing in size, shape, pigmentation, elevation and margin were selected.

2.3 Purification and maintenance of bacterial isolates

Individual bacterial colonies were aseptically picked and streaked repeatedly onto fresh nutrient agar plates until pure cultures were obtained. The purified isolates were designated as MRB-1 to MRB-26 and maintained on nutrient agar slants at 4 °C for short-term preservation.

2.4 Antibiotic Susceptibility Testing

The antibiotic susceptibility profiles of the bacterial isolates were determined by the Kirby-Bauer disc diffusion

technique using nutrient agar as the growth medium, following the standard procedure described by Bauer *et al.* (1966). The isolates were tested against six antibiotics, namely erythromycin, colistin, polymyxin, nadifloxacin, methicillin, and rifampicin. Briefly, freshly grown bacterial cultures were uniformly inoculated onto the surface of nutrient agar plates, followed by the placement of antibiotic discs at appropriate intervals. The inoculated plates were incubated at 28 ± 2 °C for 24 h. After incubation, the diameter of the zones of inhibition surrounding each antibiotic disc was measured and recorded in millimetres. Based on the presence or absence of an inhibition zone and the adopted interpretive criteria, the bacterial isolates were classified as sensitive or resistant to the respective antibiotics.

2.5 Protease Activity Assay

Proteolytic activity of the bacterial isolates was screened using skim milk agar medium. Actively growing cultures were spot-inoculated onto the prepared medium and incubated at 28 ± 2 °C for 24-48 h. Protease-producing isolates were identified by the formation of distinct clear zones surrounding the bacterial colonies due to hydrolysis of casein present in the medium. The proteolytic activity of the positive isolates was evaluated by measuring the diameter of the clear hydrolysis zone in millimetres (Smibert *et al.*, 1994) [8].

2.6 Urease Activity Assay

Urease production by the bacterial isolates was evaluated using Christensen's urea agar medium following the method of Christensen (1946) [2]. The isolates were inoculated onto the medium and incubated under suitable conditions. Urease-producing isolates hydrolysed urea, resulting in an increase in the pH of the medium and a characteristic colour change from yellow to pink. Isolates exhibiting this colour change were recorded as urease-positive, whereas those showing no colour change were considered urease-negative.

2.7 Data analysis

All experiments were performed with appropriate biological or technical replicates. The observations obtained from antibiotic susceptibility and enzyme screening assays were recorded and expressed as mean $\pm$ standard error, wherever applicable. The percentage of susceptible bacterial isolates was calculated using the total number of isolates tested as the denominator. For enzyme screening, isolates were categorized as positive or negative based on the development of characteristic hydrolysis or colour-change reactions, while quantitative measurements of hydrolysis zones were expressed in millimetres.

3.1 Antibiotic susceptibility of maize rhizosphere bacterial isolates

The antibiotic susceptibility profiles of the 26 maize rhizosphere bacterial isolates from fall armyworm-infested plants are presented in Figure 1, while the percentage susceptibility to individual antibiotics is shown in Figure 2. Considerable variation was observed in the susceptibility patterns of the bacterial isolates to the six antibiotics tested. Nadifloxacin exhibited the highest proportion of susceptible isolates (92.3%), followed by colistin and polymyxin, each with 73.1% susceptibility. Erythromycin showed an intermediate level of susceptibility, with 46.2% of the

isolates classified as susceptible, whereas 34.6% were susceptible to rifampicin. Methicillin showed the lowest proportion of susceptible isolates (11.5%), indicating that most of the isolates were resistant to this antibiotic. Overall, the isolates exhibited differential susceptibility to the antibiotics tested, with nadifloxacin showing the highest proportion of susceptible isolates and methicillin the lowest.

3.2 Screening of Bacterial Isolates for Protease Assay

Proteolytic activity was detected in seven of the 26 maize rhizosphere bacterial isolates, namely MRB-5, MRB-7, MRB-9, MRB-10, MRB-20, MRB-23 and MRB-24 (Table 1). Among the protease-positive isolates, MRB-10 recorded the highest hydrolysis zone (20.00 ± 0.36 mm), followed by MRB-5 (18.33 ± 0.15 mm), MRB-9 (16.33 ± 0.21 mm), MRB-24 (14.66 ± 0.23 mm), MRB-7 (14.00 ± 0.24 mm), MRB-23 (10.66 ± 0.37 mm) and MRB-20 (9.00 ± 0.19 mm). The remaining 19 isolates did not exhibit detectable proteolytic activity under the conditions tested.

3.2 Screening of Bacterial Isolates for Urease Assay

Urease production was detected in four of the 26 bacterial isolates, namely MRB-1, MRB-10, MRB-17 and MRB-20, as indicated by a positive colour reaction on Christensen’s urea agar (Table 1). The remaining 22 isolates showed no detectable urease activity. Among the urease-positive isolates, MRB-10 and MRB-20 also exhibited proteolytic activity, whereas MRB-1 and MRB-17 were positive only for urease activity.

4. Discussion

The present study demonstrates that the rhizosphere of FAW-infested maize harbours cultivable bacteria with diverse antibiotic susceptibility and functional characteristics. Variation in antibiotic response may be associated with differences in bacterial composition, intrinsic resistance mechanisms and adaptation to the rhizosphere environment. Such functional diversity indicates that the maize rhizosphere can serve as an important reservoir of metabolically versatile bacteria with potential ecological and biocontrol significance. The occurrence of protease-producing bacteria indicates their potential involvement in extracellular protein degradation and nutrient cycling. Protease production may also serve as an important preliminary trait for identifying bacteria with insecticidal potential. Similar observations were reported by Ranjith *et al.* (2022)^[5] and Ranjith *et al.* (2023), who demonstrated that maize-associated bacteria could influence plant defence and FAW feeding behaviour. More importantly, Winssy *et al.* (2024)^[9] reported chitinase- and protease-producing bacteria associated with *S. frugiperda*, and selected *Bacillus licheniformis* and *Enterobacter cloacae* isolates adversely affected larval growth and food utilization. These findings support the importance of extracellular enzyme-producing bacteria as

potential candidates for further FAW biocontrol screening. The detection of urease activity indicates the ability of selected isolates to hydrolyse urea and suggests a potential role in nitrogen turnover that warrants further investigation. The coexistence of different functional traits within the bacterial community may enhance their ecological adaptability and interactions with maize. Recent studies have also shown that maize-associated microorganisms can influence FAW performance and plant defence, emphasizing the importance of plant-microbe-insect interactions (Qi *et al.*, 2024; Ma *et al.*, 2024)^[4, 3]. Overall, the functional traits observed in the present isolates indicate their potential as multifunctional rhizosphere bacteria, although further molecular identification, quantitative enzyme assays and direct larval bioassays are required to confirm their suitability as microbial biocontrol agents.

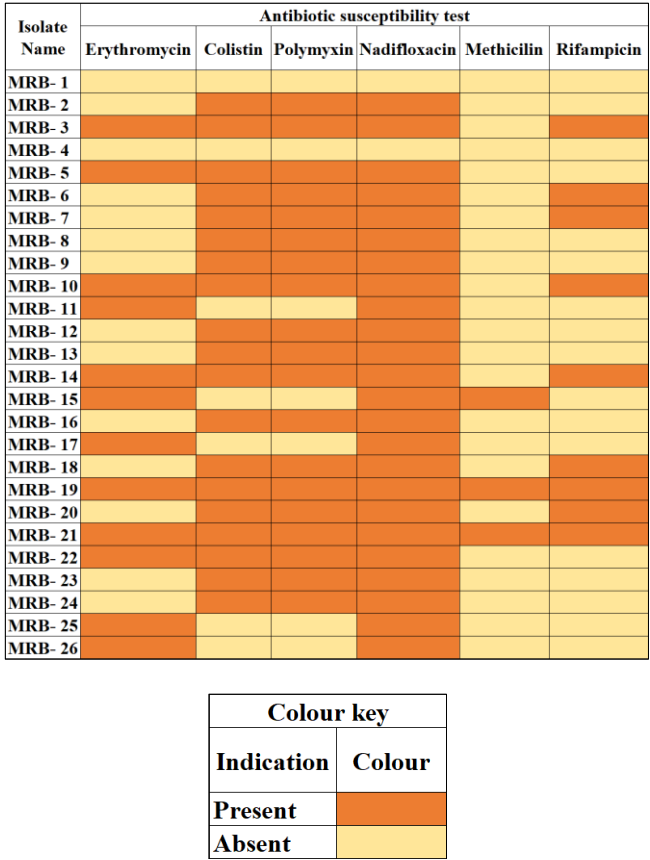


Fig 1: Antibiotic susceptibility patterns of maize rhizosphere bacterial isolates from Fall Armyworm-Infested Plants

Light yellow (S) = Susceptible; Orange (R) = Resistant.

The antibiotic susceptibility of the 26 maize rhizosphere bacterial isolates from Fall Armyworm-infested plants was determined against erythromycin, colistin, polymyxin, nadifloxacin, methicillin, and rifampicin using the disc diffusion method.

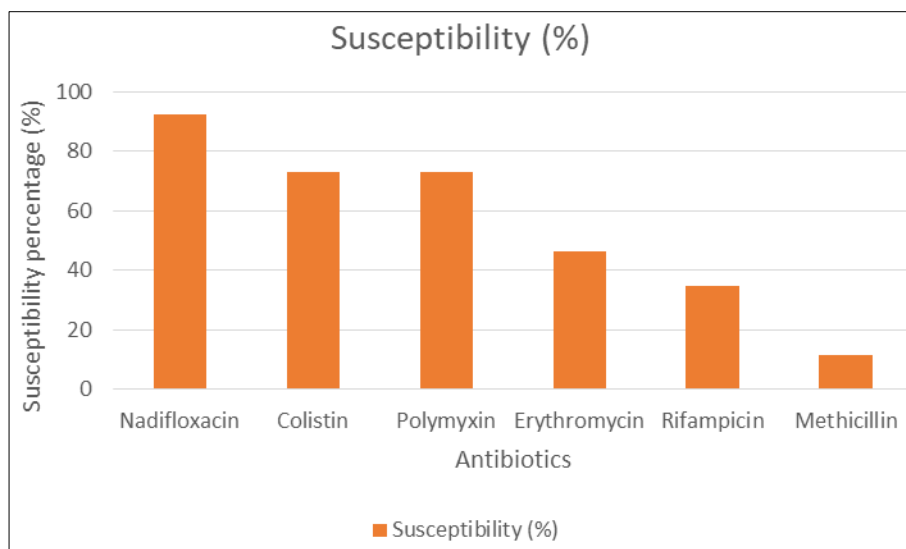


Fig 2: Percentage antibiotic susceptibility of maize rhizosphere bacterial isolates from Fall Armyworm-infested plants

Values represent the antibiotic susceptibility of 26 maize rhizosphere bacterial isolates from Fall Armyworm-infested plants ($n = 26$), determined using the Kirby-Bauer disc diffusion method. Susceptibility testing was performed on Nutrient Agar, and the inhibition response was recorded after incubation at $28 \pm 2^\circ\text{C}$ for 24 h. Light yellow indicates susceptible (S), and orange indicates resistant (R).

Table 1: Protease and urease activities of maize rhizosphere bacterial isolates from Fall Armyworm-Infested Plants

Isolate Name	Protease activity (mm)	Urease activity
MRB-1	0.00	+
MRB-2	0.00	–
MRB-3	0.00	–
MRB-4	0.00	–
MRB-5	18.33 ± 0.15	–
MRB-6	0.00	–
MRB-7	14.00 ± 0.24	–
MRB-8	0.00	–
MRB-9	16.33 ± 0.21	–
MRB-10	20.00 ± 0.36	+
MRB-11	0.00	–
MRB-12	0.00	–
MRB-13	0.00	–
MRB-14	0.00	–
MRB-15	0.00	–
MRB-16	0.00	–
MRB-17	0.00	+
MRB-18	0.00	–
MRB-19	0.00	–
MRB-20	9.00 ± 0.19	+
MRB-21	0.00	–
MRB-22	0.00	–
MRB-23	10.66 ± 0.37	–
MRB-24	14.66 ± 0.23	–
MRB-25	0.00	–
MRB-26	0.00	–

Values in each column represent the mean of three independent replications ($\pm$ standard error). Protease activity was evaluated on skim milk agar by measuring the diameter of the clear hydrolysis zone surrounding bacterial colonies. Urease activity was determined using Christensen's urea agar, where (+) indicates a positive urease reaction and (–) indicates a negative urease reaction.

5. Conclusion

The present study demonstrated that the rhizosphere of FAW-infested maize harbours a diverse population of cultivable bacteria exhibiting variable antibiotic susceptibility and functional enzyme activities. The occurrence of protease- and urease-producing isolates indicates their potential involvement in nutrient transformation and other beneficial microbial interactions. The presence of proteolytic activity is particularly relevant because hydrolytic enzyme-producing bacteria have been associated with the biocontrol of *Spodoptera frugiperda*. Overall, the isolates obtained in this study represent a promising microbial resource for further screening. Molecular characterization, evaluation of additional plant growth-promoting and insecticidal traits, and laboratory and greenhouse bioassays against FAW are required to identify effective candidates for developing sustainable microbial-based strategies for FAW management.

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