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Article

Isolation, conventional and molecular identification of *Fusarium proliferatum* responsible to bulb rot of garlic and potential biological control by new bacterial strains

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Abstract

The purpose of the present study was to isolate and identify *Fusarium proliferatum* from infected garlic bulbs, using conventional and molecular identification techniques, as well as to evaluate the *in vitro* effect of new bacterial strains on the mycelial growth of *F. proliferatum*. Infected garlic bulb samples were collected and the phytopathogenic fungi isolated and identified using standardized conventional and molecular methods. The effect of the bacterial strains isolated from the different substrates and the filtrates of their liquid culture on the mycelial growth of *F. proliferatum* was studied using the method of direct confrontation between *F. proliferatum* and the microorganism or the filtrate which could be antagonistic. Molecular analysis definitively identified *F. proliferatum* as the causal agent of garlic bulb rot. The corresponding nucleotide sequence has been deposited in the National Centre for Biotechnology Information (NCBI) GenBank database under accession number OP820542. Three bacterial isolates with a significant inhibitory effect on the growth of *F. proliferatum* were isolated and selected. These isolates were identified as *Bacillus subtilis* (Isolat A4), *Bacillus tequilensis* (Isolat A11) and *Pseudomonas sp*. (Isolat A12) which exhibited inhibition percentages of 54.00 ± 1.00 %, 59.00 ± 1.00 %, 67.33 ± 1.70 % respectively in comparison with fluconazole 59.17 ± 0.76 %. The current study will contribute to the understanding of garlic bulb rot and to the exploration of new methods of biological control of this disease, as part of a strategy of sustainability and pesticide reduction.

Keywords: *Fusarium proliferatum*; garlic bulb rot; biological control; bacterial strains; molecular identification.

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1. Introduction

The garlic bulb rot, caused by various pathogens, represents a significant threat to agricultural production and crop quality (Anum et al., 2024). Among these pathogens, *F. proliferatum* stands out as one of the main species responsible for this disease (Le et al., 2021). Due to the increase in trade and intensive agricultural practices, the incidence of this disease has expanded at an alarming rate, leading to considerable economic losses worldwide (Combe et al., 2023). *F. proliferatum* is a fungal phytopathogen

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widely distributed in foods and often isolated from several plants as the main phytopathogenic agent, as in garlic and onion bulb rot, in citrus, where it causes fruit rot, and in palms, where it is an agent of seedling blight, it generally causes root tip and tissue rot, as well as yellowing and wilting of leaves, leading to death (Wang et al., 2022). Also, *F. proliferatum* produces a large number of mycotoxins, including fumonisins, which have a considerable impact on human and animal health (Li et al., 2017b).

The accurate detection and identification of pathogens such as *F. proliferatum* is essential for the implementation of effective control strategies. Traditionally, the identification of *Fusarium* species has been based on morphological and physiological techniques, but these methods can have limitations in terms of specificity and sensitivity (Nikitin et al., 2023). Integrating molecular techniques into the identification process offers significant advantages in terms of speed, accuracy and specificity (Chethana et al., 2021). The use of molecular markers not only enables accurate identification of species, but also provides information on the genetic diversity within fungal populations, facilitating a better understanding of their epidemiology and ecology (Oliveira and Azevedo, 2022).

Many research studies in the scientific bibliography have focused on the control of *F. proliferatum* through the use of bioactive molecules, whether of natural origin (microbial and/or vegetal) or of chemical origin. The need for these substances is increasing because of the resistance that pathogenic and phytopathogenic microorganisms can develop against available antibiotics and antifungals. Plant substances, in particular essential oils and plant extracts, are highly effective against harmful microorganisms (Kamal et al., 2019; Yi et al., 2018), however, the toxicity of these substances remains a major problem (Gaire et al., 2019). As far as chemical substances are concerned, their use is appreciable in terms of cost, but they risk causing problems for the environment and human health (Raymaekers et al., 2020). Meanwhile, the use of antagonistic microorganisms, such as bacterial strains, represents a promising approach to the biological control of fungal diseases (Mesguida et al., 2023). These antagonistic agents have the potential to limit the growth and spread of pathogens while offering advantages in terms of environmental safety and sustainability (Asghar et al., 2024). Accordingly, the aim of the current study is to isolate and identify *F. proliferatum* using conventional and molecular techniques in order to understand its genetic diversity and its role in garlic bulb rot. Additionally, it explores the potential of new bacterial strains as biocontrol agents to limit the harmful impact of this disease on garlic crop.

2. Materials and Methods

2.1. Isolation, conventional and molecular identification of F. proliferatum

The phytopathogenic fungus *F. proliferatum* was isolated from the bulb rot of garlic bought on the market in Fez, Morocco, by the suspension dilution method, using Malt Agar Extract medium and incubating at 30°C for 7 days in an atmospherically saturated humidity. The identification of the fungus was based on conventional identification (macro and microscopic characterization) and molecular identification.

2.2. Conventional identification

The fungal colony isolated and then purified was identified. Standard methods were used: macroscopic characteristics (on the front of the plate: colour, consistency (hairless, fluffy, powdery, plastery, silky, etc.), surface (flat, domed, wrinkled, cerebriform, etc.), on the back of the plate: colour, deep arborisation, pigment, etc.) and microscopic characteristics, based on the description of the mycelium, the sporiferous apparatus and the spores (El Barnossi and Iraqi, 2023a). To determine the genus of the fungal colony obtained, the Saccardo classification system was adopted (Barnette et Hunter, 1972; Jedidi et al., 2018). Species were identified by reference to various identification keys (Botton et al., 1990; Domsch et al., 1980; Ellis, 1976, 1971; Hoffmann et al., 2008; Nelson et al., 1983; Wang et Zabel, 1990).

2.3. Molecular identification

2.3.1. DNA extraction

DNA was extracted from the fungal isolate using the automated Isolate II Genomic DNA Kit.

2.3.2. Amplification of extracted DNA by PCR

2.3.2.1. Primers used

Universal primers (Table 1) amplifying the ITS region (ITS1 and ITS4) were used to amplify the DNA of the fungal isolate.

Table 1. Primer sequences for fungal DNA amplification.

Region	Primers	Direction	Sequences
ITS	ITS1	Forward	5'-TCCGTAGGTGAACCTGCGG-3'
	ITS4	Revers	5'-TCCGCAGGTTCACCTACGGA-3'

Table 2. Composition of the reaction mixture for fungal DNA amplification.

Reagents (MyTaq DNA polymerase kit from Bioline)	Quantity for 1 tube
10 x buffer	2.5 µL
F 10 µM	1 I
R 10 µM	1 µL
Dntp	0.5 µL
Taq	0.4 µL
ADN (100 ng)	1 µL
H_2O	Vt = 25 µL

2.3.2.2. PCR reaction mixture

The PCR reactions were carried out in a total volume of 25 µL. The composition of the reaction mixture used for fungal DNA amplification is detailed in Table 2 (Rajkumar et al., 2024).

2.3.3. Revelation of amplified DNA

DNA amplified by the specific primers used was visualized by electrophoresis and staining with Ethidium Bromide (BET). PCR products were electrophoresed by migration through a 1% agarose gel in the presence of the 1 Kb molecular weight marker. The gel obtained after migration was stained with BET (0.02 µg/mL) for 25 min, rinsed with sterile distilled water for 10 min and then visualized using the "G Box" photo documentation system.

2.3.4. Sequencing and bioinformatics analysis of nucleotide sequences

All the PCR products obtained using the different steps detailed previously were sequenced using the same primers. The sequence data was cut and assembled using ChromasPro version 2.1. The assembled sequences were blasted with the genomic data, which were stored in NCBI (National Centrer for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/BLAST/) (Miller et al., 2024).

2.4. Isolation of bacterial antagonists to control F. proliferatum

To find new bacterial strains to control *F. proliferatum*, several hundred bacterial isolates were isolated by the dilution suspension method from different substrates (soil, water, decomposing household waste, manure, etc.) (El Barnossi et al., 2020b, 2020a).

2.5. Evaluation of the antifungal activity of antagonists

The selection test for bacterial antagonists against *F. proliferatum* was carried out using the direct confrontation method in accordance with Pereira et al. (2013). Pure bacterial isolates were grown on the same isolation medium (Nutrient agar medium and incubation at 30°C for 24 hours). After 24 hours incubation at 30°C, agar plugs (6 mm diameter) with a density of 106 to 108 CFU/mL were cut with a cookie cutter and placed on the surface of Malt agar medium (EMA) which was inoculated with *F. proliferatum*. Positive controls were made by following the same steps and using the antibiotic Fluconazole (15 mg/mL). Inoculated Petri dishes were incubated in the dark and in a humidity-saturated atmosphere at 30°C. Inhibition rates were determined after 7 days incubation using the following formula (Saghrouchni et al., 2023):

$$\%I = [(C_1 - C_2) / C_1)] \times 100$$

Where C_1 is the average diameter of control colonies and C_2 is the average diameter of the colonies confronted.

2.6. Evaluation of the antifungal activity of liquid culture filtrates of antagonists

The tested bacterial antagonists that showed promising antifungal activity against *F. proliferatum* were grown in three replicates in Erlenmeyer flasks (250 mL) each containing 100 mL of isolation medium for each antagonist. After 20 days incubation at 30°C in the dark. The suspensions were centrifuged and then filtered through Wattman paper and Millipore filters under aseptic conditions. Evaluation of the anti-*F. proliferatum* activity of the filtrates from the liquid cultures of our antagonists was carried out using the disk diffusion method, following the same steps as for the agar plug diffusion method (Balouiri et al., 2016; El Barnossi et al., 2020). Petri dishes containing EMA medium were inoculated with *F. proliferatum*. Wattman paper discs (6 mm diameter) were then placed on the surface of the inoculated media and impregnated with 20 µL of the liquid culture filtrate of each antagonist. The inoculated Petri dishes were incubated at 30°C and the inhibition rate was determined after 7 days of incubation using the following formula (Saghrouchni et al., 2023):

$$\%I = [(C_1 - C_2) / C_1)] \times 100$$

Where C_1 is the average diameter of control colonies and C_2 is the average diameter of the colonies confronted.

2.7. Identification of bacterial antagonists

2.7.1. Morphological and cultural characterization

The bacterial antagonists isolated were purified by successive subculturing until stabilization of their morphological characteristics (macro and microscopic), then identified according to the protocol described by Prevot, (1938). For each bacterial antagonist, Gram staining, motility test, spore staining, pigmentation, cilia staining, growth in nutrient agar medium at pH 4 to 10 (pH was adjusted by KOH(N) or H3PO4(N)), growth at 4, 25, 30, 37, 44 and 65°C and growth at different NaCl concentrations (0, 2, 4, 6, 8 and 10%) were revealed (El Barnossi et al., 2020).

2.7.2. Biochemical characterisation

The biochemical characteristics of the bacterial antagonists were carried out by the catalase test and the oxidase test and also by the API®20NE gallery (bioMérieux®) in accordance with Bakki et al., (2024) and Kilonzi and Otieno (2024).

2.8. Statistical analysis

Quantitative data were presented as the mean values from three identical experiments, with the standard deviation included. The statistical significance of the difference between means was assessed using analysis of variance (1-factor ANOVA). Tukey's multiple range tests were performed using GraphPad Prism 8.0.1 (Graph Pad Software Inc., San Diego, USA) (El Barnossi and Iraqi, 2023b).

3. Results and discussion

3.1. Macro and microscopic characteristics of the fungal isolate

The macro and microscopic characteristics of the harmful fungus tested are shown in the various photos in Figure 1. Isolate P2 is characterized by a rapidly growing thallus (6.7 mm/day), with white mycelium with violet to dark pigments. Macroconidia slightly curved, with a distinct foot cell, 30 to 50 µm long, with three to five septa, unicellular and slightly pyriform microconidia, and chlamydospore absent. From these characteristics isolate P2 is identified as *F. proliferatum*.

3.2. Molecular characteristics of the harmful fungus tested

Classical identifications, as they are carried out, may in some cases lead to errors, which we have overcome by using molecular identification to confirm that we have indeed isolated the phytopathogenic fungus *F. proliferatum* responsible for wing bulb rot. The fungal isolate classically identified as *F. proliferatum* was identified by sequencing the ITS region, then the nucleotide sequences were subjected to a BLAST search in the NCBI database before being deposited in the gene bank under accession number: OP820542 for *F. proliferatum* with a percentage identity of 100%.

3.3. Cultural, microscopic and biochemical characteristics of isolated bacterial antagonists

The cultural, microscopic and biochemical characteristics of bacterial antagonists are listed in Table 3. The majority of the bacterial antagonists were Gram-positive, capsulated, rod-shaped bacilli, grouped in pairs and chains, constituting endospores, and reacting positively to the biochemical tests performed. In addition, they were able to grow at temperatures between 4°C and 50°C, and over a wide pH range from 4 to 10 and NaCl concentrations from 0 to 4%. On the basis of the cultural, microscopic and biochemical characteristics of each isolate, isolate A4 was identified as Bacillus subtilis, isolate A11 as *Bacillus tequilensis*, isolate A12 as *Pseudomonas sp*.

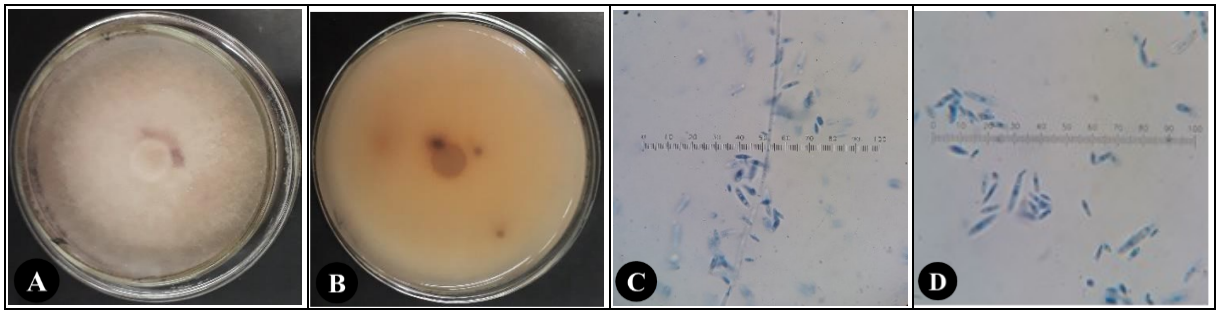

Figure 1. Macro and microscopic characteristics of isolate P2 identified as *F. proliferatum*. **A** and **B:** Macroscopic characteristics, **C** and **D:** Microscopic characteristics**.**

Table 3. Cultural, microscopic and biochemical characteristics of bacterial antagonists.

	Isolate A4	**Isolate A11**	**Isolate A12**
Gram type	+	+	-
Endospore	+	+	-
Mobility	+	+	+

Pigmentation	+	+	+
Cilia	+	+	+
Growth at			
65 °C	-	-	-
50 °C	+	+	-
44 °C	+	+	+
37 °C	+	+	+
30 °C	+	+	+
25 °C	+	+	+
4 °C	-	-	+
Growth at pH			
4	-	-	-
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	-	-	-
NaCl growth (%)			
0	+	+	+
2	+	+	+
4	+	+	+
6	-	-	-
8	-	-	-
10	-	-	-
Biochemical characteristics			
NO_2	-	-	-
N_2	+	+	+
TRP	-	-	-
GLU	-	±	-
ADH	+	+	+
URE	+	+	+
ESC	+	+	+
GEL	+	+	+
PNPG	-	-	-
GLU	+	+	+
ARA	+	+	+
MNE	+	+	+
MAN	+	+	-
NAG	+	+	+
MAL	+	+	+
GNT	+	+	+
CAP	-	-	-
ADI	-	-	-
MLT	+	+	+
CIT	-	-	+
PAC	-	-	-
Oxydase	+	+	+
Cytochrome-oxydase	+	+	+

Catalase	+	+	+
Identification	*Bacillus subtilis*	*Bacillus tequilensis*	*Pseudomonas sp.*

+: Presence of the activity. -: Absence of activity. NO_2: Reduction of nitrates to nitrites. N_2: Reduction of nitrates to nitrogen. TRP: Formation of indole (TRyptoPhane). GLU: Fermentation (GLUcose). ADH: Arginine DiHydrolase. URE: UREase. ESC: Hydrolysis (p-glucosidase) (ESCulin). GEL: Hydrolysis (protease) (GELatin). PNPG: P-galactosidase (Para-NitroPhenyl-13.D-Galactopyranosidase). [GLU]: Assimilation (GLUcose). [ARA]: Assimilation (ARAbinose). [MNE]: Assimilation (ManNosE). [NAG]: Assimilation (N-Acetyl-Glucosamine). [MAL]: Assimilation (MALtose). [CAP]: Assimilation (CAPric acid). [ADI]: Assimilation (ADlpic acid). [MLT]: Assimilation (MaLaTe). [CIT]: Assimilation (trisodium CITrate). [PAC]: Assimilation (Phenylacetic acid).

3.4. Antifungal activity of bacterial antagonists and filtrates of their liquid cultures against F. proliferatum

During the course of this research, three bacterial antagonists with considerable antifungal activity against *F. proliferatum* were isolated. The results of the antifungal activities carried out by the three antagonists against the above-mentioned phytopathogenic fungus are presented in Table 4 and the photos in Figure 2.

The results obtained show a promising anti *F. proliferatum* activity using *Bacillus subtilis* (Isolat A4) with an inhibition percentage of 54.00 ± 1.00 %, *Bacillus tequilensis* (Isolat A11) with an inhibition percentage of 59. 00 ± 1.00 %, *Pseudomonas sp*. (Isolat A12) with a percentage inhibition of 67.33 ± 1.70 % in comparison with the antibiotic Fluconazole which showed a percentage inhibition of 59.17 ± 0.76 %.

The antifungal activity of the filtrate of the liquid culture of each bacterial antagonist isolated and identified and which showed antifungal activity against the harmful fungus assayed was also tested, and the results obtained are summarized in Table 5. The results obtained show that the filtrate from the liquid culture of *B. subtilis* (Isolat A4), *B. tequilensis* (Isolat A11) and *Pseudomonas sp*. (Isolat A12) exhibited significant antifungal activity against *F. proliferatum* with inhibition percentages of 54.00 ± 1.00 %, 59.00 ± 1.00 % and 67.33 ± 1.70 % respectively in comparison with Fluconazole which presents an inhibition percentage of 59.17 ± 0.76%.

Many researchers have been interested in the control of the phytopathogenic fungus tested in our research by substances of microbial origin. Bjelić et al. (2018) reported the isolation of three bacteria; *B. subtilis* B5, *B. subtilis* B13 and *B. subtilis* B32 that showed antifungal activity against *F. proliferatum* BL16 with an inhibition rate of 43.1 %, 27.3 % and 44.3 %, respectively. Ju et al. (2014) reported that *B. subtilis* strain Y-1 exhibited a high antifungal effect on the mycelial growth of *F. proliferatum* with an inhibition rate of 54.52 %. Geng et al. (2022) showed that *Bacillus sp*. had significant antifungal activity against *F. proliferatum* and *A. alternata* with an inhibition rate of 81.56 % and 75.31 %, respectively.

The use of *Pseudomonas* species in biological control is well documented in the literature, some studies can be cited such as the study by Liu et al. (2021b) which reported significant antifungal activity of *Pseudomonas eucalypticola* sp. nov. against five phytopathogenic fungi: *Calonectria pseudoreteaudii*, *Fusarium graminearum*, *F. proliferatum*, *Magnaporthe oryzae*, and *Sclerotinia sclerotiorum*. It is clear from the scientific literature that our results are better than those obtained by the aforementioned studies, we have isolated bacterial antagonists that are more effective than those mentioned in the bibliography, and we have succeeded in controlling *F. proliferatum* OP820542.

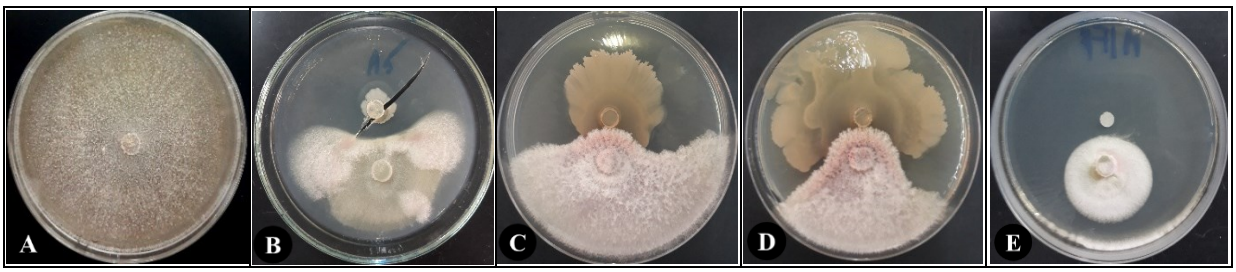

Figure 2. Antifungal activity of the three bacterial antagonists against *F. proliferatum* OP820542. **A:** *F. proliferatum* control, **B:** Confrontation between *B. subtilis* (Isolat A4) and *F. proliferatum*, **C:** Confrontation between *B. tequilensis* (Isolat A11) and *F.*

proliferatum, **D:** Confrontation between *Pseudomonas sp*. (Isolat A12) and *F. proliferatum*, **E:** Confrontation between Fluconazole and *F. proliferatum*.

Table 4. Antifungal activity of three bacterial antagonists isolated against *F. proliferatum* OP820542 compared with Fluconazole.

	Inhibition percentage (%)
***Bacillus subtilis* (Isolat A4)**	54.00 ± 1.00 [a]
***Bacillus tequilensis* (Isolat A11)**	59.00 ± 1.00 [b]
***Pseudomonas sp.* (Isolat A12)**	67.33 ± 1.70 [c]
Fluconazole	59.17 ± 0.76 [b]

Means (± standard deviation, n=3) followed by distinct letters within the same column denote a significant difference (ANOVA I, Tukey tests at $p < 0.05$).

Table 5. Antifungal activity of liquid culture filtrates of three bacterial antagonists isolated against *F. proliferatum* OP820542 compared with Fluconazole.

	Inhibition percentage (%)
***Bacillus subtilis* (Isolat A4)**	38.67 ± 1.53 [a]
***Bacillus tequilensis* (Isolat A11)**	40.33 ± 1.53 [a]
***Pseudomonas sp.* (Isolat A12)**	47.53 ± 2.34 [b]
Fluconazole	59.17 ± 0.76 [d]

Means (± standard deviation, n=3) followed by distinct letters within the same column denote a significant difference (ANOVA I, Tukey tests at $p < 0.05$).

4. Conclusion

The present study allowed the isolation, conventional and molecular identification of *F. proliferatum* OP820542, the responsible agent of garlic bulb rot, which was successfully isolated and identified from infected garlic bulbs in Morocco. The inhibitory effect of three bacterial strains on the mycelial growth of *F. proliferatum* OP820542 was quantitatively demonstrated. The results of the present study confirm the potential of biological control as a promising strategy for managing garlic bulb rot caused by *F. proliferatum* OP820542. The bacterial strains identified in the present study are promising biocontrol agents for more sustainable and environmentally-friendly agriculture. Further research is needed to optimize the conditions under which the bacterial strains are applied *in vivo* and to assess their long-term efficacy under growing conditions variety. Furthermore, elucidation of the mechanisms of action involved in biocontrol will make it possible to improve the efficacy and reliability of the above-mentioned microbial antagonists.

Finding

This research received no external funding

Conflicts of Interest

The authors declare no conflicts of interest.

Data availability statement

The data was not deposited in public repositories.

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