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First Report of Antifungal Activity of Microbulbifer Halophilus 201y Against Fusarium Verticilloides Czld Strain Isolated from Livestock Feed in Algeria

Souad Menazel[1]*, Souad Righi[1], Larbi Larous[1] and Mohamed Mihoub Zerroug[1,2]
[1.] Laboratory of Applied Microbiology, Department of Microbiology, University Ferhat Abbas Setif -1, Setif, Algeria.
[2.] Department of Microbiology and Biochemistry, Faculty of Natural and Life Sciences, University Batna 2, Batna, Algeria
***E-mail :** souad.menazel@univ-setif.dz / menazelsouad@yahoo.com

Abstract: *Fungal contamination of livestock feed causes economic losses and health risks. Biological control using beneficial microorganisms, especially antifungal bacteria, is a safe and sustainable alternative. This study aims to isolate and identify Fusarium verticilloides from livestock feed, and the in vitro investigation for the first time of antifungal activity of Microbulbifer halophilus 201Y. First, fungus and antagonistic bacterium were isolated from livestock feed. Phenotypic and genotypic identification were used to identify and confirm the fungal and bacterial isolates. Hydrolytic enzymes assays and the antifungal activity of 201Y were subsequently tested. The results indicated that 201Y was capable of reducing fungal growth in the dual culture test; the inhibition rate was estimated between 53% and 56%. Moreover, volatile compounds produced by this strain reduced fungal growth by 64%. Although the strain 201Y did not produce any tested hydrolytic enzymes. This study is the first to suggest that 201Y can be used as a biological control agent and highlights its potential for future biological control applications.*
Key words*: Antifungal activity, F. vertivillioides, Livestock feed, M. halophilus.*

INTRODUCTION

Livestock feed is frequently contaminated by a variety of factors, such as environmental pollution, insects and microorganisms, which include phytopathogenic fungi that are able to contaminate livestock feed before, after harvest and also during transportation or storage [1, 2] These fungi can affect the feed quality, and nutritional value, in addition to their capacity to produce mycotoxins which are considered as sources of serious health risks for both people and animals [3]. Several studies had reported high contamination of livestock feed with fungi and mycotoxins [4, 5, 6, 7]. In general, fungal and mycotoxin contamination affects a wide range of raw materials and final feed intended for livestock production [8]. Fusarium verticillioides is one of the most important filamentous pathogenic fungi with a wide range of grain cereal hosts [9], which are integrated into human food, livestock feed, raw materials for industrial production, and sources of biomass for energy production [10].To reduce the risk of phytopathogens, chemical control was widely applied, however, the interest of researchers has converted towards the alternative strategies using potential antagonistic microorganisms [11] due to their environmentally safety and low-cost [12]. Several researches reported the biocontrol of F. verticillioides by bacteria [13, 14].
Microbulbifer are typically isolated from high-salinity environments [15], and characterized by their capacity to degrade polysaccharides including chitin, cellulose [16], agar [17], alginate [18], and plastics [19]. It has been reported that Microbulbifer strain A4B-17 was able to prevent the growth of Gram-positive bacteria, yeasts, Aspergillus niger and Penicillium chrysogenum [20].
This study aims to isolate and identify F. verticillioides from livestock feed and to evaluate its biological control using M. halophilus, which has not been previously reported as a biocontrol agent.

MATERIALS AND METHODS

ISOLATION AND IDENTIFICATION OF F. VERTICILLOIDES

Fusarium was isolated from animal feed samples collected from Eastern Poultry Group (OEB) of Sétif. 10 g of each sample was added to Erlenmeyer containing 90ml of 0.1% peptone water. After shaking of mixture, serial dilutions to 10^{-3} were prepared for each sample. One ml of 10^{-3} dilution was inoculated in duplicates into Potato Dextrose Agar (PDA) supplemented with 0.05 mg/ml chloramphenicol. Plates were incubated for 5 to 14 days at room temperature [21]. Pure culture of the different colonies (based on morphology) was obtained by sub-culture of the isolates on potato dextrose agar plates.
Obtained fungal isolates were identified at the genus/species level upon the macroscopic and microscopic characteristics from pure cultures. Identification of Fusarium isolates was carried out using a standardized media and incubation conditions. The general characters of Fusarium species were the size and shape of macroconidia and microconidia, the presence or absence chlamydospores, manner of production of microconidia, and color of colony on PDA medium [22].
The most common strain of Fusarium species was molecularly identified.

DNA EXTRACTION, PCR AMPLIFICATION AND SEQUENCING OF F. VERTICILLIOIDES

DNA extraction of F. verticillioides was performed using a NucleoSpin Plant II commercial kit (Macherey-Nagel Germany). The F. verticiloides isolate was inoculated onto the PDA medium and incubated at 28°C for seven days. Genomic DNA was extracted after that using the a NucleoSpin Plant II commercial kit (Macherey-Nagel Germany) as described in the manufacturer's protocol, and then the extracted DNA was stored at -20°C until use.

Molecular characterization was carried out based on conserved ribosomal internal transcribed spacer (ITS) region, the universal primers pairs used was ITS1 (5′CTT GGT CAT TTA GAG GAA GTA A3′) Gardes & Bruns (1993); ITS4 (5′TCCTCCGCTTATTGATATGC 3′), and the Elongation factor 1-alpha was EF-728F (5′ CAT YGA GAA GTT CGA GAA GG 3′); EF-2 (5′GGA RGT ACC AGT SAT CAT GTT 3′) Carbone and Kohn [23]. Amplification was performed in a 25 µl reaction volume containing 5 µl Taq Promega Buffer, 1.5 µl MgCl2, 0.2 µl dNTP, 1µl primer F, 1µl primer R, 0.2 µl Taq polymérase Promega, 2 µl genomic DNA. The amplification cycle consists of an initial denaturation at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 s, 55-52°C for 30 s, and 72 °C for 45s and a final extension at 72 °C for 7 min. The amplification products were revealed after electrophoresis on 1.5% agarose gel of a deposit of 10µl of PCR products. Migration is followed by staining in an ethidium bromide bath (0.5µg/ml). Afterwards, the DNA was visualized and photographed under UV using the Gel doc system from biorad (USA). The PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit from Macherey-Nagel (Germany) following the protocol described by the supplier.

DNA SEQUENCING, ALIGNMENT AND PHYLOGENETIC ANALYSIS OF F. VERTICILLIOIDES CZLD

The isolated and purified PCR products were sequenced using the Sanger technique, the BigDye v3.1 kit from Applied Biosystems and the PCR primers used for the amplification of the fragments of interest. The sequences obtained were analyzed and cleaned using the CHROMAS PRO software. The final sequences are then compared with those of the GeneBank database using the NCBI BLAST Program (https://blast.ncbi.nlm.nih.gov/Blast.cgi Blast) for the identification of the isolate studied.

ISOLATION AND IDENTIFICATION OF M. HALOPHYLUS STRAIN 201Y

Samples of livestock feed were mixed, serial dilution of mixed samples were made in physiological water and diluted to 10^{-3}. 1mL of 10^{-3} was inoculated in nutrient agar medium. Plats were incubated at 37°C for 3-5 days [24]. The isolated bacteria were purified and tested for their ability to inhibit fungal growth [25], bacterial strains demonstrated inhibitory activity against the fungus were selected for further studies (morphological, physiological, and molecular characteristics, its antifungal activity were previously described.

The bacterial strain 201Y was characterized according to morphological and chemical analyses, Gram staining, colony and cell morphology, motility, and spore formation, catalase and oxidase tests. Genetic analysis was conducted to confirm the isolate).

SCREENING OF EXTRACELLULAR ENZYMES ACTIVITY OF M. HALOPHILUS 201Y

Amylase activity was assessed by culturing the bacterial strain on minimal agar medium supplemented with KH_2PO_4 (0.1%), yeast extract (0.2%), $MgSO_4$ (0.5%), and soluble starch (0.5%, w/v). The plates were incubated at 30°C for 48 h. After incubation, Lugol's iodine solution was applied to the plates, and starch hydrolysis was evidenced by the formation of a clear zone surrounding the bacterial colonies [27]. Cellulolytic activity was assessed by growing the bacterial strain on a minimal agar medium plates containing KH_2PO_4 (0.1%), yeast extract (0.2%), $MgSO_4$ (0.5%), and carboxymethylcellulose (CMC, 0.5%) as a soluble cellulose substrate. The plates were incubated at 30°C for 48 h. After treatment with an aqueous Lugol's iodine solution, cellulose hydrolysis was visualized by the appearance of clear halos around the colonies, indicating the ability of the bacterium to hydrolyze CMC. Chitinolytic activity was assessed by culturing the bacterial strain on chitin agar medium containing (per liter): chitin (4 g), $KHPO_4$ (0.7 g), $FeSO_4$ (0.01 g), KH_2PO_4 (0.3 g), $MgSO_4{\cdot}H_2O$ (0.5 g), $ZnSO_4{\cdot}7H_2O$ (0.01 g), $MnCl_2$ (0.001 g), NaCl (0.3 g), yeast extract (0.2 g), and agar (20 g). The plates were incubated at 30 °C for 5 days. Following incubation, the cultures were flooded with Lugol's iodine solution. The formation of clear zones around the bacterial colonies indicated chitin hydrolysis, confirming the chitinolytic activity of the bacterium [28]. Protease activity was tested by culturing bacterium on skim milk agar (SMA) plates (2.0% skim milk, 1.5% agar, and 1.0% glucose). Plates were incubated for 48 hours at 30°C. A positive proteolytic activity was shown by the observation of bacterial growth on plates [29].

MOLECULAR IDENTIFICATION OF M. HALOPHILUS 201Y

The extraction of bacterial genomic DNA was performed using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies Sdn Bhd, Selangor DE, Malaysia) according to the manufacturer's instructions. Extracted DNA was stored at 4°C until required for PCR. PCR amplification was achieved using the primer set of 16S rRNA gene (27F: 5' – AGA GTT TGA TCC TGG CTC AG – 3' and 1492R 5'– CCG TCA ATT CCT TTG AGT TT- 3'). Reaction mixture containing 1X PCR buffer (Solis Biodyne, Estonia), 1.5 mM Magnesium chloride (Solis Biodyne, Estonia), 0.2 mM of each dNTP (Solis Biodyne, Estonia), 2 U Taq DNA Polymerase (Solis Biodyne, Estonia). PCR reaction mixture contained 25 µl of master mix (1.25 U Taq DNA polymerase (Solis Biodyne, Estonia), 3 µl of DNA template, 5 µl of each primer and made up to 50 µl reaction volume with distilled H_20. The PCR runs were as follows: Initial denaturation at 94°C (2 min), denaturation at 94°C (1 min), annealing at 55°C (1 min), and extension at 72°C (1 min). The amplification was repeated in 30 cycles followed by a final extension at 72°C (7 minutes). PCR were carried out using a thermocycler (icycler Bio-Rad, USA). In this work, the DNA concentrations were checked using Nanodrop Spectrophotometer (NanoDropTM 2000, USA). Agarose gel electrophoresis: After the PCR reaction, PCR product was separated on a 1.5% agarose gel (Sigma-Aldrich, USA). One hundred base pair (100 bp) DNA ladder (Solis Biodyne, Estonia) was used as DNA molecular weight marker. Electrophoresis was done at 80 V for 1 h 30 min, and the gel was viewed under UV light after staining with Midori Green Advance (Nippon Genetics, Japan) and inspected with a UV transilluminator. The PCR products were electrophoresed and purified (Clean-Up kit,Vivantis) and sent to a sequencing agency (Apical scientific Sdn. Bhd.).

DNA SEQUENCE ANALYSIS AND PHYLOGENETIC TREE OF M. HALOPHILUS 201Y

Purified PCR products were sequenced in the forward and reverse direction in separate reactions and in duplicate. Each reaction contained 40 µg template DNA, 2 µl of the appropriate PCR primer, 10 µl water and 2 µl BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems). Each reaction was heated to 96°C for 1 min, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 s. The sequencing products were purified using an ethanol precipitation method to remove unincorporated reagents and ensure a neutral charge. Briefly, sequencing products were washed in 80 µl ethanol precipitation mix (3 µl NaAc, 62.5 µl 95% ethanol and 14.5 µl water) and the DNA was pelleted by centrifugation. The pellet was again washed in 200 µl 75% ethanol and centrifuged. The pelleted DNA was air-dried and rehydrated in 15 µl formamide and then loaded onto a 3130 Genetic Analyzer Capillary Array for detection (Applied Biosystems). Two forward and two reverse sequences for each sample were aligned using Bionumerics v3.5 (Applied Maths) to obtain a composite sequence. The quality of each sequence trace was manually evaluated through visual inception. Low-quality sequences were edited and removed. The resulting sequences were analyzed using BLASTn which available at NCBI website.

ANTIFUNGAL EFFICACY OF STRAIN 201Y AGAINST F. VERTICILLOIDES CZLD
DUAL CULTURE TEST

The antifungal efficacy test of strain 201Y against CZLD was conducted using two methods. In the first method, the strain was cultured in nutrient broth and incubated at 30°C for 24 hours. A bacterial streak was then made in the center of each Petri dish containing PDA, and then a disc of 5 mm of mycelium was taken from the edge of a 7-olde colony of F. verticilloides and placed at 2.5 cm from bacterial strip. Plates were incubated for 7days at 28°C. Inhibition percentage was calculated by the following formula:
I= (R1 –R2) / R1 * 100
Where: R1: the radial growth of fungal colony from center to the edge of the Petri dishes. R2: the radial growth of the colony from the center to the bacterial strip. The test was established with three repetitions [30].
The second method involved placing a 5-mm mycelial disk, taken from a 7-day-old culture, at the center of a Potato Dextrose Agar (PDA) plate, and the antagonistic isolate was streaked approximately 3 cm away from fungus disk. Plates with fungus only served as controls, and three repetitions were established for each treatment. All Petri dishes were incubated at 28°C in the dark for 7 days. Inhibition percentage was calculated by the following formula:
Inhibition percentage (%) I= (C -T) / C * 100. Where, C: the radial growth of pathogenic mycelia without bacteria, T: the radial growth of pathogenic mycelia with bacteria [31].

EVALUATION OF ACTIVITY OF VOLATILE COMPOUNDS PRODUCED BY STRAIN 201Y AGAINST F. VERTICILIOIDES CZLD

To evaluate the inhibitory effect of volatile compounds (VOCs), a double Petri dish assay was used. In brief, 100 µl of bacterial suspension of 10^8 (bacteria/ml) taken from a 24h nutrient agar culture was speared on PDA, the plate cover was then replaced by a PDA plate previously cultured in the center by a disk of (5mm) of Fusarium. Both plates were directly sealed with Parafilm, and incubated for 7days at

28°C. Plates of PDA with only Fusarium culture were saved as control [32]. Each experiment was conducted three times. The antifungal activity was calculated by the following formula:
Inhibition percentage (%) = ((A1 − A2)/ A1) *100. Where,
A1: the radial growth of pathogenic mycelia without 201Ystrain,
A2: the radial growth of pathogenic mycelia with 201Ystrain.

Statistical analysis was performed using Microsoft Excel, with inhibition rates expressed as mean ± standard deviation (SD) based on three replicates (n=3)

RESULTS

ISOLATION AND IDENTIFICATION OF THE F. VERTICILLIOIDES CZLD

Our study showed that all livestock feed samples was contaminated with fungi. The level of contamination ranged from 4 10^3 to 1. 8 10^4 CFU/g. This study was focused on the Fusarium fungus, especially on the most prevalent F. verticillioides isolates.

On PDA medium, F. verticilloides colonies were low to moderately deep, mycelium colored white, pale salmon, and powdery with microconidia. After a long period of incubation, the center of colony changes to deep violet, paler at the margins. Microconidia abundant, fusiforme, aseptate, produced from phyalide, the distinctive features of this fungus are the production of microconidia in fals haeds from polyphyalides or monophialides, positioned in long chains, and the absence of chlamydoconidia (Fig 1).

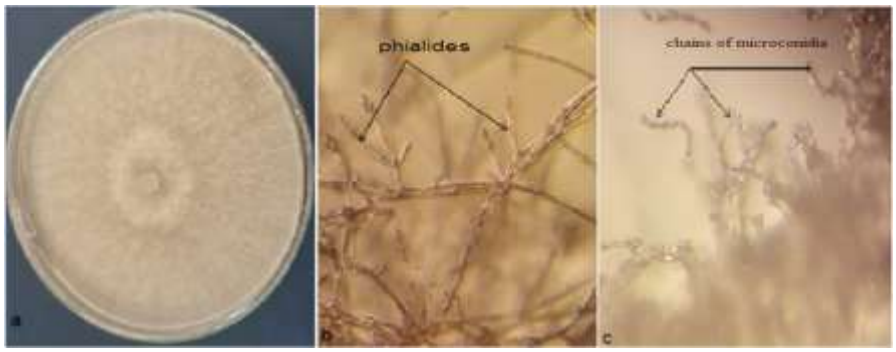

Fig.1. Macroscopic and microscopic characteristics of F. verticillioides CZLD (a: colony in PDA culture medium, b: phialides, c: chains of microconidia)

The final sequences of the strain were then compared with those in the GeneBank database using the NCBI BLAST Program (https://blast.ncbi.nlm.nih.gov/Blast.cgi Blast) for the identification of the isolate studied based on the % homology with the reference strains. The closest isolate was F. verticillioides strain CZLD, with a similarity rate of 99.61%. To construct phylogenetic tree, the Molecular Evolutionary Genetic Analysis (MEGA-11) software was used (Fig2).

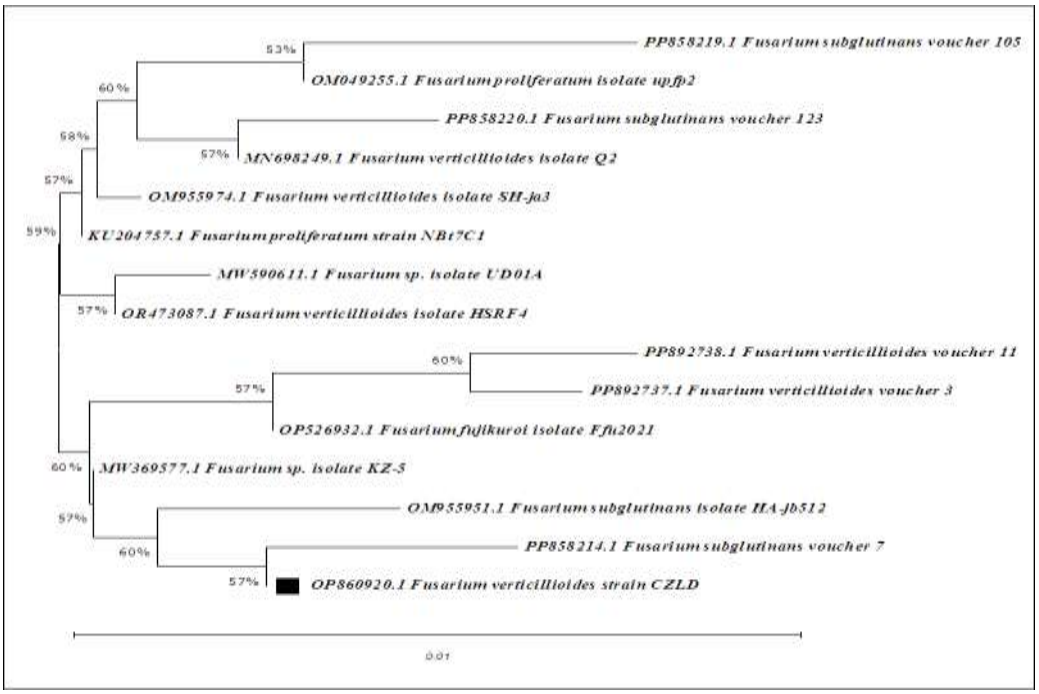

Fig.2. Phylogenic tree of F. verticillioides CZLD constructed with MEGA 11using neighbor joining method, Phylogeny Booststrap method, with node IDs. Bar 0. 01.

IDENTIFICATION OF ANTAGONISTIC STAIN 201Y AND PHYLOGENETIC TREE

The study was focused on bacteria that exhibited a preliminary inhibitory effect on fungal growth. According to the morphological characteristics, antagonistic strains 201Y was rods, single, Gram negative, non-sporulation, colonies were circular, opaque, white. The strain 201Y was negative oxidase and positive catalase. Genetic analysis was conducted to confirm the isolate.

After comparing consensus sequences with those of the Gene Bank database using the NCBI BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi Blast), our isolate was identified as Microbilbufer halophilus strain 201Y witch a similarity rate of (99.79%). The molecular Evolutionary Genetic Analysis (MEGA-11) software was used to make phylogenetic tree (Fig.3).

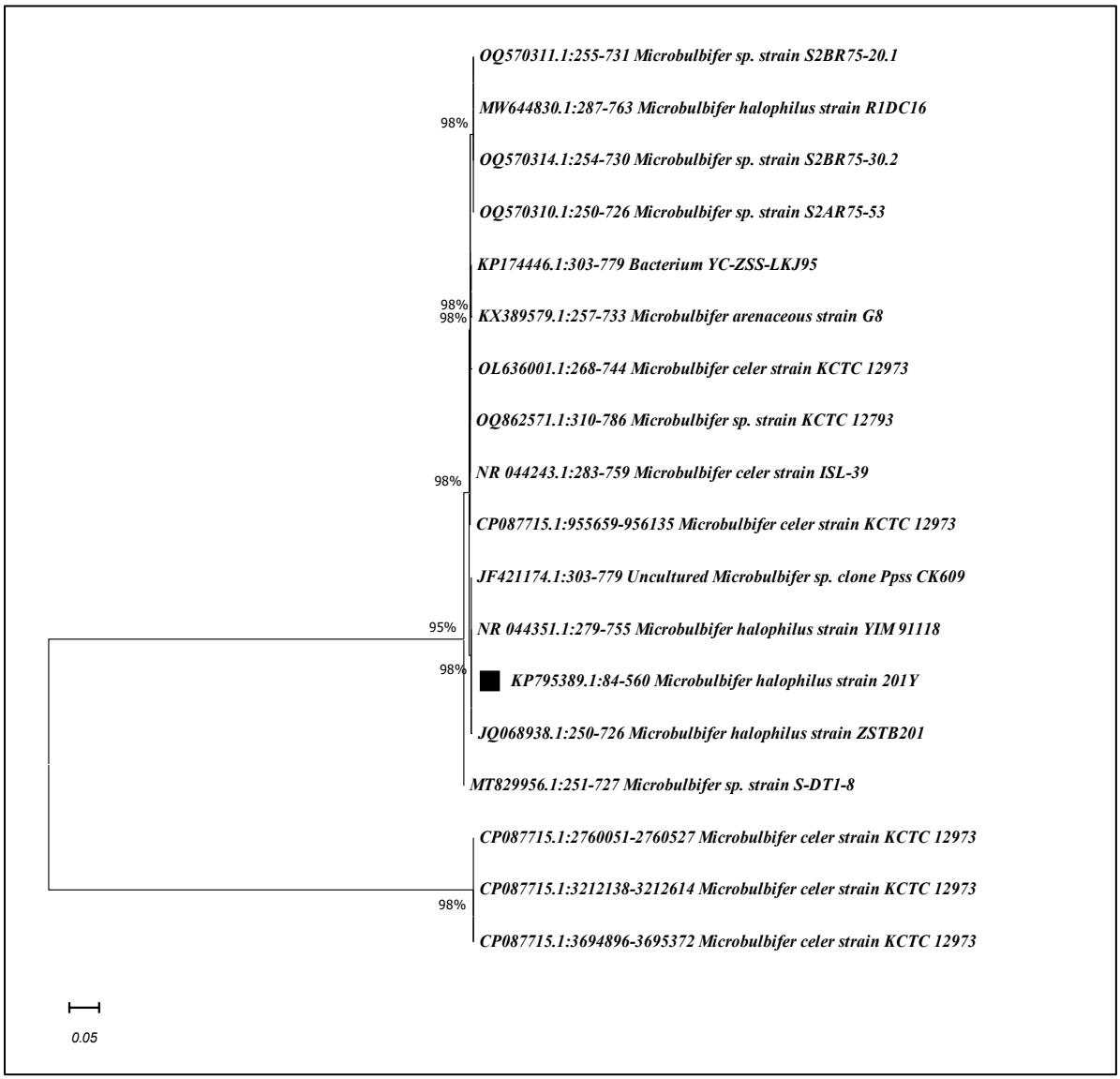

Fig 3: Phylogenetic tree of M. Halophilus strain 201Y based on 16S rRNA gene sequences showing the relationship of the strain with species that are genetically similar. Tree was constructed by the neighbor-joining method. Bootstrap values (%) based on 1000 replications are given at nodes. Bar. 0.05.

The enzymatic assays revealed that M. halophilus showed negative results for hydrolytic enzyme production, as no detectable activity of amylase, cellulase, protease, or chitinase was observed under the tested conditions.

ANTAGONISTIC ACTIVITY OF M. HALOPHILUS STRAIN 201Y

Antagonistic tests were performed to assess the ability of the strain 201Y to inhibit the growth of F. verticillioides CZLD. According to the first method [30], the strain 201Y showed an inhibitory effect the

growth of F. verticillioides CZLD, the inhibition rate was estimated at 53 %. However, when using the second method [31], the inhibition rate was estimated at 56 % (Fig4).

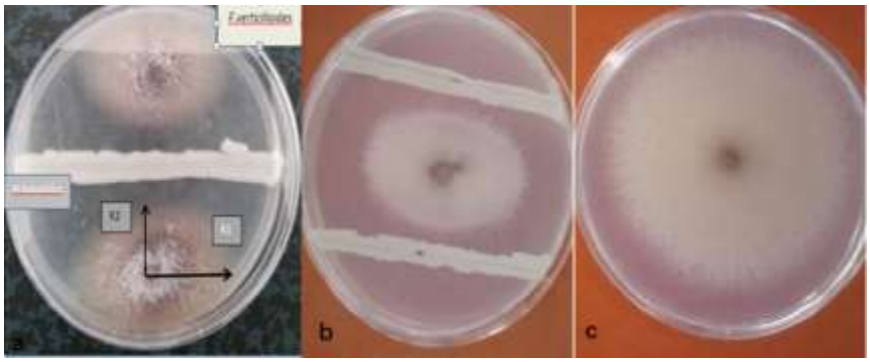

Fig 4: Antagonistic activity of M. halophilus 201Y against F. verticillioides CZLD in dual culture test. a: according to the first method [30]; b: according to the second method; c: control.

ANTIFUNGAL ACTIVITY OF VOCs PRODUCED BY STRAIN 201Y AGAINST F. VERTICILIOIDES CZLD

Regarding the test of volatile compounds on fungal growth, the strain 201Y demonstrated its ability to reducing growth of pathogen with percentage inhibitory of 64 % (Fig5).

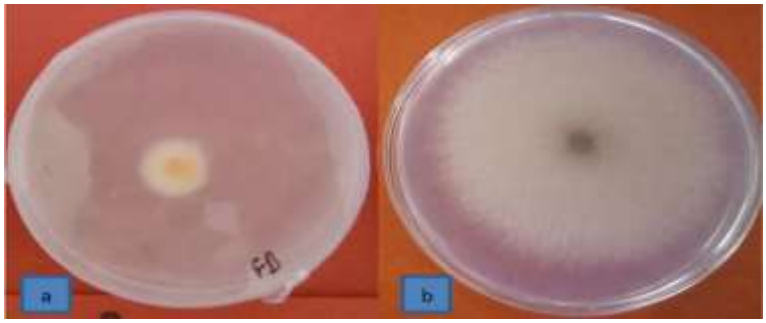

Fig. 5. Effects of the antifungal volatiles produced by the strains201Y on mycelial growth of F. verticilioides CZLD (a; treated plate, b; control)

DISCUSSION

All feed samples were contaminated with fungi which is line previous results reported by several researchers in other regions, such as Iran [1], Nigeria [21], Italy [33], and Algeria [34]. Contamination values was ranged from 4 10^3 to 1.8 10^4 CFU/g. In comparison to published results our samples showed nearly the same fungal contamination with other results obtained from Algeria [34] and Iran [1]. Fusarium species were the predominant fungi linked to cereals worldwide, F. verticillioides being the most commonly isolated species from cereals [35]. Bacterial and fungal agents are among the known contaminants of poultry feeds [36]. The contamination of feed with these microorganisms may originate from raw feed ingredients, stored feed products, during feed processing feed processing and handling in addition to other environmental sources.

Biological control strategy can be used to reduce the contamination by fungi [37]. Several bacterial species were proved their capacity to reduce fungal growth in vitro such as Bacillus subtilis [38], B. megaterium and B. cereus [39].

This study is the first that indicates the presence and the use of the M. halophilus as biological control in Algeria. Some species were isolated and identified like Microbulbifer sp. ALW1 [40], Microbulbife strain C4-6, isolated [41] from Nagasaki, Japan. The bacterial strain was isolated from livestock feed in our study, likely originating from the concentrated mineral supplements used in feed production. These minerals are typically sourced from diverse natural reservoirs including terrestrial deposits (soil and rocks) and marine environments [42]. It could also be due to contamination from air, soil, or water used during the manufacturing process [36]. Moreover, this bacterium has the ability to grow in low-salinity culture media, such as PDA, Nutrient Agar. The strain 201Y is Gram-negative, non-spore-forming, and motile. Colonies are white, round, and opaque. These cells test negative for oxidase and positive for catalase. M. halophilus is a Gram-negative bacterium, some are yellowish-brown like YIM 91118T strain isolated from north-west China, this strain was oxidase and catalase positive [43].

In dual culture test strain 201Y showed an inhibitory effect on growth of F. verticillioides CZLD with a percentage of inhibition ranged between 53 to 56 %, and also by the production of volatile compounds. Some species of the genus Microbulbifer have been reported to produce hydrolytic enzymes, including chitinase [40], and cellulase [44]. However, in the present study, M. halophilus did not exhibit detectable amylase, cellulase, protease, or chitinase activity under the tested conditions. Despite the absence of these enzymatic activities, the bacterium demonstrated a clear inhibitory effect against fungal growth. This observation suggests that the antifungal activity of M. halophilus is likely not mediated by hydrolytic enzymes, but rather attributed to the production of other bioactive molecules, such as secondary metabolites or antifungal compounds, which may play a key role in fungal growth suppression. In a previous study, the strain Microbulbifer sp. WMMC-695 exhibited antimicrobial activity against E. coli [45]. It has been reported that the production of secondary metabolite 4HBA appears to be a common characteristic of the genus Microbulbifer. In the same time, the alkyl esters of 4HBA produced by strain A4B-17 showed an inhibitory effect on growth of Aspergillus niger and Penicillium chrysogenum [20]. A previous study reported that compounds produced by Microbulbifer sp, namely bulbiferates A and B, showed antibacterial activity against E. coli and methicillin-sensitive Staphylococcus aureus [45]. Similarly, other compounds, bulbimidazoles A–C, isolated from Microbulbifer sp., demonstrated broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungi [46]. The results of this study indicate that the investigated bacterial isolate, which has not previously been used as a biocontrol agent, was able to inhibit fungal growth through the production of volatile compounds, with an inhibition rate reaching 64 %. These findings provide preliminary evidence of the specific effectiveness of this isolate against this fungus and highlight its potential for future biological control applications.

CONCLUSION

In this study, F. verticillioides and an antagonistic bacterial isolate 201Y were isolated from livestock feed and genetically identified. The ability of the bacterial isolate to inhibit fungal growth was evaluated, and the results demonstrated for the first time, that this isolate can suppress F. verticillioides growth both through the production of inhibitory metabolites and via volatile organic compounds, whose role had not previously been reported for this isolate. These findings highlight the potential of this bacterium as a promising source of biological control agents, particularly in light of the growing need for safe and sustainable alternatives to chemical treatments in feed protection and animal health. Moreover, this work contributes to expanding current knowledge on the use of bacteria and their secondary metabolites to reduce feed-contaminating fungi.

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interest.

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