

In vitro Evaluation of Some Biological Agents in Controlling *Neoscytalidium dimidiatum*, the Causal Agent of Apple Wilt and Stem Blackening

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Abstract

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This study aimed to isolate and diagnose the fungal pathogen causing apple wilt and stem blackening disease. The results obtained identified the pathogenic fungus *Neoscytalidium dimidiatum*, with a similarity of 99% with the previously diagnosed Chinese isolate, as the causal agent of apple and stem blackening disease, and for its ability to produce several enzymes that permit the fungus to attack its host, including pectinase, protease, and amylase. Results obtained also showed that the antagonistic ability of *Bacillus subtilis* bacteria, at a concentration of 22.8×10^8 CFU/ml reached 100% inhibition. In addition, the fungus *Trichoderma harzianum* gave a high antagonistic activity against the pathogenic fungal isolate on PDA medium, reached 98.61% inhibition, seven days after treatment.

Keywords: Apple, wilt, *B. subtilis*, *N. dimidiatum*, *T. Harzianum*.

Introduction

Apples, *Malus domestica* are fruit-bearing trees, historically known since ancient times in Mesopotamia, dating back to the late fifth millennium B.C. (Al-Naimi & Youssef, 1980). These fruits originated in the western foothills of the Himalayas, central Asia, and the Caucasus, where wild apple forests are found (Brite, 2021). Fruit trees are susceptible to various diseases, including ulcer-causing pathogens, stem rot, and dieback, resulting in the death of branches, bark, and internal tissues (Abdul-Karim & Aljarah, 2021; Intana *et al.*, 2023). Pathogenic fungi produce a spectrum of enzymes such as amylase, protease, lipase, cellulase, phenol oxidase, peroxidase, and tyrosinase, which play a crucial role in causing plant diseases by breaking down plant cell components and affecting cellular functions (Abbas, 2005; Mohd *et al.*, 2013).

Due to the adverse effects of chemical pesticides on the environment, human health, and non-target organisms (Lorenz, 2009), there has been a shift towards using beneficial microorganisms to suppress plant pathogens while enhancing crop productivity, known as plant growth promoting rhizobacteria (PGPR). These microorganisms include bacteria such as *Pseudomonas*, *Bacillus*, *Azotobacter chroococcum*, and the fungus *Trichoderma harzianum* (Abdulmoohsin *et al.*, 2019; Lahuf *et al.*, 2019a; Matloob, 2012).

This study aimed to isolate and diagnose the fungus causing apple wilt and stem blackening disease, determine its enzymatic activity spectrum and assess the antagonistic potential of two bio-control organisms, the fungus *Trichoderma harzianum* and the bacterium *Bacillus subtilis* against the pathogenic fungus under laboratory conditions.

Materials and Methods

Isolation and diagnosis

The fungus was isolated from an apple farm in Mahaweel district, Babylon province, Iraq, from several samples from apple tree stems that showed symptoms of wilting, necrosis, cracking, and discoloration of brown wood using a procedure reported earlier by Jaber & Lahuf (2020). The isolated fungus was identified at the genus level based on morphological characteristics (Barnett & Hunter, 1972).

Molecular diagnosis of *Neoscytalidium* spp.

DNA extraction of the fungus was performed using the ZR-fungal/bacterial DNA MiniPrep™ kit from Zymo Research following the manufacturer's instructions. Agarose gel electrophoresis was performed using 0.8% (w/v) agarose gel to confirm the quality of the extracted DNA. The gel was prepared according to Sambrook *et al.* (1989). and gel bands were visualized using a UV transilluminator at a wavelength of 360 nanometers.

A specialized primer set targeting the internal transcribed spacer (ITS) region (Table 1), comprising of the forward primer ITS1 and reverse primer ITS4, was used for PCR amplification. The gene was amplified using a PCR reaction kit from INTRON following the manufacturer's protocol.

Table 1. Sequence of the specialized primers used for the detection of the ITS gene region in the *Neoscytalidium dimidiatum* fungal pathogen.

Primer	Sequence 5'-3'	T _m (°C)	Product size (bp)
Forward	TCCGTAGGTGAACCTGCGG	60.3	500-650
Reverse	TCCTCCGCTTATTGATATGC	57.8	

Sequencing

The PCR product was sent to the Korean company Macrogen to identify the fungus's specific DNA sequence and identify it to the genus and species level. The sequence obtained was analyzed using bioinformatics related tools and submitted to BLAST analysis at the global online platform of the National Center for Biotechnology Information (NCBI). The phylogenetic tree was built using molecular evolutionary genetics analysis (MEGA X) software applying the neighbour-joining method (Lahuf *et al.*, 2019b).

Pathogenicity testing of the fungal isolate *Neoscytalidium dimidiatum* using radish seeds

The pathogenicity of the fungal isolate *N. dimidiatum* was assessed using radish seeds following the method of Bolkan & Butler (1974), which also calculated the seed germination rate.

Enzymatic activity spectrum of the pathogenic fungus *N. dimidiatum*

This test is closely related to the development of symptoms caused by this phytopathogenic agent such as wilting of apple leaves and the blackening of stems. The following enzymatic activities were determined as described below:

Pectinase enzyme activity- The ability of the pathogenic fungus *N. dimidiatum* to produce pectinase enzyme was tested using Zapek-Dox agar medium. Orange peels were ground and added to the medium using the method of Rangarajan *et al.* (2010). The medium was sterilized, poured into Petri dishes, and then inoculated with *N. dimidiatum*. The dishes were then incubated at 30°C for five days. Logule's iodine solution was used to detect pectinase enzyme production (Al-Shamary & Kadem, 2012).

Protease enzyme production test- Abbas (2005) method was used to prepare a gelatin-containing medium to detect protein-degrading enzyme production. Nutrient Agar supplemented with 0.4% gelatin was prepared. The gelatin solution was sterilized separately and added to the nutrient agar. The medium was inoculated with *N. dimidiatum* and then incubated at 30°C for seven days. Frazier's reagent was used to detect protease enzyme activity.

Amylase enzyme activity- Amylase enzyme production by *N. dimidiatum* was detected using a medium containing soluble starch, yeast extract, agar, and water. The inoculated plates were incubated at 30°C for seven days, followed by adding potassium iodide solution to detect amylase enzyme production (Abbas, 2005).

Antagonistic activity of *B. subtilis* bacteria and *T. harzianum* biofungus and the effect of chemical pesticide beltanol against the pathogenic fungus *N. dimidiatum*

The antagonistic ability of *B. subtilis* against *N. dimidiatum* was tested by adding 1 ml of the previously grown bacterial suspension to Petri dishes containing PDA medium, and distributed uniformly by gentle swirling. 0.5 cm discs of 7-day-old *N. dimidiatum* culture were then added to four

replicates, and four plates were left without the addition of bacteria (*N. dimidiatum*) as control. The inhibition rate was calculated using the formula by Montealegre *et al.* (2003) as follows:

$$\text{Inhibition (\%)} = \frac{\text{The average diameter of control colony} - \text{the average diameter of the treated colony}}{\text{The average diameter of control colony}} \times 100$$

The dual culture method was also used to determine the antagonistic activity of *T. harzianum* against *N. dimidiatum*. Four replicate plates were incubated at 25±2°C, and the inhibition rate was calculated using the same formula mentioned above.

The efficacy of the fungicide Beltanol was tested using the poisoned food technique. PDA medium was sterilized in a glass flask, and the fungicide was then added at 1 ml/L medium before solidifying. The mixture was stirred in a regular circular motion to ensure even fungicide distribution. The medium was then poured into Petri dishes, inoculated with a 0.5 cm diameter disk taken from the edge of a 5-day-old colony of the pathogenic fungus.

The experiment was carried out with four replicates, and four dishes were left as a control treatment (pathogenic fungus alone). The plates were incubated at 25±2°C. After the pathogenic fungus in the control treatment had grown to the dish's edge, the inhibition rate was calculated based on the formula developed by Montealegre *et al.* (2003).

Results and Discussion

Isolation and phenotypic diagnosis of the pathogenic fungus *N. dimidiatum*

The pathogenic *N. dimidiatum* fungal isolate was characterized by woolly growth, white to grey colour at the beginning, then it completely turned into a darker black colour (Figure 1-A) (Dy *et al.*, 2022). Al-Dabagh & Al-Murad (2014) reported this pathogen on Cypress trees in Iraq, where spores are characterized by three cells, darker in color in the middle than in the peripheral cells, and with pointed ends. When the infection progresses and the disease develops, ulcers appear on the infected plants, and the affected areas begin to crack longitudinally, and black powder appeared on the affected parts.

Molecular diagnosis of pathogenic fungus

The migration of PCR products on agarose gel showed that the amplified band size was 500-650 bp, when forward and reverse primers were used (Figure 2). The alignment was matched with the sequences of the global isolates of the fungal pathogen *N. dimidiatum* registered in the database of the National Center for Biotechnology Information (NCBI). Results obtained showed nucleotide sequence homology of 99% as compared to a Chinese isolate (accession number KF612310.1). This result was identical to a previous study used these primers for DNA amplification (Al-Tamimi, 2019).

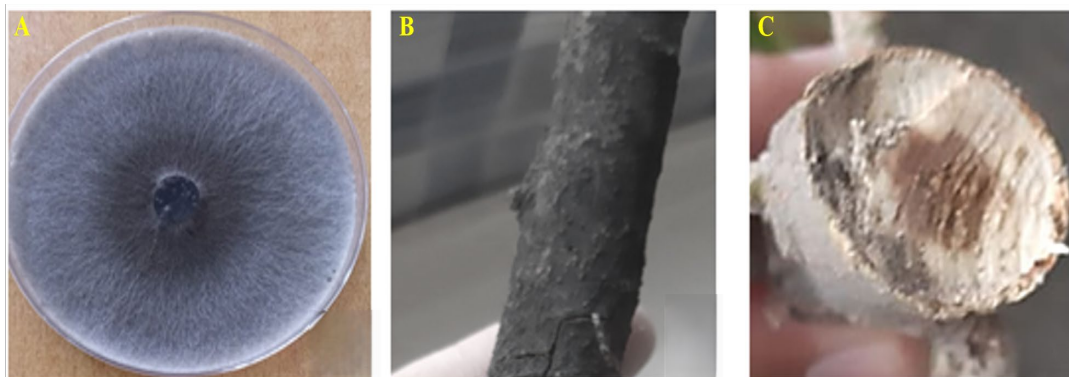


Figure 1. The symptoms of wilt disease and pathogenic fungus colony growth. (A) The fungus *N. dimidiatum*, (B) Infection with the pathogenic fungus on apple stem, (C) Brown discoloration of the vascular bundle.

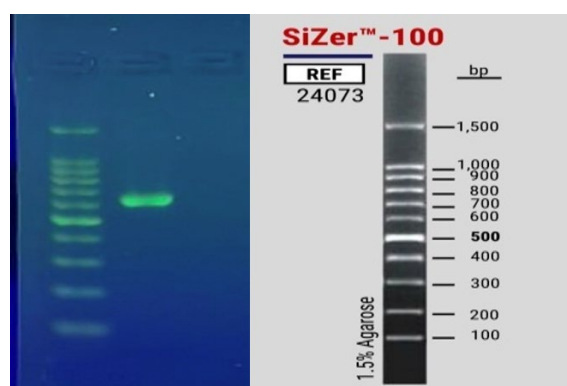


Figure 2. PCR amplified band of *N. dimidiatum* genomic DNA separated on agarose gel.

The developed neighbor-joining tree (Figure 3) showed that the *N. dimidiatum* isolate appeared in a separate clade when compared to other isolates of the same fungus. The results obtained also showed genetic convergence and divergence between the sequences of the Iraqi isolate and the global isolates registered at the NCBI. This analysis included seven nucleotide sequences from 261 sites registered in the final data set. The Iraqi isolate was genetically closer than the isolates from China, Egypt, Thailand, and the USA and more distant from the isolates of Taiwan and Türkiye. Thus, the Iraqi *N. dimidiatum* isolate was genetically different from the other worldwide isolates available in the NCBI data base, and was registered under the accession number OP846026.

Pathogenicity test of *Neoscytalidium dimidiatum* isolates using radish seeds

Results obtained showed that the pathogenic *N. dimidiatum* fungal isolate caused a significant decrease in germination rate reached 0.0% compared to the control treatment, in which the germination rate was 100%. The reduction in germination rate of radish seeds may be due to the fungus secretion of some toxins and enzymes by the pathogen, which affected the normal function of the host cells (Mandava *et al.*, 1980).

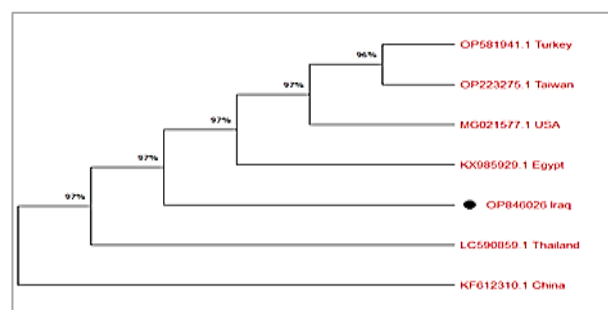


Figure 3. Neighbor-Joining tree shows the genetic relationship of the fungus isolates isolated in this study when compared to the global isolates.

Ability of the pathogenic fungus *Neoscytalidium dimidiatum* to produce pectinase, protease and amylase enzymes

Results obtained showed the ability of the *N. dimidiatum* fungus to secrete many pathogenic enzymes that the fungus uses to attack the plant host, such as the production of pectin enzyme (Figure 4-A and 4-B), which decomposed the pectin present in the culture medium, following the use of Logule's reagent. Pectinase enzymes break the glycosidic bond of the long carbon chain, one of the basic components of plant cell walls (Davis & Blevins, 1979). Pectinase enzymes include a group of enzymes such as pectinase, pectinase, pectin esterase, and polygalacturonase (Reda *et al.*, 2008). The ability of the pathogenic fungus *N. dimidiatum* to produce the protease enzyme is shown in Figure 4-C. This was demonstrated by the formation of a transparent halo around the colony after adding the reagent and leaving it for few minutes, then pouring it out, and the diameter of the halo obtained reflected the extent of its effectiveness. Thus, the degradation caused by proteolytic enzymes significantly impacts host cells (Sandhya & Kurup, 2013). In addition, results showed the secretion of the amylase enzyme by the pathogenic fungus *N. dimidiatum*, by observing the formation of a yellow halo around the colony of the fungus and the coloring of the rest of the medium in blue (Figure 4-D), as most plant pathogens are characterized by their ability to hydrolyze starch with the amylase enzyme and use as a source of carbon for its metabolic activities (Abbas, 2005; Agrios, 2005).

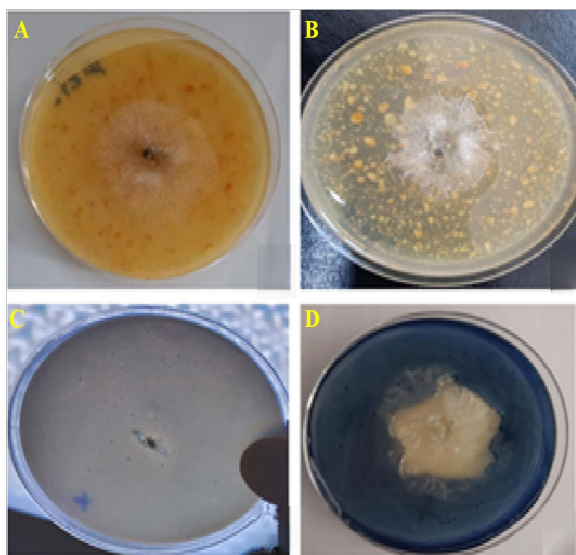


Figure 4. *In vitro* investigation of enzymatic activities in *Neoscytalidium dimidiatum*. (A) Pectinase medium before adding reagent, (B) Detection of pectinase, (C) Detection of protease, (D) Detection of amylase.

In vitro* antagonistic potential of the microbial agents studied and Beltanol on the mycelial growth of *Neoscytalidium dimidiatum

Results obtained (Table 2) showed the ability of *B. subtilis* bacteria to inhibit the pathogenic fungus *N. dimidiatum* isolate at a concentration of 22.8×10^8 CFU/ml, and the inhibition rate reached 100%, as compared to 0.0% for the control treatment (Figure 5-A). This could be due to the ability of *B. subtilis* to secrete antifungal compounds, including the production of antibiotics such as subtiline, bacitracin, bacillin and bacillomycin that act on the decomposition of fungal hyphae cytoplasm as well as bacterial production of volatile antifungal compounds (Montealegre *et al.*, 2003). Results obtained also showed the high antagonistic ability of the fungus *T. harzianum* against the pathogenic isolate giving an inhibition rate of 98.61%, compared to 0.00 % for the control treatment (Figure 5-B). These results are in agreement with what was reported earlier by Rusmarini *et al.* (2017) and confirmed that the fungus *T. harzianum* can inhibit the growth of the pathogenic fungus *N. dimidiatum* isolated from fig fruits in Malaysia in the laboratory. The antagonistic ability of the bio-control fungus *T. harzianum* could be due to various mechanisms by which it affects the pathogenic fungus (Harman, 2000). Results

obtained also showed that the use of the chemical pesticide Beltanol at a concentration of 1 ml/litre led to 100% inhibition of the growth of the pathogenic fungal isolate *N. Dimidiatum*, compared to 0.00 % for the control treatment (Figure 5-C). Such result was consistent with many previous studies that demonstrated the effectiveness of the chemical pesticide Beltanol against this fungus (AL-Tememe *et al.*, 2019).

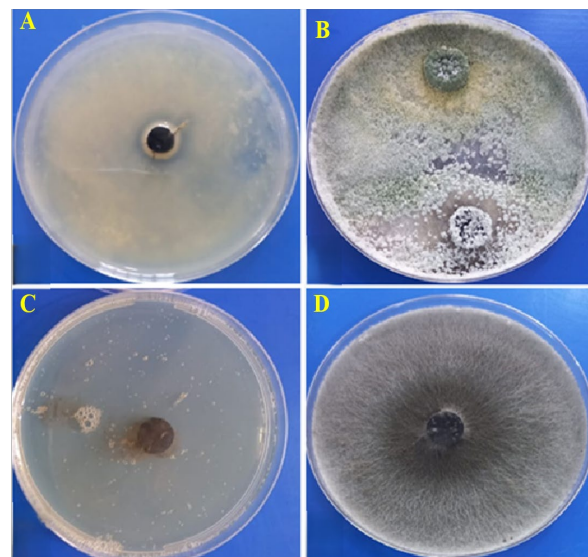


Figure 5. The antagonistic potential of microbial agents and the fungicidal power of Beltanol tested against *Neoscytalidium dimidiatum*. (A) the ability of *B. subtilis* bacteria to inhibit the pathogenic fungus, (B) *T. harzianum* against the pathogenic isolate, (C) chemical pesticide Beltanol inhibition of the growth of the pathogenic fungal isolate, (D) control *N. dimidiatum* alone.

It can be concluded from this study that the fungus *N. dimidiatum* is a pathogenic fungus that infects apple trees in Iraq and causes charring and blackening of apple stems. It can secrete many enzymes that work to decompose plant cells and contribute to disease development, including pectinase, protease, and amylase enzymes. The fungus *T. harzianum* and the bacteria *B. subtilis* can be considered as potential bio-control agents for this pathogen in the laboratory. However, field testing is essential to confirm their use in farmers' fields.

Table 2. Testing the antagonistic potential of the bio-control factors' resistance elements and the effect of Beltanol fungicide against the pathogenic fungus *Neoscytalidium dimidiatum*.

Type of treatment	Diagonal growth rate of the pathogenic fungus (cm)	Pathogenic fungus inhibition rate (%)
<i>N. dimidiatum</i> + <i>B. subtilis</i>	0.000	100.000
<i>N. dimidiatum</i> + <i>T. harzianum</i>	0.125	98.610
<i>N. dimidiatum</i> + Beltanol	0.000	100.000
<i>N. dimidiatum</i> alone (control)	9.000	0.000
LSD _{0.05}	0.193	2.142

Each value in the table represents an average of four replicates.

المخلص

الكرعاوي، كاظم زغير خضير. 2026. التقييم المختبري لبعض العوامل الحيوية في السيطرة على الفطر *Neoscytalidium dimidiatum* المسبب لمرض ذبول واسوداد ساق التفاح. مجلة وقاية النبات العربية، 44(3):390-395. <https://doi.org/10.22268/AJPP-001411>

هدفت هذه الدراسة إلى عزل وتشخيص الفطر الممرض المسبب لمرض ذبول واسوداد ساق التفاح، وتحديد نشاطه الأنزيمي. حددت النتائج التي تم الحصول عليها بأن الفطر الممرض هو *Neoscytalidium dimidiatum*، والذي يتشابه بنسبة 99% مع العزلة الصينية التي تم تشخيصها سابقاً، كمسبب لمرض ذبول واسوداد ساق التفاح، وقدرته على إنتاج العديد من الإنزيمات التي تسمح للفطر بمهاجمة مضيفه، مثل البروتياز والبكتيناز والأميلاز. كما أظهرت النتائج المتحصل عليها أن القدرة التضادية للبكتيريا *Bacillus subtilis* بتركيز 10×22.8 وحدة مكونة للمستعمرة/مل وصلت إلى نسبة تثبيط 100%، بالإضافة إلى ذلك أعطى الفطر *Trichoderma harzianum* نشاطاً تضادياً عالياً ضد العزلة الفطرية المسببة للمرض على وسط PDA، حيث حقق نسبة تثبيط 98.61% بعد سبعة أيام من المعاملة.

كلمات مفتاحية: تفاح، ذبول، *T. Harzianum*، *N. Dimidiatum*، *B. subtilis*.

عناوين الباحثين: كاظم زغير خضير الكرعاوي، قسم تقنيات المقاومة الاحيائية، الكلية التقنية المسيب، جامعة الفرات الأوسط التقنية، العراق. البريد الإلكتروني للباحث kadhim.zghir@atu.edu.iq المراسل

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