Efficacy of ecofriendly biocontrol *Azotobacter chroococcum* and *Lactobacillus rhamnosus* for enhancing plant growth and reducing infection by *Neoscytalidium* spp. in fig (*Ficus carica* L.) saplings

Sabah Lateef Alwan **Hawraa N. Hussein**
Professor **Assistant lecturer**
Plant Protection Department, Agriculture College, University of Kufa
Email: hawraan.alkaaby@uokufa.edu.iq

Abstract:

The aim of the research was to use the environment-friendly agents to reduce the effect of *Neoscytalidium dimidiatum* and *Neoscytalidium novaehollandiae*, that cause dieback and blacking stem on several agricultural crops. This disease was the first record on fig trees in Iraq by this study and registered in GenBank under accession numbers : MF682357 , MF682358, in addition to its involvement in causing dermatomycosis to human. In order to reduce environmental pollution due to chemical pesticides, two antagonistic bacteria *Lactobacillus rhamnosus* (isolated from yoghurt) and *Azotobacter chroococcum* (isolated from soil) were used to against pathogenic fungi *N. dimidiatum* and *N. novahollandiae*. The *in vitro* tests showed that both bioagents bacteria were highly antagonistic to both pathogenic fungal reducing their radial growth to 44 and 75% respectively . Results of greenhouse experiments in pot showed that both *A. chroococcum* and *L.rhamnosus* decreased severity of infection by pathogenic fungi and enhanced plant health and growth. All the growth parameters of fig trees including leaf area, content of total chlorophyll, catalase and peroxidase activities were significantly higher compared to infected untreated control.

Keywords: Neoscytalidium, Azotobacter, Lactobacillus , dieback, fig trees .

Introduction:

Most of the endophytic fungi of the class Dothideomycetes represent major opportunistic plant pathogens that cause canker, dieback and black stem in various types of trees (7,10, and 29) To date, only a limited number of Neoscytalidium (*Hendersonula* previously) species has been described based on several morphological and molecular characteristics. Studies on these few species have reported that Neoscytalidium dimidiatum is closely related to both Neoscytalidium novaehollandiae (25) and Scytalidium hyalinum (18) . The severity disease of these fungi is associated with the temperature and soil moisture (22). This fungus was reported to be pathogenic and virulent to many plant trees due to its enzymatic system (e.g. cellulases, pectinase, proteases and amylase) with high ability to degrade the plant cell wall (primary and secondary cell walls). Infection can disrupt plant transportation activities and nutrients uptake (3,and12). *N.dimidiatum* registered as dermatomycosis and Tinea and there is no oral therapy or topical treatment for this fungal infection (5) lungs were also reported to be infected by this fungus (9) Infection is caused by contact with soil or plant material contaminated with this fungus. This disease is contagious and it is

often transmitted to the people with immunodeficiency are more vulnerable to infection (27and 8). Despite little information available on *N. dimidiatum*, it has been reported to be a pathogen of sinusitis in Jamaica, Brazil, Algeria, Canada, the United States, and the United Kingdom (6). In Iraq, this fungus was reported with high incidence of occurrence which was 45% in Saladdin. It was also found to cause trees canker that was extended to 18.53 mm and 16.11 mm on eucalyptus and poplar, respectively in Duhok (North of Iraq) In Basrah (south of Iraq) grapevines suffer from dieback by Lasiodiplodia theobromae and *N. dimidiatu*m (1,13and 4) Due to the importance of the disease on fig trees Ficus carica L. in the Middle Euphrates region of Iraq, and to reduce the environmental pollution due to chemical pesticides, the study aimed to reduce the effect of namely blackening stem of fig by using two eco-friendly agents *A.chroocuccum* and *L.rhamnosus*.

Materials and methods

Isolation and diagnosis of *Neoscytalidium spp*.

Surveys on trees orchards was performed during 2016 and 2017 for recording and collecting plant material infected with *Neoscytalidium*. A total of 100 naturally canker, dieback and black stem -infected fig (*Ficus carica* L.) trees aged between 4 and 5 years were scanned randomly from nine locations in the middle Euphrates region in Iraq. Infected plant materials were used for species initial isolates(stem and shoots). The fungal isolates were then identified to the genus level (Figer 1), and the characteristics of the fungal isolates were identified using the morphological and microbiological characterizations described by Crous et al. (24) Based on the pathogenicity test results, the most pathogenic and aggressive isolates were selected and further used in this study. Out of 100 identified isolates were used in this study. For the species level, *Neoscytalidium* was identified by tendency to be characterized either *N.dimidiatum* or *N.novahollandae*. Both species were very close and difficult for clearly discrimination. Therefore, the downstream molecular genomics characterization was followed to ovoid troubleshoot for these cumbersome similarities.

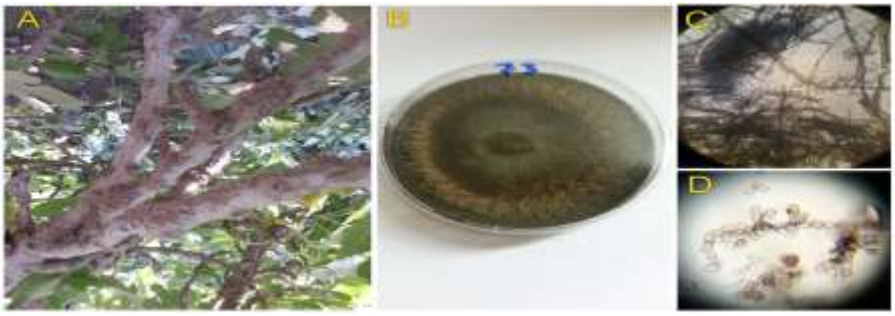

Figure 1: A- Fig tree's barks and branches showing symptoms of canker and dieback caused by *Neoscytalidium*. B- *Neoscytalidium* hyphal growth on PDA medium. C- Fungal mycelia and D-arthrospore of *Neoscytalidium* under the light microscope at magnification of 40 X

Effect of *Neoscytalidium* spp. on fig saplings

According to Koch's postulates, the 100 isolates of *Neoscytalidium* were tested for their pathogenicity on 300 fig saplings (3 replicates for each isolate) which already grown from cuttings for 5 months. A scale from 1-4 was the pathogenicity according to disease index described by Jimenez-Diaz and Trapero-Casas (16) that was measured based on percent growth of fungal extended within the stem, where 1= 0-25% , 2=26-50% , 3=51-75% and 4=76-100%. The pathogenicity test was designed as a randomized complete block design (RCBD) experiment with three replicates.

Cellulase Assay

Seven isolates of Neoscytalidium (N1, N2, N17, N24, N26, N27, N73) which caused the highest severity of infection were incubated at 40-°C and pH6 ,then the cellulase activity was estimated by both filter paper activity (FPA) and glucose production activity (GPA) according to Wood and Bhat (28). Centrifugation for 10 min at 6000 rpm were performed on 0.17g mycelium in 2ml distill water. The crude liquid was placed in 50mg filter paper with 4 ml acetate buffer 0.05M (pH 4.8) was placed in a test tube and culture filtrate was added. The reaction mixture was incubated at 50˚C for 1 hour and then was terminated by adding 3 ml glucose oxidase reagent. The tubes were heated at 100˚C in a boiling water bath for 15 min and then cooled at room temperature. The absorbance was measured at 500 nm using spectrophotometer (Optima-Japan).

Antagonistic test of *A. chroococcum*, *L.rhamnosus* to *Neoscytalidium*

Antagonistic of *A. chroococcum* isolated from soil ($5 \times {}^{8}10$ C.F.U.ml^{-1}) and *L.rhamnosus* isolated from yoghurt (6×10^{7} C.F.U.ml^{-1}) were tested against three isolates of *N.dimidiatum* (N17, N24, N26) and four isolates of *N.novahollandiae* (N1, N2, N27, N73) on PDA. According to Montealegre and et al. (20) the inhibition rate of fungal growth was estimated for each isolate with each bio control bacteria. This laboratory experiment was designed according to complete randomized design (CRD) with three replicates for each treatment **.**

Effect of the biological agents on infection severity, growth parameters, and enzymes activity of fig saplings

Each fig sapling aged one year was sprayed with 100 ml of pathogenic fungus based on each treatment . Whereas *A. chorococcum* and *L. rhamnosus* drenched to the soil with irrigation water at 100 ml.plant^{-1} (23and 21) one week prior to inoculation with the pathogenic fungus. While, plant treated with bacteria alone served as a control. A scale was used to assess infection severity depended on Jimenez-Diaz and Trapero-Casas (16).

Data collection

The seven pathogenic fungal isolates were compared among each other in terms of their cellulase activities. Effect of antagonistic bacteria *A. chorococcum* and *L. rhamnosus* on growth of the pathogenic fungi (radial growth and percent inhibition) was recorded and compared between the two bacteria among the pathogenic fungal isolates. Spraying with biocontrol bacteria was also compared among treatments for their effects on plant growth parameters (leaf area and total chlorophyll content) and

defense enzymes activities (peroxidase and catalase) of fig saplings at 6 months post inoculation with *N.dimidiatum* or *N.novahollandiae*. Severity caused by the pathogenic fungi was also recorded and compared across to each treatment.

Statistical analysis

Data were analyzed and analysis of variance ANOVA was performed using Gen-Stat package 2009 (12th edition), version 12.1.0.3278 (www.vsni.co.uk). Means were compared with the least-significant-difference (LSD) whenever appropriate at ($P \leq 0.05$).

Results and Discussions

Diagnosis of *Neoscytalidium* species

In this study, we presented the exploitation of the fungal ITS4–ITS5 internal transcribed spacer sequences to explore the nature of phylogenetic relationships among *Neoscytalidium* isolates. In the present study, a powerful ability of ITS4–ITS5 PCR amplicon in differentiating between two *Neoscytalidium* species was revealed(Figure 2). It was discovered thatITS4–ITS5 PCR primer pair exhibited extremely high efficiency in the amplification of *Neoscytalidium*. The overall sensitivity of this identification method was 100%, since all 100 isolates were detected successfully, yielding distinguishable PCR amplicons of two discrete electrophoretic forms. The two electrophoretic variants of about
555 bp and 938 bp represented *N. novahollandiae* (69 isolates) and *N.dimidiatum*(31 isolates), and they were registered at NCBI under accession nmbers: MF682357 and MF682358, respectively.

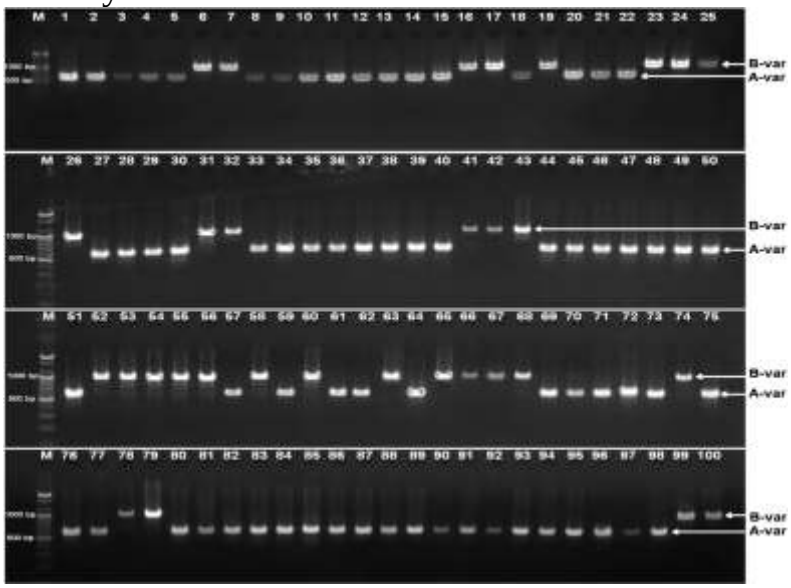

Figure2: Electrophoretic variants of PCR amplification.

Effect of *Neoscytalidium* on fig saplings

Growth of fig saplings was decreased due to infection with *Neoscytalidium* spp. The severity of infection ranged from 15.32 (N95) to 98.75% (N73) as shown in Figure 2. This may be due to the differences among the fungal isolates in terms of their virulence (15), or may be due to genetic differences among isolates that affects pro-

ducing important enzymes such as cellulose, pectinase, protease, and amylase which play essential roles in penetration of the plant tissue and further pathogenicity (3,15, and 26).

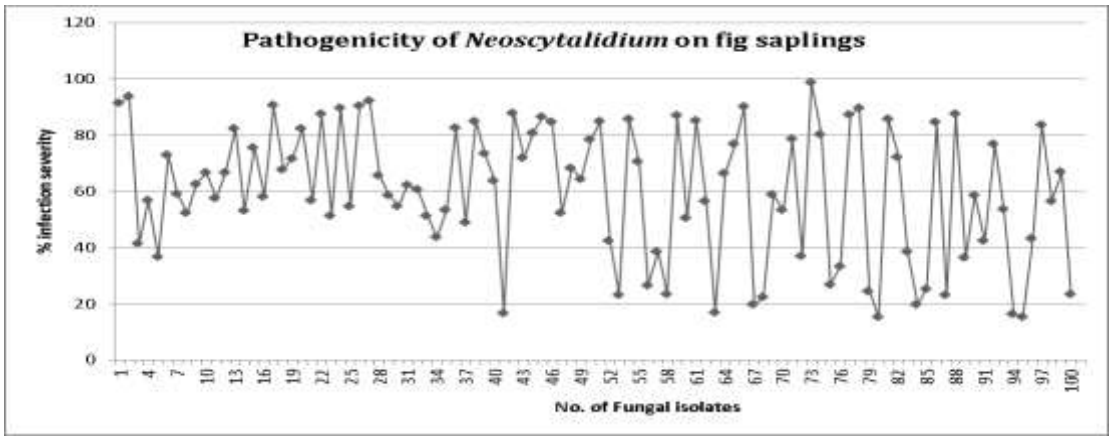

Figure3: Severity of infection Neoscytalidium on fig saplings

Cellulase Assay

The results revealed that all isolates had cellulase activity ranging from 0.161 U . ml^{-1}. min^{-1} in isolate N27(*N.dimidiatum*) to 0.201 U. $mg^{-1}.min^{-1}$ in isolate N73 (*N.novahollandiae*) with significant differences among all the seven tested isolates (Table1).

Table 1: Cellulase activity in the biomass of pathogenic *Neoscytalidium*

No.isolates	Species of isolates	Cellulose activity U . ml^{-1}. min^{-1}
N1	***N.novahollandiae***	**0.192**
N2	***N.novahollandiae***	**0.185**
N17	***N.dimidiatum***	**0.164**
N24	***N.dimidiatum***	**0.142**
N26	***N.dimidiatum***	**0.163**
N27	***N.novahollandiae***	**0.161**
N73	***N.novahollandiae***	**0.201**
L.S.D 0.0012		

The fungi were cultured in 9 cm Petri- dishes with standard PDA medium for the period of 3 days at 25±3° C. The walls of plant cells consist of three types of poly-saccharides: cellulose, hemicellose, and pectin as well as lignin in most of the trees. These substances can be degraded by pathogens producing enzymes such as cellulase, pectinase, protease, xylanase, and ligninase which facilitate the fungus to penetrate the vascular tissues. They are believed to be more important in the occurrence of disease because it is responsible for the weakness and degeneration of the infected tissues, as these pathogens can dominate the central plate resulting in cell death. The effectiveness of these enzymes depends on the number of genes encoded for this enzyme which are varying from pathogen to another. This may explain our findings that some isolates did not cause severe symptoms. In contrast, the plant contains resistance genes (*R* gene) according to the 'gene for gene interaction', which activates a

series of resistance genes in the plant that lead to the production of phenolic , toxic, and oxidative compounds as reactions to fungi attack (2).

Antagonism of *A. chroococcum* and *L.rhamnosus* to *Neoscytalidium*

Radial growth and inhibition rate of a pathogenic fungus is frequently used to indicate efficiency of a biocontrol agent. In this study *A. chroococcum* was significantly effective to reduce pathogenic fungal growth rather than *L.rhamnosus* (Table2). The effect of these bacteria, in case of *A. chroococcum* may be due to their ability to produce a number of enzymes that breakdown cell wall of pathogens, such as chitinase, laminarinase, glucanase and antibiotics like pyoluteorin, herbicolin, phenazin, (HCN) produced. The presence of such compounds at high concentrations inhibits the growth of pathogenic fungi (14). While the mode of action in case of *L.rhamnosus* is related to their ability to produce antibiotics included acidophilin, lactocidin, lactolin, nisin, lactic acid, acetic acid, probionic acids, bacteriocins and many other antimicrobial compounds that affect pathogen's physiological activities including cell division, synthesis of nucleic acid DNA and RNA and protein synthesis.

Table2: .Effects of *A. chroococcum* and *L. rhamnosus* on growth of seven isolates of pathogenic *Neoscytalidium* on PDA medium

Treatments	No.isolates	Fungal growth(cm)	Inhibition(%)
N.novahollandiaeA.chroococcum	**N1**	**3.80**	**57.78**
N.novahollandiae+L.rhamnosus	**N1**	**5.04**	**44.00**
***N.novahollandiae* alone**	**N1**	**9.00**	**0.00**
N.novahollandiae+A.chroococcm	**N2**	**3.41**	**62.12**
N.novahollandiae+L.rhamnosus	**N2**	**4.05**	**55.00**
***N.novahollandiae* alone**	**N2**	**9.00**	**0.00**
N.dimidiatum+A.chroococcum	**N17**	**2.11**	**75.67**
N.dimidiatum+L.rhamnosus	**N17**	**3.16**	**63.56**
***N.dimidiatum* alone**	**N17**	**8.67**	**0.00**
N.dimidiatum+A.chroococcum	**N24**	**2.15**	**75.21**
N.dimidiatum+L.rhamnosus	**N24**	**3.74**	**56.87**
***N.dimidiatum* alone**	**N24**	**8.67**	**0.00**
N.dimidiatum+A.chroococcum	**N26**	**2.83**	**68.56**
N.dimidiatum+L.rhamnosus	**N26**	**3.63**	**59.67**
***N.dimidiatum* alone**	**N26**	**9.00**	**0.00**
N.novahollandiae+A.chroococcm	**N27**	**3.44**	**61.78**
N.novahollandiae+L.rhamnosus	**N27**	**4.27**	**52.56**
***N.novahollandiae* alone**	**N27**	**9.00**	**0.00**
N.novahollandiae+A.chroococcm	**N73**	**3.41**	**62.12**
N.novahollandiae+L.rhamnosus	**N73**	**4.12**	**54.23**
***N.novahollandiae* alone**	**N73**	**9.00**	**0.00**
L.S.D		**0.160**	**1.520**

Effect of biological agents on infection severity, growth parameters and enzymes activities of fig saplings infected with *Neoscytalidium*

Results of this experiment (Table3) showed high efficiency of *A. chrocooccum* in promoting growth parameters and enzymes activities of figs regardless the presence of pathogenic fungus. This treatment resulted in highest leaf area, chlorophyll con-

tent, peroxidase and catalase. Both *A. chrocooccum* and *L. rhamnosus* reduced infection severity, in order, by 75% and 66% due to pathogenic fungi. The high effectiveness of *A. chrocooccum* is because of its high competence on nutrients over the pathogenic fungus as well as due to its ability to produce Sidorphores compounds that absorbed triangular iron and not available for fungus, and consequently causing fungus death and decomposition (19). As for the treatment of *L. rhamnosus* alone, the results showed a good efficiency for increasing growth parameters, which is possibly due to the production of Indol acetic acid (IAA) leading to elongation of apical zone of cells. The negative effects of *N. dimidiatum* was more reduced by the biocontrol bacteria compared to that of *N.novahollandiae*, as plant growth parameters were significantly increased in plants infected with first rather than those infected with the last. These results were consistent with those from severity of infection experiment. Confirming that *N.novahollandiae* were more aggressive and causing severe infection than *N.dimidiatum* isolates .

Table3:.Effect of biological agents treatments on growth parameters and enzymes activities of fig saplings infected with *Neoscytalidium*.

Treatments	Severity of infection%	Paper area (Cm^2)	Chlorophyll content (spad)	Peroxidase activity ($U.mg^{-1}. min^{-1}$)	Catalase activity ($U.mg^{-1}. min^{-1}$)
N1+*A.chroococcum*	25.10	861	15.46	34.76	28.56
N2+*A.chroococcum*	27.68	873	12.53	35.10	30.76
N17+*A.chroococcum*	23.33	1021	30.36	46.60	32.20
N24+*A.chroococcum*	30.78	1033	32.03	36.66	31.50
N26+*A.chroococcum*	28.16	980	35.33	38.20	32.83
N27+*A.chroococcum*	31.22	923	28.50	31.70	27.13
N73+*A.chroococcum*	25.00	905	22.43	28.36	21.26
N1+*L.rhamnosus*	37.90	711	22.00	29.16	22.86
N2+*L.rhamnosus*	41.56	728	20.56	28.46	22.56
N17+*L.rhamnosus*	45.78	848	23.53	31.56	23.13
N24+*L.rhamnosus*	35.66	851	23.40	26.80	21.20
N26+*L.rhamnosus*	38.92	823	35.40	24.63	18.16
N27+*L.rhamnosus*	40.12	816	38.50	25.63	18.93
N73+*L.rhamnosus*	38.85	609	29.40	22.80	17.16
N1	91.54	50	0.46	3.76	2.70
N2	95.33	57	1.20	4.80	5.33
N17	80.93	62	3.10	15.63	11.20
N24	83.86	65	5.23	12.56	10.33
N26	90.22	65	5.40	6.50	5.86
N27	87.59	52	2.06	9.33	8.30
N73	98.31	57	5.26	12.33	9.53
A.chroococcum alone	0.00	1830	47.76	18.66	12.93
L.rhamnosus alone	0.00	1575	40.16	8.90	7.13
Control	0.00	860	22.40	14.43	8.03
L.S.D.	1.264	2.33	4.62	17.54	5.89

Means are average of three replicates. N1, N2, N27,N73 are *N.novahollandiae* and N17, N24,N26 are *N.dimidiatum* isolates.

Conclusion

N. dimidiatum and *N. novaehollandiae*, caused canker, dieback, and blacking stem on various trees in world wide finding of this research confirmed the efficiency of using bio controlling agents (*L.rhamnosus* and *A. chroococcum*) to reduce the infection by pathogenic fungi, enhance plant growth and health and reduce environmental hazard and harmfulness .

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