Molecular Characterization and Pathogenicity of *Cylindrocarpon destructans* isolates from grapevines in Duhok, North Iraq

RAED A. HALEEM , SAMIR K. ABDULLAH[1] and JALADAT M. S. JUBRAEL[2]

Plant Protection Department, Faculty of Agriculture and Forestry, University of Duhok,

[1] Department of Biology, Faculty of Science, University of Zakho, Iraq

[2] Scientific Research Center, University of Duhok, Iraq

samer_abdalh@yahoo.com

abstruct

Ten *Cylindrocarpon* isolates were detected from roots of declined grapevines (*Vitis vinifera* L.) plants collected from five vineyards in Duhok governorate, North Iraq. The symptoms of the declined host including reduce vigor with small-sized trunks ,reduction in root biomass, black discoloration and brown to black streaks in wood mainly at the base of rootstock .Based on cultural and morphological characteristics, all isolates were identified as *C.destrunctans*. These isolates were subjected to species-specific PCR assay. Total genomic DNA was isolated from pure cultures of the isolates. The average DNA yields ranged between 1.5-6.7 µg/ml with a purity 1.6-1.8. The specific primers for *C. destructans* (400 bp) were used to amplify the ITS region of nuclear ribosomal DNA (rDNA) containing ITS1, ITS2 and the intervening 5.8 rRNA genes of *Cylindrocaropon* produced appropriate and successful results with the selected isolates confirmed that all isolates were correctly identified as *C. destructans*. Pathogenicity of *C. destructans* was tested under greenhouse conditions. One – year old dormant rooted cuttings of the two grape cultivars, Reshmew and Taefi, were inoculated by dipping their roots for 30 min in the conidial suspension (1×10^6 conidia ml^{-1}). Infected vines showed reduced vigour with small leaves, interveinal chlorosis and necrosis. Other symptoms included a reduction in root biomass and root hairs. The severity

of root mass reduction reached to 0.41 on Taefi (cv.) after five months of inoculation with significant difference compared with control treatment. *C. destructans* caused significant decrease in the fresh and dry weight of grapevine shoots and roots. This fungus is reported for the first time in Iraq during this study.

Key words: *Cylindrocarpon destructans,* grapevines ,pathogenicity, PCR ,Iraq

Introduction

Species of *Cylindrocarpon* Wollenw. are common soil inhabitants, saprobes on dead plant materials, root colonizers or pathogens often associated with roots of herbaceous and woody plants(Brayford,1993).

Cylindrocarpon destructans(Zinnsm.)Scholten anamorph of *IIyonecteria radicicola*(Gerlach&L.Nilsson)Chaverri&C.salgado and *C.obtusisporum*(Cooke&Harkn.)Wollenw. have been described as the agents of root rots of various hosts and a black foot disease of grapevines (Seifert *et al.*2003,Chaverri *et al*.2011). Grasso & Magnano di San Lio (1975) described black foot symptoms from nursery plants with black discoloration and gum inclusions in xylem vessels of affected rootstocks. Scheck *et al.* (1998) also described dark-brown to black streaking in the vascular tissue of young (2–5 years-old) grapevines.

The impact of this disease on nursery seedling production is twofold. First, these fungi can cause seedling mortality in nurseries up to 50% (Anderson *et al.* 1962 & Anonymous, 1993). Second, infected seedlings have a lower survival rate after out planting to reforestation sites. An important factor compounding this problem is that in some cases the symptoms are not visible on infected seedlings but disease can develop after transplantation.

Detection of plant pathogens directly from infected tissues has been reported for several agricultural plant pathogens (Le´vesque *et al.* 1994 : O'Gorman *et al.* 1994). Also, PCR has been used to detect soil pathogens directly from infested soil (Henson *et al*., 1993). These molecular detection approaches are ideally suited for the study of root rot organisms because of the difficulty in isolating and identifying some of these fungi. Hamelin *et al*. (1996) designed species-specific primers (Dest1 and Dest4) to detect *Cylindrocarpondestructans* from conifer seedlings. Using these primers in direct PCR assays on DNA extracted from *C. destructans* cultures isolated from grapevines in Portugal, obtained a DNA fragment of 400 bp; The universal primer ITS4 and the fungus-specific primer ITS1F were used in a first-stage fungus-specific amplification, followed by a second-stage amplification with the primers Dest1 and Dest4 using the PCR product from stage one. This is a simple and reliable method for detection of *Cylindrocarpon* spp. directly from infected grapevines (Nascimento *et al*., 2001).

Rego *et al*. (2000) conducted pathogenicity studies with rooted cuttings of '99R' rootstock by dipping the roots in a conidial spore suspension of *C. destructans*. Typical black foot symptoms including root lesions, vascular discolouration and necrosis developed within two months. Similar results were obtained in studies conducted with rooted cuttings of Seara Nova cv. (Oliveira *et al*., 1998) and Periquita cv. (Rego *et al*., 2001). However, in the latter study 13 *C. destructans* isolates collected over a period of seven years were used. Although all the isolates were pathogenic, variation in virulence was observed and it was not correlated with the age of the cultures. All the isolates significantly reduced plant height and most significantly reduced the number of roots. In most cases the stunting could be explained by the

shortened internodes, although it appeared as the most virulent strains reduced the number of internodes. Inoculation of six-month-old potted grapevine rootstocks ('Ramsey') with *C. destructans*, *C. macrodidymum*, *Campyl. fasciculare* and *Campyl. pseudofasciculare* resulted in death and reduction of root and shoot mass of inoculated plants (Halleen *et al*., 2004).

The purpose of this study was to isolate, identify *Cylindrocarpon destructans* the causal agent of grapevine black foot disease in Iraq based on PCR and determine its pathogenicity on grapevines cultivars.

Material and Methods

Fungal isolation : Isolation was done in two methods:

1- Isolation from vine roots.

Small pieces of vine roots collected from five locations in Duhok governorate (Bajelor, Badi, College nursery, Malta nursery and Nizarke) were surface sterilized by placing in 70% ethanol for 30 s, 1% NaOCl for 1 min and again in 70% ethanol for 30 s and then dried by filter papers. Sterilized tissues pieces were plated onto 2% potato dextrose agar (PDA) (Himedia Laboratories Pvt. Ltd. - India) containing 0.25 mg/ml chloramphenicol. Hyphae growing out from the tissue pieces were cut and sub cultured onto fresh PDA plates, and incubated at 25±2 °C (Van Niekerk*et al*, 2004).

2- Moist chamber method.

Cuttings were made from vine roots and placed in 90 mm petridishes containing sterilized moist filter paper. Plates were incubated at room temperature until fungal growth observed. Propagules (spores, mycelia) were transferred to Potato-dextrose-Agar (PDA) plates. Pure cultures of each isolate were obtained by excising a hyphal tip from colony margins and plating it onto fresh PDA.

Phenotypical characterization

All isolates were grown on PDA and MEA at 25°C in darkness or under NUV + fluorescent illumination with a 12-h photoperiod (Philips /36W) for 10 days until cultures sporulated. The colonies were further incubated for 20 days to determine the presence or absence of chlamydosporcs. The diameter of 20 chlamydospores per isolate was measured. Length and width of 40 conidia (microconidia and one-, two-, and three-septate macroconidia) were measured. Isolated fungus was identified based on the characters in culture and on natural substrates (Domsch *et al*, 1980; Watanabe, 2002; & Petit and Gubler, 2005).

DNA extraction and PCR amplification of *Cylindrocarpon destructans*.

- **Fungal Isolates**

Ten isolates (DC1-DC10) were selected to confirm the identification by a specific primer of the ITS region. Isolates were collected from five locations in Duhok governorate (Bajelor, Badi, College nursery, Malta nursery and Nizarke).

- **Total genomic DNA extraction and PCR amplification of ITS region.**

Genomic DNA was extracted according to a method reported by Borges *et al.* (1990). The specific primers of *Cylindrocarpon destructans* were used to amplify the ITS region of nuclear ribosomalDNA (rDNA), containing ITS1, ITS2 andthe intervening 5.8 rRNA gene (Dest1 5'-TTGTTGCCTCGGCGGTGCCTG-3', Dest4 5'-GGTTTAACGGCGTGGCCGCGCTGTT-3') (Hamelin *et al.*, 1996). The PCR reactions were carried out in a totalvolume of 25 µl, in thin-walled, 0.5 µl Eppendorftubes.Master mix was prepared for 12 samples of each fungus (10 isolates plus 2 control) by mixing 30 µl of 10XPCR, 30 µl of dNTPs, 24 µl forward primer, 24 µl

Reverse primer, 12 µl MgCL2, 4.8 µl of Taq polymerase enzyme and de-ionized distilled water was added to a final volume of 252µl. The solution mixed and spun for 10 second in a microcentrifuge. Later, the mixture was dispensed in PCR tubes. All these steps were done on ice. Amplification was carried out in an automated thermal cycler (Delphy 1000, Oracle Biosystems, MJ Research Inc., Watertown, MA, USA) according to the following program: An initial denaturation at 94°C for 4 min, after which 30 cycles of denaturation (1 min at 94°C), primer annealing (1 min at 58°C) and primer extension (1.5 min at 72°C) were performed (Alaniz *et al.,* 2007). A final extension was performed at 72°C for 10 min Amplification reactions were conducted at least twice, in two separate experiments. For each isolate, 5 µl of PCR products were mixed with 7µl loading buffer and then analyzed by electrophoresis in 2% (w:v) agarose gels with 1xTBE buffer visualized by UV fluorescence.

Pathogenicity test

Inoculum was prepared from one month old culture grown on PDA at 25°C, by flooding the agar surface with 10 ml of sterile distilledwater (SDW) and scraping with a spatula. The resultingspore suspension was filtered through two layers of cheesecloth. Spore concentration was calculated with haemocytometer, and then adjusted by dilution with SDW to 1×10^6conidia ml^{-1}. One – year old dormant rooted cuttings of Reshmew and Taefi grape cultivars, planted in pots containing 20 kg sterilized sandy loam soil- petmoss (3:1) in a Greenhouse were used for pathogenicity test. Before inoculation, roots were trimmed and disinfested by immersion for 2 min in 1.5% sodium hypochlorite solution,and washed twice with SDW. Each plant was inoculated by dipping its roots for 30

min in the conidial suspension of *C. destructans* (Isolate DC1). Control plants were dipped in SDW. Rooted cuttings were then planted in a greenhouse at 25 to 30 °C. One month later, each plant was again drenched with 40 ml of the corresponding spore suspension (1×10^6 conidia ml^{-1}). After 5 months of incubation, all plants were gently uprooted and washed free of soil (Alaniz *et al*, 2007). Root symptoms of each individual plant were evaluated depending on the grade of roots discoloration and mass reduction in Figure (1) using the following scale:

0 = healthy with no lesions.

1 = slight discoloration with 1 to 10% root mass reduction.

2 = slight discoloration with 11 to 25% root mass reduction.

3 = moderate discoloration with 26 to 50% root mass reduction.

4 = severe discoloration with >50% root mass reduction.

5 = dead plant.

Disease severity was calculated following the index described by Michenny. (1923) .Dry and fresh weights of roots of each plant were recorded.

Symptomatic roots were aseptically plated on MEA to reisolate the fungus.

The layout of the trail was factorial in a Complete Randomized Design (CRD) with three replications; each replicate had three plants. The data obtained from this trial were analyzed using SAS program and means were compared using Duncan's multiple range test (SAS Institute Inc., Gary, NC, USA, 1999).

Results and Discussion

Phenotypical characterization of *Cylindrocarpon destructans*

Cylindrocarpon destructans (Zinssm.) Scholten, Neth. J. Plant. Path. 70 (Suppl. 2) 9 (1964). Figure (2) A – C. Telemorph: *Ilyonectria radicicola* (Gerlach & L. Nillsson) P.Chaverri&C.Salgado.Studies in Mycology 68.71(2011).

Colonies on MEA reached a diameter of 78 mm on PDA and 80 mm on MEA after 20 days at 25°C. Colony surface slimy to felty; aerial mycelium typically sparse to felty, white to buff or a shade of brown. Colony reverse was orange to dark brown. Conidiogenous cells formed apically on densely, irregularly branching clusters of cells borne laterally on otherwise undifferentiated vegetative hyphae. Conidiophores 65 µm tall. Macroconidia cylindrical, mainly 4-celled, 18 – 45 (- 47) × 4 – 8 µm. Microconidia, cylindrical, 1-celled, 8 – 11 (-12) × 3 – 4 µm. Chlamydospores yellowish brown, ovate to ellipsoidal, a few in a chain, 8 – 10 (-15) µm in diameter. Conidial dimensions were in concordance with the previous identification (Petit & Gubler, 2005).

Molecular detection of *C. destructans*Genomic DNA isolation and purification.

Suitable yields of genomic DNA were obtained from repeated experiments with an average yield of 1.5-6.70 µg/ml and a purity of about (1.6-1.8) determined by spect-rophotometer ratio A260/A280. The molecular weight of DNA samples was estimated using 1% agarose gel electrophoresis containing ג DNA sample as control (Figure 3). Ratios above 2.0 correspond to RNA contamination, while ratios below 1.6 suggest protein contamination (Sinha *et al.*, 2001).

Species specific primers

All isolates of *C. destructans* collected from different locations of Duhok Governorate were amplified by two specific primers (Dest1, Dest4) which were designed by (Hamelin *et al*, 1996). A PCR fragment of about 400-bp was obtained for all of them. The agarose gel electrophoresis of amplified products with this specific primer is shown in Figure (4). Other investigators have used the same primers to amplify the ITS region of *C. destructans* (Hamelin *et al.*, 1996 & Alaniz *et al.*, 2007).

This is the first molecular detection work on *C. destructans* in Iraq. In this study, the differences in the intensity of bands were not taken in consideration; despite they may reflect the differences in copy number of the priming sites in the individual genome. The band intensity may also be attributed to the difference of DNA concentration of individual isolates. This method did not require going through all classical methods and only in a few hours the results could be obtained. This approach is particularly well suited to soil organisms that are difficult to identify or isolate because of the presence of other aggressive species.

Pathogenicity test

Affected vines showed reduced vigour with small-sized trunks, shortened internodes, sparse foliage, and small leaves with interveinal chlorosis and necrosis (Figure 5). Other symptoms included a reduction in root biomass and secondary roots with sunken, necrotic root lesions. Black discolouration and brown to dark streaks in wood was observed, mainly at the base of the rootstock. The pith of the affected vines was also compacted and discoloured. This description is in agreement with previous results (Scheck *et al*., 1998; and Rego *et al*., 2000). The examination of symptomatic tissue revealed that the xylem vessels may be plugged with thick-walled tyloses or brown gum; furthermore the presence of fungal hyphae in the ray cells of the phloem and younger xylem. Starch reserves are mainly stored in the ray cells, providing a readily metabolisable carbon source for *Cylindrocarpon destructans*, which can produce extracellular amylases (Sweetingham, 1983). The severity of root mass reduction by *C.destructans* reached to 0.35 after 5 months of inoculation with significant difference compared with control treatment. The highest disease severity (0.41) was recorded on Taefi (cv.) inoculated by *C. destructans* with significant difference when compared

to the control, but did not show significant difference compared with Rashmew (cv.) (Figure 6). Similar results were reported by Rego *et al*., (2001), and Halleen *et al*., (2004) in the roots mass reduction of inoculated plants by *C. destructans*.

This pathogen caused significant decrease in the fresh and dry weight of grapevine shoots (151.14 g , 44.91g respectively) after 5 months of planting. The reduction in fresh and dry weight of grapevine root was also varied significantly compared with control (Figure 7, 8).

These results are in agreement with that published by Alaniz *et al*, (2007) .

Figure (1). Grapevine roots showing different grades of discoloration and mass reduction, from left to right (scale 0 – 4).

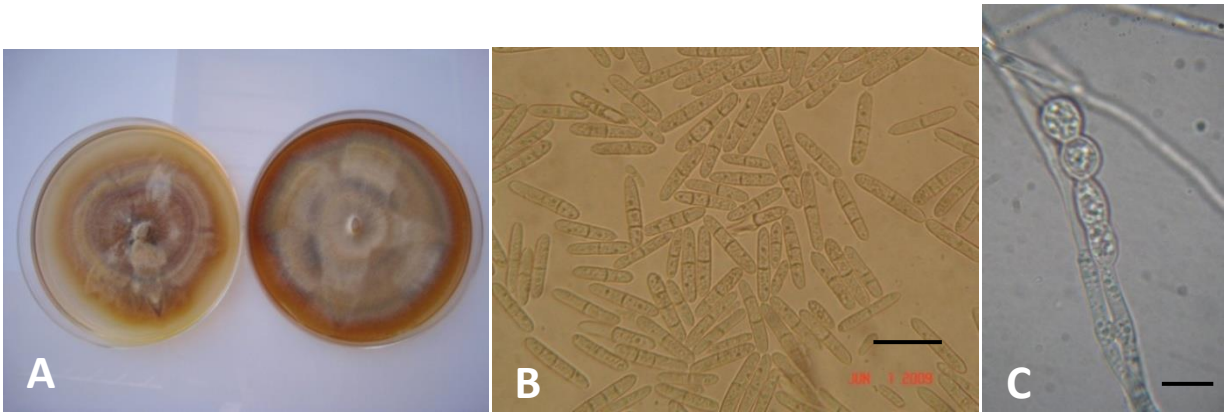

Figure (2) *Cylindrocarpon destructans*. A) Twenty- day old colony on PDA-left, and MEA-right. B) Microconidia and macroconidia. Scale bar: 30 µm. C) Mycelia and Chlamydospores, Scale bar: 15 µm

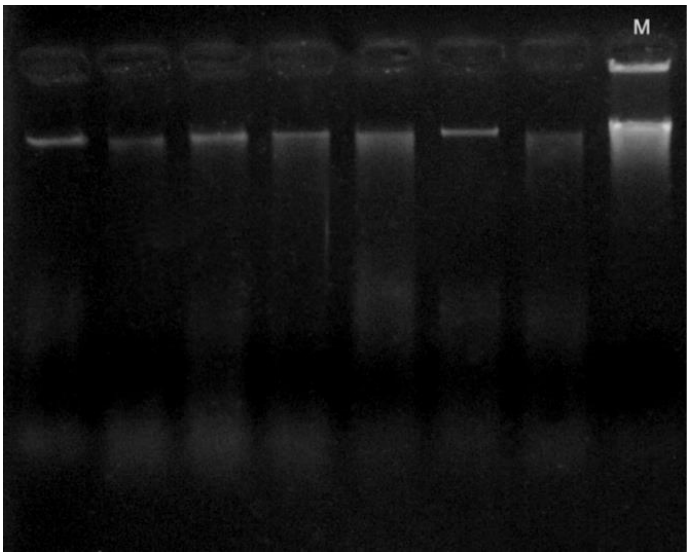

Fig. (3): Agarose gel electrophoresis 1% at 70 volt for 45 minutes. M: represents unrestricted λ DNA as a standard molecular weight marker.Lane1- 7: Whole Genomic DNA of some *C. destructans* isolates isolated from different locations of Duhok Governorate.

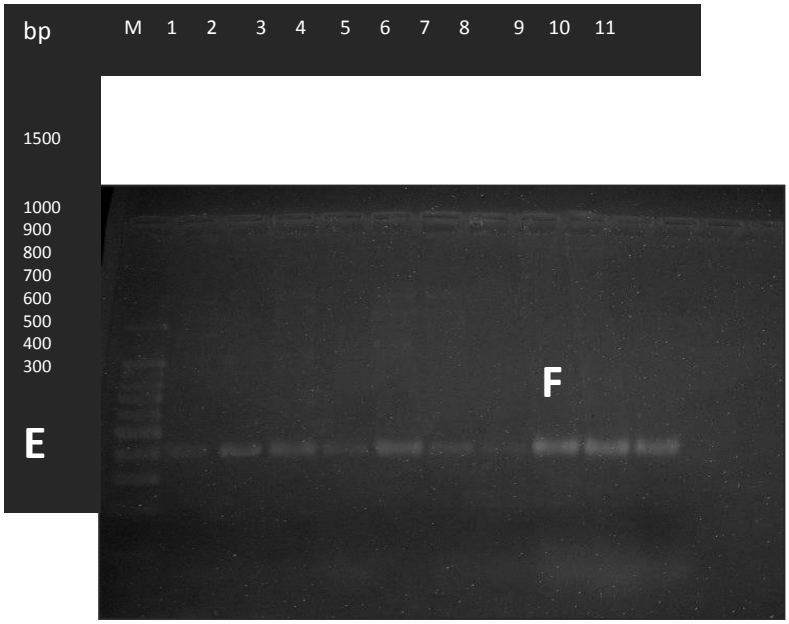

Figure (4). Agarose gel of the PCR products using primer pairs Dest1 – Dest4. Lanes 1 (DC1)-10(DC10), *C. destructans* isolates. Lane 11, negative control of sterile distilled water; lane M, 1Kb Plus DNA Ladder.

Figure (5): Symptoms of *C. destructans* on the Rashmew cv.: A) Sparse foliage with small leaves and internal chlorosis. B) Reduction in secondary roots with sunken, necrotic root lesions. C) Black discoloration and dark streaks in longitudinal section of inoculated roots (left). D) Discolored pith of the inoculated roots under greenhouse conditions (right).

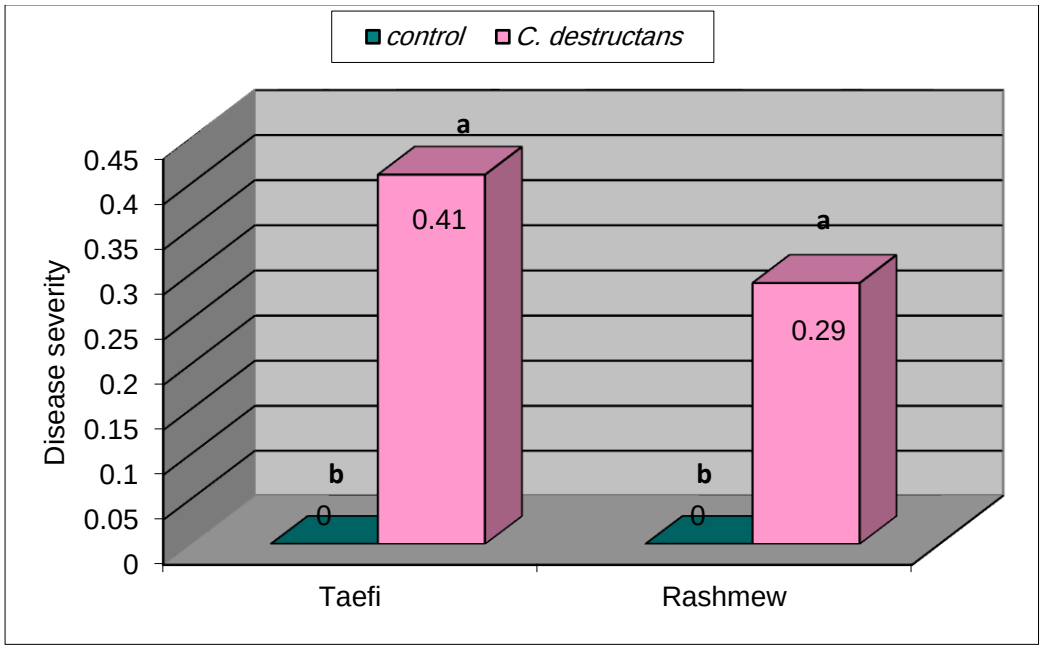

Figure (6): Disease severity of *C. destructans* on Taefi and Rashmew cultivars after 5 months of incubation.

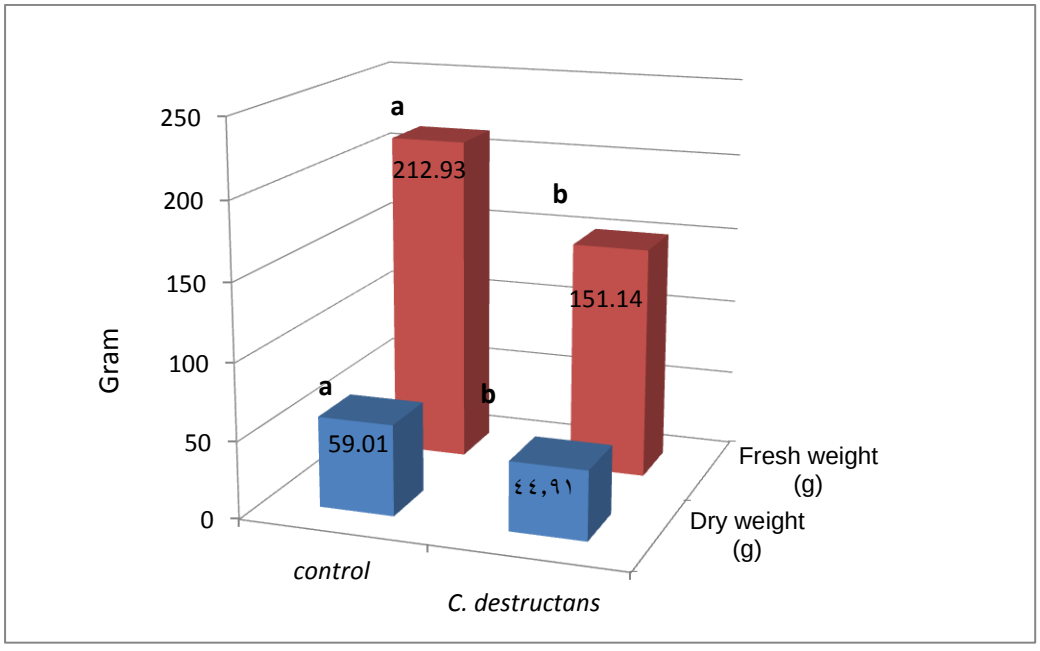

Figure (7): Effect of *C. destructans* on the fresh and dry weight of Grapevine shoots.

* Means followed by different letters in each column are significantly different based on Duncan's Multiple Range test (P=0.05).

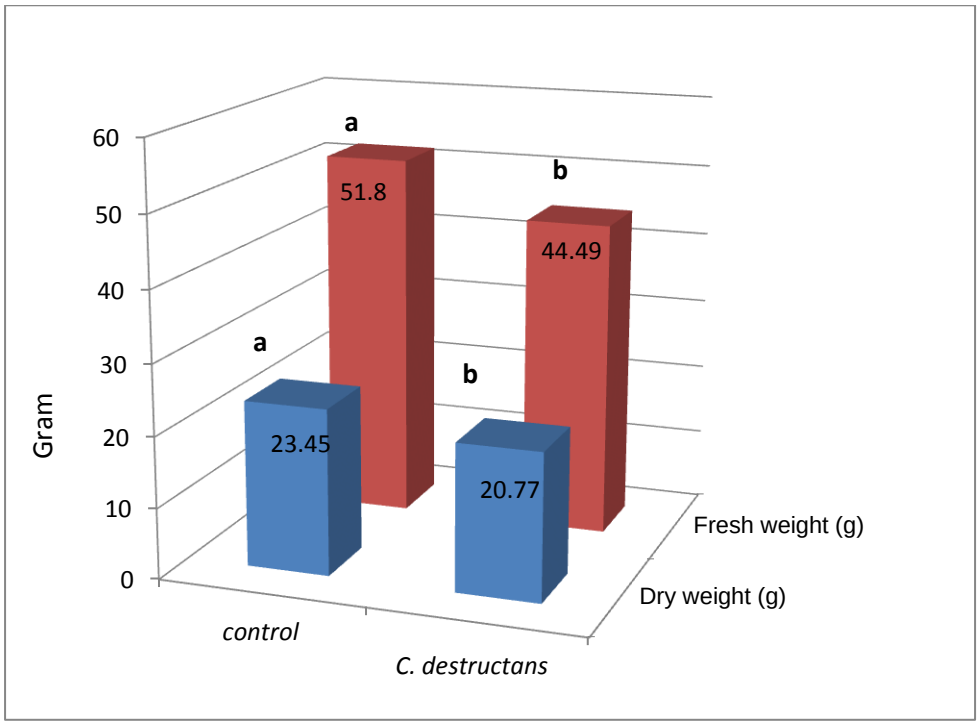

Figure (8): Effect of *C. destructans* on the fresh and dry weight of Grapevine roots.

* Means followed by different letters in each column are significantly different based on Duncan's Multiple Range test (P=0.05).

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التوصيف الجزيئي وأمراضية الفطر *Cylindrocarpon destructans* المعزول من جذور كرمات العنب في دهوك شمال العراق

رائد عبدالجبار حليم [1]سمير خلف عبدالله [2]جلادت محمد صالح جبرائيل

قسم وقاية النبات كلية الزراعة والغابات جامعة دهوك العراق

[1]قسم علوم الحياة كلية العلوم جامعة زاخو العراق

[2]مركز الابحاث العلمية كلية العلوم جامعة دهوك العراق

الخلاصة

عزلت عشر عزلات من الفطر *Cylindrocarpon* من جذور نبات كرمات العنب والتي جمعت من خمسة مزارع في محافظة دهوك شمال العراق. اظهرت النباتات المصابة اعراض شملت: قلة النمو، صغر في حجم السيقان، اختزال في كتلة الجذور ، وتخطط بلون بني الى الاسود في الاوعية خاصة عند قاعدة الساق. شخصت جميع العزلات على انها تعود للفطر *Cylindrocarpon destructans* اعتمادا" على الصفات المظهرية والزرعية. لغرض التأكد من تشخيص هذه العزلات استخدمت بادئات خاصة بالفطر لتضخيم

the ITS region of nuclear ribosomal DNA (rDNA) containing ITS1, ITS2 and the intervening 5.8 rRNA genes of *Cylindrocaropon*.

والتي ميزت بشكل صحيح النوع المشخص. أختبرت أمراضية الفطر تحت ظرف البيت الزجاجي وذلك بغمر جذور النبات لنوعين من اصناف العنب لمدة ثلاثين دقيقة في معلق سبوري للفطر (تركيز$X1^{6}$ 10 / ملم). أظهرت النباتات المصابة اعراضا مرضية تمثلت باصفرار وتنقر في الاوراق فضلا عن اختزال في الشعيرات الجذرية. تم تسجيل فرق معنوي في اختزال الوزن الجاف لكل من السيقان والجذور للنباتات المصابة مقارنة مع السيطرة. سجل هذا الفطر لاول مرة في العراق.