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Isolation and identification of *Ulocladium sp.* VL204 associated with *Pistacia atlantica* leaves from Dana biosphere reserve, Jordan

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Abstract

Endophytic fungus isolated from *Pistacia atlantica Desf.* which associated with leaves necrotic spotting was identified using nuclear ribosomal DNA internal transcribed spacer regions (ITS1-5.8S rDNA-ITS2) as *Ulocladium sp.* VL204 a fungus member of Pleosporaceae family.

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Introduction

Dana biosphere reserve is the largest reserve in Jordan had area around 292.534 km² lays in the south of Jordan. The reserve characterized by the presence of four main vegetation communities according to the climate and vegetation; Mediterranean (23% of reserve area), Irano-Turanian (22% of reserve area), Sudanian (48% of reserve area) and Saharo-Arabian (7% of reserve area). Hence, the reserve native flora is exposed to different abiotic and biotic environmental stresses.

The genus *Pistacia* belongs to the family *Anacardiaceae* that comprise about 70 genera and over 600 species. A mix of *Pistacia atlantica Desf.* and *Juniperus phoenica L.* occupied around 1.17 km² of the reserve and they form around 0.4 % of the plant cover of the reserve, grown up on altitude between 1000 to 1300m above the sea level (The royal society for the conservation of nature (RSCN)-Dana biosphere reserve, 2008).

Bozorgi *et al.*, (2013) reviewed wide phytochemical and pharmacological properties from various parts of *Pistacia* species since they utilized by people widely for different nutritional and medicinal proposes. *P. atlantica* leaves extracts showed antioxidant, antimicrobial activities and antidiabetic (Benhammou *et al.*, 2008; Hamdan and Afifi, 2004; Kasabri *et al.*, 2011; Peksel, 2008).

Ulocladium genus belongs to the family *Pleosporaceae* of the Ascomycota. Comprehensive systematic analysis of the *Ulocladium* genus using three genetic loci also included related *Alternaria*, *Embellisia*, and *Stemphylium* spp. the findings were the taxonomic status of *Ulocladium* as a monophyletic clade. In addition, to provide final resolution of this clade other more informative loci may provide additional support for this resolution (Runa *et al.*, 2009).

Ulocladium species cause leaf spots, suppress sporulation and other diseases of different agricultural crops and plan organs (Elmer and Köhl, 1998; Hill *et al.*, 1994; Köhl *et al.*, 2003; Wang *et al.*, 2008; Vannini and Vettraino, 2000; Zitter and Hsu 1990). The objectives of this study were to provide identity of *Ulocladium* species isolated from diseased leaves of *P. atlantica Desf.* from Dana biosphere reserve in south of Jordan.

Materials and methods

Plant sample collection

P. atlantica Desf., was used for this study because of presence of necrotic spots on the plant leaves. Plant leaves samples were collected from the 4th to 22th of May, 2014.

Handling of plant samples

Leaves samples were kept directly on ice box after cutting from randomly selected *P. atlantica Desf.* plants. In the laboratory, samples were transferred and stored at 4°C and processed within 24 h of collection. For further steps the procedure of Romero *et al.*, (2001) was used. Briefly, leaf surfaces were washed thoroughly with sterilized distilled water in order to remove the epiphytic fungal flora before surface sterilization. The washed leaves were then treated with 15% NaHOCl solution diluted with sterilized distilled water in ratio 2:1 for 5min. surface sterilized leaves were cut into small pieces of 2–5 mm squares for endophytic fungi isolation.

Isolation of endophytic fungi

The surface-sterilized leaf segments were aseptically transferred to petridishes containing potato dextrose agar (PDA) medium and incubated for 14 days at 25 °C.

The pure endophytic fungi strains were transferred to new PDA petridishes.

DNA extaction, PCR Amplification and DNA Sequencing

The procedure of White *et al.*, (1990) was used for total deoxyribonucleic acid (DNA) extraction from isolated fungus strain. Mycelia DNA for extraction were scraped from 12-day-old PDA cultures of pure isolate. Followed by polymerase chain reaction (PCR)

amplification of nuclear ribosomal DNA internal transcribed spacer (ITS) regions (ITS1-5.8S rDNA-ITS2) then sequenced using primers ITS5 and ITS4 that designed also by White *et al.*, (1990) . Primer pair ITS4 and ITS5 were used to amplify the 5.8S gene and flanking ITS1 and ITS2 regions.

Primer extension sequencing was performed by GENEWIZ, Inc. (South Plainfield, NJ) using Applied Biosystems BigDye version 3.1. Both forward and reverse strands were sequenced. The reactions were then run on Applied Biosystem's 3730xl DNA Analyzer.

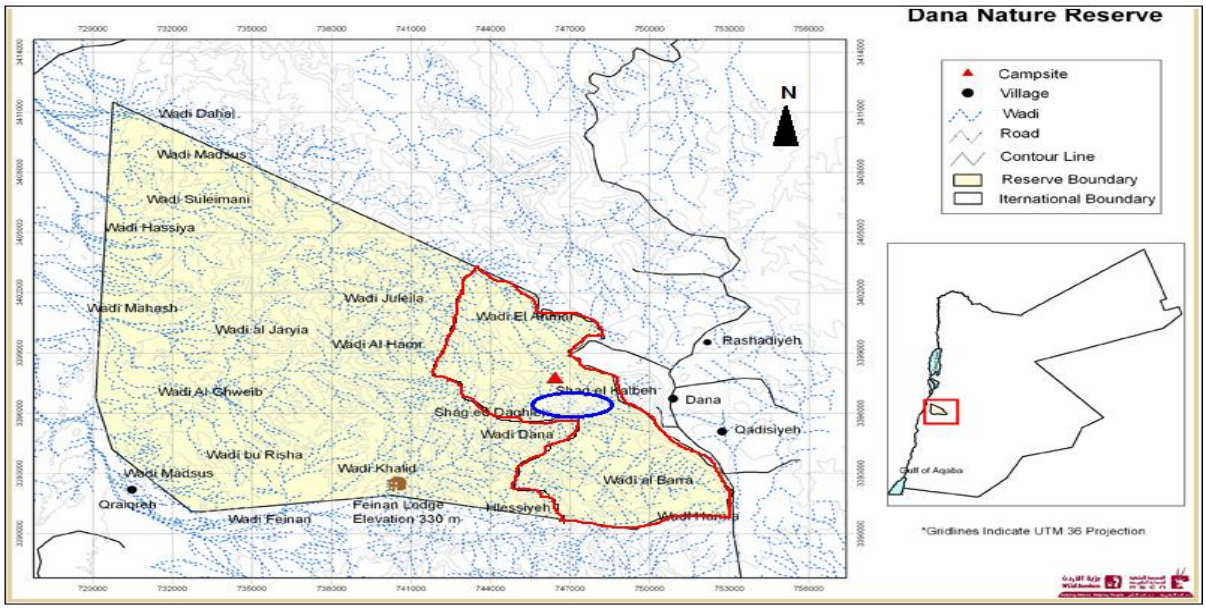

Fig. 1. Dana reserve map with area of study. Red lined area represent the reserve area with mediterranean climate and area with blue circle is the study area.

Soil electric conductivity and soil pH

Soil samples were collected using a soil auger. Four sub-samples were taken from the sample area at different soil depth (20cm, 40cm and 60cm) and mixed thoroughly to take representative soil sample then samples dried for three days.

10g of soil sample that collected was dissolved in 25 ml dH_2O and incubated at 25 °C with shaking at 150 rpm for 1h. The electric conductivity (EC) measured of the soil slurry using conductivity meter 4310 (JENWAY) and the pH readings was taken by using WTW pH 330i pH meter.

Dana reserve climate

Information related to soil type, temperature and rainfalls of the study area and Dana reserve were extracted from RSCN reports (2008). Typical Mediterranean soil was of the study area with most of it is calcareous soil. The eastern part of the reserve is characterized by semi –arid mediterranean climate with winter temperature reached to -10 °C and rainfall average around 100-350mm/ year and dry moderate summer. The western part of the reserve is characterized by dry desert climate (arid climate) with rainfall average around 50 mm/ year (RSCN, 2008).

Results

Area of study

Figure 1 represent the map of Dana biosphere reserve with the area lined with red color characterized with mediterranean climate and soil and the blue circle is the studied area that occupied with a mixture of *P. atlantica Desf.* and *J. phoenica L.*

Soil of the study area characterized with pH above 8.0 even in depth around 60 cm below the surface. Where the soil EC was also above 300 µS/cm reaching to 420 µS/cm at depth of 60 cm (Table 1).

Table 1. Soil electric conductivity (EC) and pH; Data were represent means ± SD, n=3.

Soil character Soil depth	EC (µS/cm)	pH
20cm	368±1.4	8.1±0.14
40cm	311.33±2.6	8.053±0.0094
60cm	420±0.47	8.03±0.008

Etymology

Colonies grow moderately rapidly on PDA, attaining 45-50 mm diam in 12 d at 25°C, at first whitish, later becoming gray, with abundant dark hyphal bundles in central part, reverse black (Fig. 2A).

The conidiophores varied from long, flexuous, and simple to short, geniculate, and branched. Conidia were spherical to ellipsoidal, dark brown, verrucose and many conidia were septate were seen (Fig. 2A & C).

Sequence analysis

Nucleotide-nucleotide BLAST (Megablast) search using the sequence of the 549 bp amplicon against the nr database of NCBI (www.ncbi.nlm.nih.gov) suggested the isolated strain was *Ulocladium sp.* VL204 with 99% significant alignments and the gene bank accession number is JF440622.1.

Table 2. Species, strains number and Sequences retrieved from GenBank accession numbers for the construction of phylogenetic tree. * indicate that unpuplished in journals.

Species	Strain number	GenBank accession No. (ITS)	Citation
Alternaria petroselini	EGS 09-159	AF229454.1	Pryor and Gilbertson, 2000
Alternaria smyrnii	EGS 37-093	AF229456.1	Pryor and Gilbertson, 2000
Ulocladium chartarum	ATCC 18044	AF229488.1	Pryor and Gilbertson, 2000
Alternaria alternata	EGS 34-016	AF347031.1	Pryor and Michailides, 2002
Coniothyrium palmarum	CBS 400.71	AY720708	Lennox *et al.*, 2004
Alternaria brassicae	RGT-S32	HQ674659.1	*
Ulocladium sp.	VL204	JF440622.1	*
Aureobasidium melanogenum	CBS 105.22	FJ150886.1	Zalar *et al.*, 2008
Edenia gomezpompae	CBS 124106	FJ839619	Crous *et al.*, 2009
Stagonosporopsis cucurbitacearum	CBS 233.52	EU167573.1	Simon *et al.*, 2009
Leptospora rubella	CPC 11006	DQ195780	Crous *et al.*, 2006
Ulocladium chartarum	U13-8	JQ585683.1	*
Ulocladium alternariae	BMP 31-41-05	AF229485.1	Pryor and Gilbertson, 2000
Ulocladium consortiale	U13-2	JQ585682.1	*

Phylogenetic analyses

The sequence analysis of the isolated fungus *Ulocladium sp.* VL204 was confirmed by phylogenetic tree constructed with the sequences of *Ulocladium sp.* VL204 and similar taxa retrieved by BLAST search (Table 2). The software MEGA version 4.0 (Tamura *et al.*, 2007) was used for the phylogenetic analyzing processing (neighbor joining method) (Fig. 3).

Discussion

Isolated *Ulocladium sp.* from *P. atlantica Desf.* leaves of Dana biosphere reserve considered the first reported study. The study was influenced by clear death of the apical part of the *Pistacia* trees and the

necrotic spots on the leaves in particular the older one.

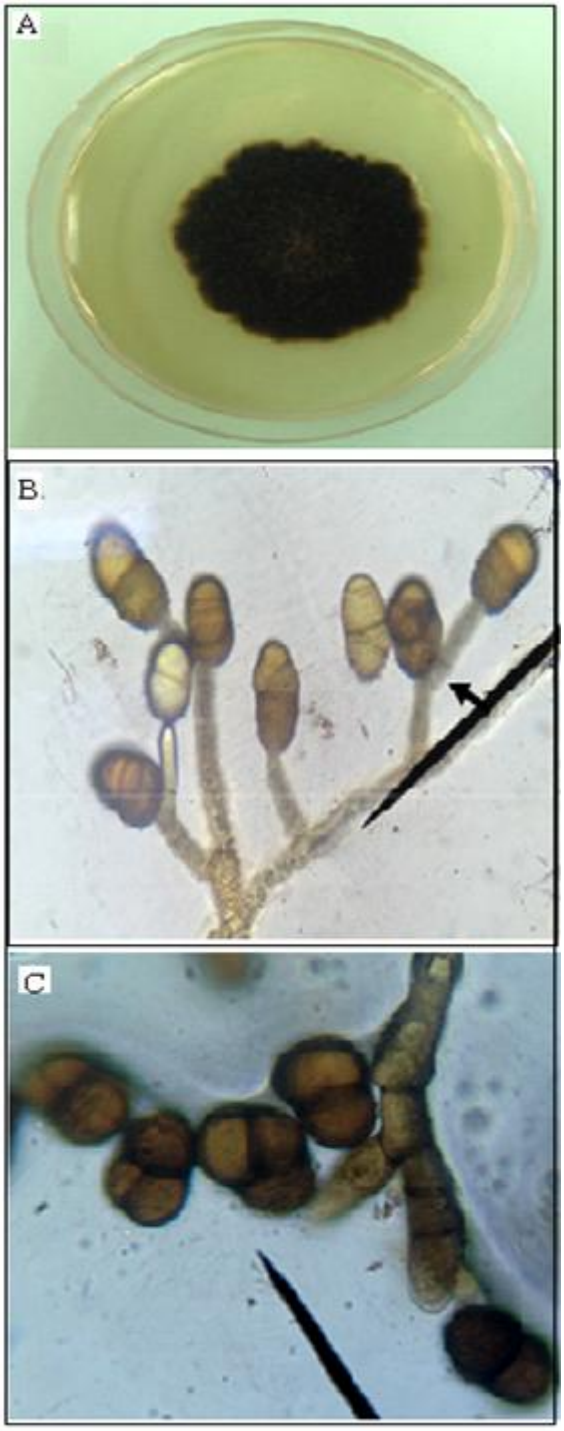

Fig. 2. A) *Ulocladium sp.* colony appearance on PDA after 12 days incubation at 25°C. B) Micrographs of the isolate. Flexuous conidiophores with conidia, secondary conidiophore (arrow) and conidia (magnification, 400). (C) Verrucose conidia. Septate conidium is indicated by an arrow.

Soil pH values at different depths showed the normal pH value of calcareous soil that forms most of Mediterranean soil of the study area that considered moderately alkaline, however, soils with pH as high as 8.0 are consider productive and are commonly planted (Sibbett, 1995). The EC values at levels far away from the salinity soil EC values that used as indirect indicator of water content and water-soluble nutrients available for plant uptake and also without affecting on soil microorganism activity which is vital for soil processes such as respiration, residue decomposition, nitrification, and denitrification (Adviento-Borbe *et al.*, 2006; Smith and Doran, 1996).

The phylogenetic analysis of the isolate fungus *Ulocladium sp.* VL204 after sequenced DNA internal transcribed spacer regions (ITS1-5.8S rDNA-ITS2) using genera of the same family indicates its very close to *Alternaria brassicae* strain RGT-S32 with high bootstrap, a pathogen that affect most cruciferous crops and influenced by climate with most effective in mild, wet seasons and in areas with relatively high rainfall (Humpherson-Jones and Phelps, 1989). The species grows in the vascular system and rapidly infects the entire plant (Valkonen and Koponen, 1990). However, also previous finding reported by Vannini and Vettraino, (2000) as result of wet summers, *U. chartarum* which is associated with high incidence of leaf spotting. However, low bootstrap was with *Coniothyrium palmarum* strain CBS 400.71 which is one of Pleosporales genera. Also the low bootstrap with other *Alternaria* sp.

Woudenberg *et al.*, (2013) delineated the phylogenetic lineages within *Alternaria* and allied genera, and created a robust taxonomy into 24 section based on nucleotide sequence data of parts of the 18S nrDNA, 28S nrDNA, ITS, GAPDH, RPB2 and TEF1-alpha gene regions. *A. brassicae* was not assigned to one of the 24 *Alternaria* sections and is treated as separate, single species and lineage. However, these study along with others as Runa *et al.*, (2009) improved that the include of other more informative loci may provide additional support for this resolution and also the use of metabolites

analysis (Andersen and Hollensted, 2008) to distinguish these related genera which do not always correlate to species-groups based upon morphological characteristics. Using strain *Aureobasidium melanogenum* strain CBS 105.22 sequence was chosen as out group which clear that it is not close to other genera by low bootstrap.

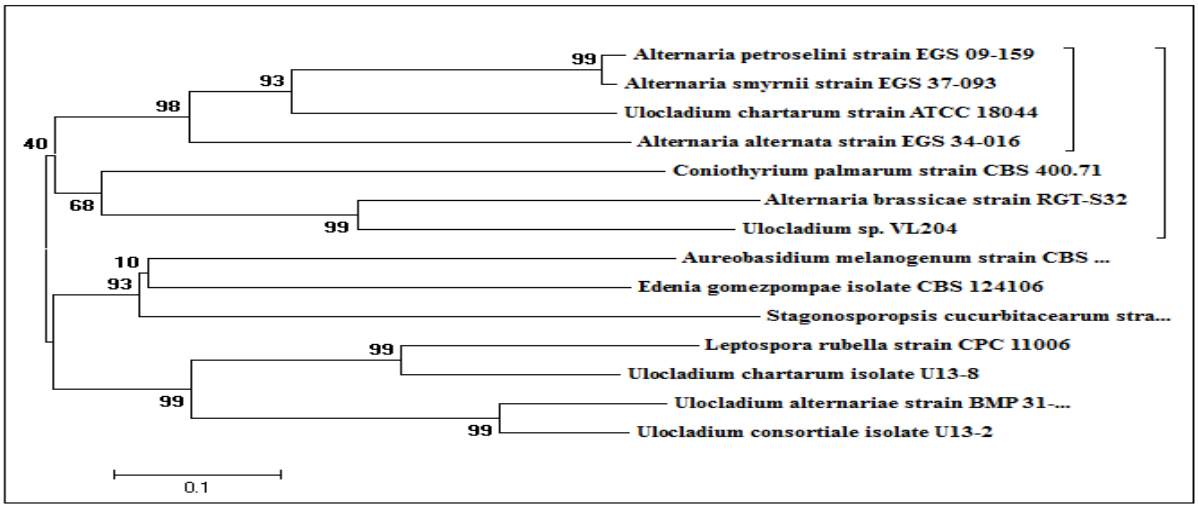

Fig. 3. The optimal Neighbor-Joining tree constructed using sequences of Pleosporales genera and that of *Ulocladium* sp. VL204.

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