

# Identification and Occurrence of *Trichoderma harzianum* Associated with Cork Oak in Tunisia

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## ABSTRACT

**Yangui, I., Zouaoui Boutiti, M., Hlaiem, S., Vettraino, A.M., Vannini, A., Ben Jamaâ, M.L., and Messaoud, C. 2018. Identification and occurrence of *Trichoderma harzianum* associated with cork oak in Tunisia. Tunisian Journal of Plant Protection 13 (si): 39-48.**

*Trichoderma harzianum* is an endophyte fungus of considerable interest because of its effectiveness as a biocontrol agent against various plant pathogenic fungi. In this study, *T. harzianum* was isolated from cork oak trees in three forests in northwest Tunisia. Initially, the fungal characterization was carried out based on macroscopic and microscopic features. Sequencing of the internal transcribed spacers 1 and 2 of the DNAr was carried out to confirm fungus identification at the species level. The aims of this work were to study the occurrence of *T. harzianum*, to understand its relationship with the host plant, and to quantitatively investigate its distribution on the different organs of cork oak trees across three sites (Babouch, Ain snoussi, Ain zana). *T. harzianum* frequency varied significantly ( $P < 0.001$ ) among the surveyed forests. The fungus was more common at Babouch forest and was rarely encountered at Ain zana. Correlation analysis was used to determine the relationship between the dendrometric parameters, the phytosanitary status of the investigated trees and the abundance of *T. harzianum*. The results showed a significant and positive correlation between the fungus frequency and the tree height. A negative and significant correlation was noted between the trees' chlorosis index and fungus abundance. These findings may afford a contribution to the knowledge of *T. harzianum* in Tunisian forests and its relationship with cork oak trees which could help to develop control strategies using *Trichoderma* strains.

**Keywords:** Cork oak, morphological and molecular identification, occurrence, *Trichoderma harzianum*, Tunisia

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Species of the genus *Trichoderma* are filamentous fungi that inhabit diverse environments. They are generally abundant in soils, roots, and woods. It is

well known that many *Trichoderma* species are endowed with high ability to establish various heterotrophic interactions with other organisms such as decomposition, parasitism, and opportunistic endophytism (Druzhinina et al. 2006; Zeilinger et al. 2016). Due to their antagonistic properties against a wide range of bacteria, yeasts, and fungi,

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*Trichoderma* strains are able to survive in a highly competitive environment with other microorganisms (Reino et al. 2008). Dominance of *Trichoderma* species is mainly due to their ability to synthesize a wide array of secondary metabolites, such as terpenoids, steroids and pyrones. Bioactive products of *Trichoderma* species have great potential for a variety of applications in agriculture for the biocontrol of various phytopathogens and for the enhancement of plant growth (Mukherjee et al. 2013; Zeilinger et al. 2016). Currently, they are largely applied as bio-fungicides to prevent several plant diseases (Kumar et al. 2014).

*T. harzianum* is the most common species of the genus. It is frequently applied on field and greenhouses as a biocontrol agent against plant pathogens (Chaverri et al. 2002). In Tunisia, many studies have been conducted about local *T. harzianum* colonizing soils (Ayed et al. 2006; Sadfi-Zouaoui et al. 2009). However, little data are available about its occurrence on cork oak (*Quercus suber*) trees in Tunisia.

In this paper, we identified a *Trichoderma* species isolated from cork oak and we investigated its distribution in three Tunisian forests and its influence on the phytosanitary status of the trees. A better understanding of the relationship

between *Trichoderma* and cork oak trees may lead to further develop new strategies to fight against cork oak diseases.

## MATERIALS AND METHODS

### Study sites and tree sampling.

In 2016, a survey was carried out in cork oak stands of the northern west of Tunisia in order to evaluate the phytosanitary status of the trees in relation with the occurrence of *Trichoderma* species. Three sites were selected in the main Tunisian cork oak forests at different altitudes: Babouch (36°835'N, 8°709'E - 207m), Ain snoussi (36°86'N, 9°014'E - 520.3m), Ain zana (36°16' N, 8°24' E - 924m) (Table 1). At each site, samples were collected from the different organs of ten cork oak trees and dendrometric parameters were noted. A distance of at least 300 m was kept between two trees in order to obtain a representative sampling for each site. Chlorosis index, defoliation index and necrosis index were noted for each tree. A categorical scale ranging from 0 (no symptoms) to 5 (very severe symptoms) was used for each parameter. Four parameters: trunk exudations (1 = presence / 0 = absence), number of cracks, number of cavities and number of cankers were also noted for trunks.

**Table 1.** Ecological parameters of the investigated cork oak sites

Site	Longitude (E)	Latitude (N)	Altitude (m)	Rainfall (mm)	Temperature (°C)
Babouch	8°709'	36°835'	207	950	20
Ain snoussi	9°014'	36°86'	520,3	1222	16
Ain zana	8°24'	36°16'	924	1780	20

### Collection of isolates.

Collected samples were soaked in alcohol (70%). Fragments of 1 mm<sup>2</sup> in size were cut from each sample and placed in Petri dishes poured with potato dextrose agar (PDA) medium. A total of 203

*Trichoderma* isolates was selected based on their morphological characteristics. *Trichoderma* isolates were grouped into morphotypes and the dominant one was proposed to be studied.

### Morphological identification.

The dominant morphotype was preliminarily identified according to the morphological characters and the microscopic traits as reported by Samuels et al. (2002). Conidia measurements were taken from colonies after a week of incubation in darkness at 25°C. One representative isolate of the dominant morphotype was selected for molecular identification.

### DNA extraction and molecular identification.

Mycelial plugs of the *Trichoderma* isolate were collected from the margin of a PDA culture and transferred into Eppendorf tubes containing 500 µl of potato dextrose broth (PDB) with three replications. PDB cultures were incubated in darkness at 25°C for 7 days. The DNA extraction was performed from the PDB cultures as described by Cenis et al. (1992). The ITS region of the nuclear rDNA gene of the *Trichoderma* isolate was amplified using primers ITS1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). PCR amplification was carried out in a volume of 25 µl containing 1 µl of DNA (100 ng), 1 µl of each primer (1:10), 12.5 µl of MyTaq HS Mix (2X) (Bioline, UK) and 9.5 µl of water using a PCR program comprised of an initial denaturation at 95°C for 1 min followed by 34 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final extension at 72°C for 7 min. The PCR products were visualized by electrophoresis on a 1.5% agarose gel containing 1% of GelRed, then purified by the Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). The resulting amplicons were sequenced (Eurofins, Italy) and a basic local alignment search tool (BLAST,

<http://www.ncbi.nlm.nih.gov/>) was used for sequence similarity searches.

### Isolation frequency.

The isolation frequency of *Trichoderma* isolates (%) from the different organs was calculated using the following formula: IF (%) =  $100 \times (\text{Ni}/\text{Nt})$ , where Ni and Nt are the number of segments colonized by the fungus and the total number of examined segments, respectively (Franceschini et al. 2005).

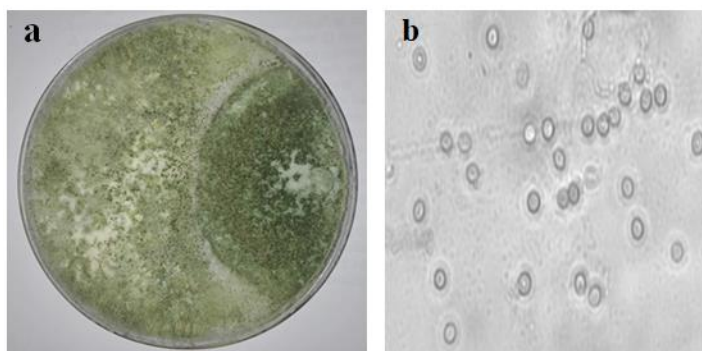
### Data analysis.

The significance of the variation in the isolation frequency of *T. harzianum* among sites or organs was determined using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using SAS version 9. Correlation analysis between the *T. harzianum* frequency, the dendrometric parameters and the phytosanitary variables was carried out using SAS.

## RESULTS

### Morphological and molecular identification.

According to the phenotypic traits of colonies and their micro-morphology, *Trichoderma* isolates, belonging to the dominant morphotype, probably belonged to *T. harzianum*. In fact, colonies grown on PDA medium were floccose with effuse conidiation covering the entire surface of the plate. Colonies grew very rapidly and reached the edge of the plate after three days of incubation. Conidia were globous and 2.4 to 2.6 µm in diameter. They began yellow and became green to dark green after one week. The conidial production started after less than 48 h. The macroscopic and microscopic characteristics of a representative isolate are illustrated in Fig. 1.



**Fig. 1.** Morphological characteristics of *Trichoderma harzianum*, a: Colony grown on PDA medium after 7 days of incubation in darkness at 25°C, b: Conidia (40×).

The BLAST searches of the obtained ITS-rDNA sequence resulted in 100% homology with *T. harzianum* isolate 153L (Accession No KF889067) described by Dawidziuk et al. (2014), and nineteen other *T. harzianum* accessions. The obtained sequence was submitted to GenBank and assigned the following accession number: MG675027.

### Site differences as measured by dendrometric parameters.

Differences in the total tree height (m) were highly significant ( $F = 30.51$ ,  $P < 0.0001$ ) among the investigated sites. Babouch was characterized by the tallest trees ( $12.1 \pm 0.87$  m). However, the trunk height (m), the trunk circumference (m) and the crown width (m) did not differ significantly between surveyed sites (Table 2).

**Table 2.** Dendrometric parameters of the investigated cork oak sites

Site	Total height (m)	Trunk height (m)	Trunk circumference (m)	Crown width (m)
Babouch	$12.1 \pm 0.87$ a	$2.77 \pm 0.28$ a	$1.35 \pm 0.17$ a	$4.38 \pm 0.19$ a
Ain snoussi	$8.2 \pm 0.30$ b	$2.69 \pm 0.21$ a	$1.29 \pm 0.15$ ab	$4.96 \pm 0.46$ a
Ain zana	$5.99 \pm 0.28$ c	$2.21 \pm 0.25$ a	$1.05 \pm 0.12$ b	$3.97 \pm 0.28$ a

For each parameter, values followed by the same letters are not significantly different based on Duncan's Multiple Range test at  $P < 0.05$ .

Among the studied phytosanitary parameters, the chlorosis index showed high significant differences among the

investigated sites ( $F = 98.28$ ,  $P < 0.0001$ ) (Table 3).

**Table 3.** Phytosanitary parameters of the investigated cork oak sites

Phytosanitary parameter	Babouch	Ain snoussi	Ain zana
Chlorosis index	0 ± 0.00 c	1.9 ± 0.1 a	0.8 ± 0.1b
Defoliation index	1.9 ± 0.38 a	1.7 ± 0.3 a	2 ± 0.36 a
Necrosis index	2.8 ± 0.18 a	2.2 ± 0.2 a	2 ± 0.39 a
Trunk exudations	0.7 ± 0.14 a	1 ± 0.00 a	0.3 ± 0.15 a
Number of cracks	0 ± 0.00 b	24.5 ± 5.39 a	0 ± 0.00 b
Number of cavities	0 ± 0.00 b	14 ± 6.18 a	2.7 ± 1.7 b
Number of cankers	1.3 ± 0.5 a	2.1 ± 0.4 a	3 ± 0.15 a

For each parameter, values followed by the same letters are not significantly different based on Duncan's Multiple Range test at  $P < 0.05$ .

### Incidence of *T. harzianum*.

The distribution of *T. harzianum* varied significantly among sites. Babouch exhibited the highest isolation frequency in all the organs ( $18.4 \pm 8.04\%$  in branches,  $14 \pm 1.63\%$  in leaves and  $3 \pm$

$1.52\%$  in trunks). However, Ain zana showed a very low frequency ( $1.6 \pm 1.06\%$ ) of *T. harzianum* in branches and a total absence in leaves and trunks (Table 4).

**Table 4.** Isolation frequency of *Trichoderma harzianum* (%)

Site	Branches	Leaves	Trunk
Babouch	$18.4 \pm 8.04$ a	$14 \pm 1.63$ a	$3 \pm 1.52$ a
Ain snoussi	$6.9 \pm 2.02$ b	$9 \pm 2.02$ b	$0 \pm 0.00$ b
Ain zana	$1.6 \pm 1.06$ c	$0 \pm 0.00$ c	$0 \pm 0.00$ b

Within each column, values followed by the same letters are not significantly different based on Duncan's Multiple Range test at  $P < 0.05$ .

Correlation analysis showed that the presence of *T. harzianum* in trunks and leaves was found to be significantly correlated to the increase in tree height and circumference. The high frequency of

*T. harzianum* appeared to be negatively correlated to the chlorosis index. However, its incidence on cork oak trees was found to be positively correlated to the degree of leaf drying (Table 5).

**Table 5.** Correlations between *Trichoderma harzianum* infestation and the dendrometric and the phytosanitary parameters

Predictor variable	Correlation coefficient (r)	Significance probability (P)
Trunk height (m)	0.715**	<.0001
Trunk circumference (m)	0.409	0.025
Crown width (m)	0.225	0.231
Chlorosis index	-0.361*	0.05
Defoliation index	0.245	0.192
Necrosis index	0.245	0.192
Trunk exudations	-0.236	0.210
Number of cracks	-0.183	0.333
Number of cavities	-0.139	0.462
Number of cankers	-0.306	0.100

\*\* highly significant at  $P \leq 0.01$ ; \* significant at  $P \leq 0.05$ .

The defoliation and the necrosis indexes were not significantly affected by the *T. harzianum* frequency. Furthermore, all parameters indicating the phytosanitary status of the trunks were not significantly influenced by the increase in *T. harzianum* incidence (Table 5).

## DISCUSSION

Morphological characterization approach is considered as a potential method to identify *Trichoderma* species (Anees et al. 2010; Gams and Bissett 2002). In our study, the selected morphotype was identified as *T. harzianum* according to colony appearance, growth rate and conidia size. In fact, the small conidia size of *T. harzianum* isolates and their capacity to grow and sporulate at 35°C are the main features that distinguish them from the other *Trichoderma* species (Samuel et al. 2002). However, only colony appearance

may not provide enough information to identify the species due to the difficulty to establish an exact description of the colony (Gams and Bissett 2002).

Since the distinguishing morphological features of a fungus are usually too limited to allow its identification, molecular techniques are required. The sequencing of the ITS1-5.8s-ITS2 region of the rDNA remains one of the most reliable methods for the identification of fungi at the species level (Kullnig-Gradinger et al. 2002). In this study, by comparing the obtained sequence of the ITS region to the sequences deposited in GenBank database, the representative isolate was confirmed as *T. harzianum* with homology percentage of 100%.

The second aim of this study was to determine the distribution of *T. harzianum* species on cork oaks in three different sites. Babouch forest, characterized by the tallest trees, revealed

the highest frequencies of *T. harzianum*. Based on correlation analysis, the tree height and the trunk circumference were correlated to *T. harzianum* frequency. Indeed, it was demonstrated in recent studies that *Trichoderma* species may influence the plant development by several mechanisms such as production of growth hormones (Chowdappa et al. 2013). Furthermore, *Trichoderma* species are known to induce large changes in the plant proteome by stimulating unregulated proteins which are involved in carbohydrate metabolism and/or in photosynthesis. These proteins enhance respiratory and photosynthetic rates which may lead to an increase in the plant growth (Shores and Harman 2008). Moreover, *Trichoderma* species participate in increasing the availability of essential elements for plants by mineralization of organic matter (Haque et al. 2012, Mukherjee et al. 2012).

Considering the phytosanitary situation of leaves, a significant difference in chlorosis index was noted between the investigated sites. Babouch forest, characterized by the highest frequency of *T. harzianum*, exhibited the lowest chlorosis index. Indeed, chlorosis is a deficiency disease due to an insufficient chlorophyll production, often as a result of a nutrient deficiency or infections by plant pathogens such as viruses, bacteria or fungi (Hale et al. 1946). In this study, the correlation analysis showed a negative correlation between the chlorosis index and *T. harzianum* infestation. Accordingly, we suggested that *T. harzianum* species may avoid chlorosis by increasing the solubility of nutrients like phosphate, zinc and iron and by inhibiting a large diversity of plant pathogens. Thus, our study supports the consideration that *Trichoderma* species are able to act as biocontrol agents by producing defense

related compounds which may enhance the plant resistance (Harman et al. 2004, Smolińska et al. 2007, Yedidia et al. 2004).

In this context, several previous studies have proved the antifungal potential of *Trichoderma* species against various plant pathogens such as *Fusarium* spp., *Alternaria* spp., *Rhizoctonia* spp., *Phytophthora* spp., *Pythium* spp., and *Sclerotinia* spp. The antifungal activity of *Trichoderma* was found to be attributed to their ability to produce a wide range of antibiotics and lytic enzymes (Toghue et al. 2016, You et al. 2016). This may explain the frequent use of *Trichoderma*-based formulations as commercial biocontrol agents for foliar application to treat fungal diseases (Oros and Naár 2017). Nevertheless, correlation analysis did not show any relationship between the phytosanitary situation of the trunks and *Trichoderma* infestation. This suggests that *T. harzianum* species may not be effective in controlling trunk damages which may probably due to infection by wood decay fungi such as *Armillaria* and *Xylaria*. This result supports the idea that most of *Trichoderma* species are more effective for controlling some pathogens, but may be largely inefficient against others (Pratella and Mari 1993). Furthermore, the bioactive agents of *Trichoderma* strains are living organisms and their activities depend heavily on several factors (Kaur et al. 2005; Li et al. 2005). Accordingly, the effective protection exerted by *Trichoderma* strains against pathogens is often unpredictable. As an overall conclusion, our study showed an eventual positive effect of *T. harzianum* species on the development of the tree and its resistance against some plant pathogens. However, further studies are required in order to conclude a causal association between *Trichoderma* species and the phytosanitary status of the host

plant. Other studies on the bioactive compounds of *Trichoderma* species are also necessary to a better understanding of their antagonistic interactions and mechanisms of actions.

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#### RESUME

**Yangui I., Zouaoui Boutiti M., Hlaïem S., Vettraino A.M., Vannini A., Ben Jamaâ M.L. et Messaoud C. 2018. Identification et occurrence de *Trichoderma harzianum* associé au chêne-liège en Tunisie. *Tunisian Journal of Plant Protection* 13 (si): 39-48.**

*Trichoderma harzianum* est un champignon endophyte d'un intérêt considérable en raison de son efficacité en tant qu'agent de lutte biologique contre divers champignons phytopathogènes. Dans cette étude, *T. harzianum* a été isolé à partir des arbres de chêne-liège du nord-ouest de la Tunisie. Initialement, la caractérisation fongique a été réalisée sur la base des caractéristiques macroscopiques et microscopiques. Le séquençage des espaceurs transcrits internes 1 et 2 de l'ADNr a été effectué pour confirmer l'identification du champignon au niveau de l'espèce. Les objectifs de ce travail étaient d'étudier l'occurrence de *T. harzianum*, de comprendre sa relation avec la plante hôte et d'étudier quantitativement sa distribution dans les différents organes des arbres de chêne-liège dans trois forêts (Babouch, Ain snoussi et Ain zana). La fréquence de *T. harzianum* a varié significativement ( $P < 0,001$ ) selon les forêts étudiées. Le champignon a été plus abondant dans la forêt de Babouch et très rare dans la forêt de Ain zana. L'analyse de la corrélation a été utilisée pour déterminer la relation entre les paramètres dendrométriques et l'état phytosanitaire des arbres étudiés et l'abondance de *T. harzianum*. Les résultats ont montré une corrélation significative et positive entre la fréquence du champignon et la hauteur des arbres. Une corrélation négative et significative a été notée entre l'indice de jaunissement des feuilles et l'abondance de *T. harzianum*. Ces résultats peuvent contribuer à la connaissance de *T. harzianum* dans les forêts tunisiennes et à sa relation avec les arbres de chêne-liège, ce qui pourrait aider à développer des stratégies de lutte en utilisant des souches de *Trichoderma*.

**Mots clés:** Chêne-liège, identification moléculaire et morphologique, occurrence *Trichoderma harzianum*, Tunisie

#### ملخص

اليانقي، إسلام ومريم زواوي بوتيتي وسوسن حليم وأنا ماريافترينو وأندريا فنيني ومحمد لحبيب بن جامع وشكري مسعود. تشخيص وانتشار الفطر *Trichoderma harzianum* المقترن ببوط الفلين (الفرنان) في تونس.

**Tunisian Journal of Plant Protection 13 (si): 39-48.**

الفطر *Trichoderma harzianum* هو فطر داخلي ذو أهمية كبيرة بسبب فعاليته كعامل مكافحة بيولوجية ضد كثير من الفطريات الضارة. في هذه الدراسة، تم عزل الفطر من أشجار بلوط الفلين (الفرنان) المتواجدة في الشمال الغربي للبلاد التونسية. في مرحلة أولى، تم تشخيص الفطر وفقا لخصائصه المضمخة والمجهريّة. تم التأكد من النوع الفطري باستعمال تسلسل الفواصل الداخلية 1 و 2 للحمض النووي. تتمثل أهداف هذه الدراسة في التحقيق في تواجد الفطر ودراسة العلاقة بينه وبين شجرة الفرنان ودراسة توزيعه الكمي على مختلف أعضاء الشجرة ثلاث غابات (ببوش وعين السنوسي وعين الزانة). تفاوت توزيع الفطر بشكل كبير بين الغابات. كان أكثر شيوعا في غابة ببوش ونادرا جدا في عين الزانة. تم استعمال تحليل الترابط لتحديد العلاقات بين مقاييس الخصائص الفيزيائية للأشجار وحالتهم الصحية ومدى انتشار الفطر. بينت النتائج وجود علاقة ايجابية بين علو الأشجار وانتشار الفطر. كما أثبتت النتائج وجود علاقة عكسية وهامة بين درجة اصفرار الأوراق ووفرة الفطر. يمكن لهذه النتائج أن تساهم في التعرف على هذا الفطر وعلى حقيقة علاقته بشجرة الفرنان مما يساعد على وضع استراتيجيات السيطرة على مسببات الأمراض وذلك باستخدام عزلات من هذا الفطر.



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