Discovering the environmental factors affecting the distribution of *Terfezia claveryi* Chatin in the Northwest of the Region of Murcia

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Master thesis:

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Abstract:

Terfezia claveryi is hypogeous fungi belonging to the family Pezizaceae. It is a widespread fungus in the arid and semi-arid soils, particularly in the Mediterranean region and Northern African countries. In Murcia, South-Eastern Spain, Particularly at (Provenicia Castellano-Maestrazgo-Manchega) where the study area taking place. Sampled points identified for sampling counted 31 samples. From the total sampled points 87% has comprised the host plants (*Helianthamum* and *Fuman* spp). Real-time PCR, was used to quantify and examines the presence or not of *T.claveryi*. qPCR soil and root analysis showed the presence of *T.claveryi* mycelium 34% and 24% of the samples accordingly. Thus, the amount of mycelium was varied between 0.04 and 0.52 µg/g in soil samples and 0.91 and 1.67 µg/g in root. Testing the results of qPCR against the environmental factors, it displayed a significant correlation with altitude and both host plants ($p=0.032$, $r=-0.45$). Whereas the precipitation has a significant correlation with *Helianthamum spp* ($p=0.028$, $r=0.81$), and *Fumana* spp ($p=0.021$, $r=0.88$). Besides, two types of soil were determined at the study area, fluvent, and xerolls, where the majority of *T.claveryi* mycelium root presence in the fluvent soil type. Finally, a spatial map analysis was made in order to determine the spread and the density of *T.claveryi* associated with the observed ecosystem of the study area showed a majority of *T.claveryi* mycelium in soil with the shrubland ecosystem at the expense of other ecosystems.

1. Introduction

1.1. Importance of fungi in Europe

People have been gathering fungi since ancient times. Currently, wild fungi provide a wide of utilities to people around the world. In ancient Greek and Roman times, edible fungi were already valued by the upper class[1]. Southern European particularly (France and Italy), and Eastern European countries value fungi. They had a strong and long tradition uses of it. However, Northern and Western Europe had much weaker tradition of collecting fungi. Thus, fungi were feared for consumption at the ancient time.

In modern Europe, the distinction between mycophilic and mycophobic countries becomes less and less clear. In addition, interest in gathering of fungi is steadily increasing across Europe. Although main reason of such shift is fungi's commercial value, influence of immigrants from fungi loving cultures has also made its contribution (Brainerd, & Doornbos, 2013).

In Spain, numerous of mycological societies were established, like Spanish Association of Mycology (AEM), Mycological Society Barakaldo, and Catalan Society of Micology[2]. Thanks to the great and the increasing interest in the collection of fungi. Such events, as field trips, workshops gastronomical meetings, which were focused on fungi, were organized every autumn. It is obvious that the collection and marketing of this non-wood forest product is an enjoyable and profitable task, which falls within the concept of sustainable development. Moreover, it can be an extremely important source of income in rural areas with few other economic possibilities (Roman & Boa 2004).

1.2. What is a desert truffle?

Desert truffles comprise a group of mycorrhizal fungi appreciated for their edible hypogeous carpofores. They include species from genera *Tuber*, *Tirmania*, and *Terfezia*

[1] Buller AHR. The fungus lores of the Greeks and Romans. Transactions of the British Mycological Society 1914; 5: 21 – 66.
[2] https://aemicol.com/, http://micologica-barakaldo.org/, http://www.micocat.org/

(Morte *et al.* 2017). Various species of these truffles are common in the Mediterranean area and considered as an important economic resource for the local population (Zotti *et al.* 2013). Desert truffles have been associated with Mediterranean cultures since ancient times. They have been traded by the Greek and Romans. Also, they imported them from Libya to be sold in the markets of the respective empires (Honrubia et al. 2007). Nowadays, desert truffles still being marketed and consumed in North Africa and southern Europe.

Among desert truffle, the genus *Terfezia* includes the most appreciated and marketed species. The genus compresses more than 20 different species (http://www.indexfungorum.org), of which only *Terfezia arenaria* (Moris) Trappe and *Terfezia claveryi* Chatin are commercially valued in Spain because of their gastronomic interest and crop yields. Two other species*, Terfezia boudieri* Chatin and *Terfezia olbiensis* Tul., which are harvested for consumption purposes, although they have a lower commercial impact. The reason is, they have a poorer taste than the other species, and thus, they have limited presence (Gutiérrez *et al*. 2001).

In general, desert truffles have a good fruit sizes and quantities. Due to environmental compatibility, wild *Terfezia,* is more collected and marketed in southern Europe. Likewise, North Africa and other countries bordering the Mediterranean Sea. However, areas where desert truffles grow naturally have gradually disappeared. Large areas of the coastal desert in Egypt and Libya were mined during the World War II. Besides, in Kuwait, the effects of the 1990-1991 Gulf War have apparently ruined many truffle-gathering areas. Whereas, the reason in Europe was the widespread of constructions. It played a critical role in preventing and occupying the 'sunny' areas over the last years (Morte *et al*. 2008).

In the Arabic countries desert truffles are calling 'Terfass','Terfess'. It is believed that the current name 'Terfezia' is coming from these names. In addition, it is known as 'sand truffles' (Khabar et al. 2001). *Helianthemum* genus (*Cistaceae* family) is considered as the most common host plants of *Terfezia*, forming mycorrhizal symbiosis.

Terfezia species fructify in the spring. However, its fruiting starts once the host plant finished flowering. So that, the production time might fluctuated according to the early or delaying of the host plant flowering (Morte *et al*. 2009).

1.3. *Tefezia claveryi* distribution.

T. claveryi is widely distributed in the arid and semiarid lands, particularly, in the Mediterranean Sea countries. It is found in Central and South-Eastern Spain, Portugal, Italy, France, Hungary, Turkey. In addition, in the North African countries from Morocco to Egypt and Syria can be found, besides, the Arabian Peninsula, Iraq, Kuwait and Iran (Marasas and Trappe 1973).

In the Iberian Peninsula, it is outspread in southern, south-eastern and central areas up to around 1.100 m a.s.l. We could find it in, carbonated and clayey soils, or in sandy soils on the coast (Honrubia, 2007).

In terms of the ecological value, *T. claveryi* has an important role due to its adaptation to grow in arid and semiarid zones in a symbiotic ectendomycorrhizal association with annual and perennial species of *Helianthemum spp.*, including chamaephytes, hemicryptophytes and therophytes, which are located in sunny scrubland, or in the meadows of mountain plains.

1.4. *T. claveryi* economical value

Thanks to the value of desert truffle, there was an interest to establish several studies for planting it. Plantation was taking place in several countries. Mediterranean basin countries Middle East, Iran, the Arabian Peninsula, Persian Gulf, Southern Africa and South American countries such as Chile and Argentina, where desert ecosystems cover large areas, are suitable places for it cultivations. It is believed that, desert truffle cultivation in these countries could might play an important role in developing the rural areas (Honrubia and Andrino 2014).

In Murcia (Spain), the first plantation of *Terfezia* mycorrhizal took place in 1999. It considers as the first successful plantation. Since then, thanks to the increasing demand

on desert truffle major studies has promoted including a new biotechnological strategies to satisfy the demand of shifting the plantation scale from experimental scale to a medium-large cultivation scale. By applying a good management and selecting productive mycorrhizal seedlings for different cultivation sites, it was possible to maintain a good productivity over time. Most of the host plants used for experimental desert truffle mycorrhization are perennial *Helianthemum* species (Morte & Andrino 2014).

Prices for *T. claveryi* in the Spanish market are similar to and range between 20 and 60 €/kg, depending on the natural production and the geographical region. This truffle is an important resource for Spanish collectors, who usually sell them to restaurants and in local markets. At the national level, its market is mostly local. However, this product is in a great demand in international markets. In the Middle East, such as, the United Arab Emirates, Kuwait, Saudi Arabia or Qatar, where they are highly valued and can reach prices up to 220 €/kg. Recently, national cultivators of *T. claveryi* are establishing pre-agreements with importers from Middle Eastern countries. Although it is a young product in terms of its commercialization, it has great potential for international export (A. Morte personnel communication).

As consequences, the research group at the University of Murcia initiated a term for *Terfezia* cultivation so-called 'turmiculture'. This term means the set of techniques and knowledge to cultivate truffles and the part of the primary sector dedicated to it; the term includes the different works of mycorrhized plant production, soil treatment, grassland cultivation and harvesting (Morte *et al.* 2008, 2009, and 2012, 2017).

1.5. The biotechnology role in developing *T.claveryi*

For the establishment of new *Terfezia* plantations in semi-arid lands, global climatic change and increasing global warming must be considered. Indeed, the recent climatic changes, with increasing mean temperatures and decreasing precipitations are affecting truffle production in Mediterranean areas (Büntgen *et al.* 2012). Anyway, correct planning and management choices are available and high yields can be achieved (Bencivenga & Baciarelli-Falini 2012).

Although the Mediterranean area exhibits a high diversity in hypogeous mushrooms, including some species of great economic importance where these truffles are essential for plant survival in arid and semi-arid climates, contributing to maintenance of diverse ecosystem. Climatic changes make Mediterranean conditions extreme with an increase of mean temperatures and a decrease in rainfalls until the end of the twenty-first century (Büntgen *et al.* 2012).

In the Middle East, hyper-arid, arid, and semi-arid areas constitute 90% of total surface. Every year, 60,000 ha become uncultivable due to the lack of respect for environment, delays in territory improvement actions, and bad utilization of agricultural soils. In this poor agricultural contest, desert truffles constitute an important economical resource, which can contribute to the development of arid areas to control desertification with proper soil management (Zambonelli et al. 2014).

As consequences, in Murcia, a research group in the University of Murcia has carried out a study. By using the GIS multivariate system. To simulate a map clarifying the distribution of the host plants. They interested in building up a distribution map. Showing the desert truffle potential areas. Associated with *Helianthemum* species, as a host plants. Besides, considering the climatic variances that corresponding with the growing of *T.claveryi.* (Honrubia et al. 2014).

It is important to know real distribution map of *T. claveryi* in the region of Murcia (Spain) and the environmental factors affecting its distribution. Besides, there is no yet any real distribution map giving information about the host plant distribution and density (*Helianthamum* or *Fumana*), which is implying the most prospective places to find wild *T.claveryi* or not.

The aim of the following work is to determine the distribution of *T. claveryi* in the region of Murcia and to create a potential map of "desert truffle resource" and to understand which environmental factor could limiting that distribution.

2. Materials and methods

2.1. Samples collection

The study took place in Murcia (South-Eastern Spain). The study area comprised the whole bioclimatic Province Castellano-Maestrazgo-Manchega" (figure1) (Alcaraz et al. 2008). According to the previous experience of the hosting group, this area was recorded several natural production points of *T. claveryi.* In addition, The province included some of the most productive man-planted plots of *T. claveryi*. Thus, according to the previous prediction study made by Honrubia et al. (2014) by GIS, it was one of the most promising bioclimatic area.

In order to assign the sampling plots, Google Earth Pro were used and a 10 km square grid has been created over the study area. Sampling points have been assigned at the closest road to the intersection between the horizontal and vertical line on the map. Besides that, the previous study, which predicted a distribution map of the desert truffle potential areas associated with the presence of different *Helianthemum* species that was carried out using a GIS multivariate system was considered (Honrubia *et al*. 2014) (figure 2).

The coordinates corresponding with these points were retrieved and shown in Table 1. Sampling collection was carried out from 16th of April until 25th of April 2018, which corresponds to the fruiting season for *T.claveryi* (April-May).

A total of 31 sample plots were set. From each sampling plot the presence or absence of any host plant was recorded (*Helianthemum* and/or *Fumana* spp). The principle was to collect from each plot about two to four plants (root with soil samples). Collected samples were transported in refrigerated bag and stored at 4°C afterwards. Eventually, during the way, while moving from sampling plot to another. Once the existence of a high density of host plants was observed. It was recorded (as coordinates).

Figure 1. Murcia map. Adopted from Alcaraz et al. (2008) http://www.floraprotegida.es/introduccion-flora-protegida.php

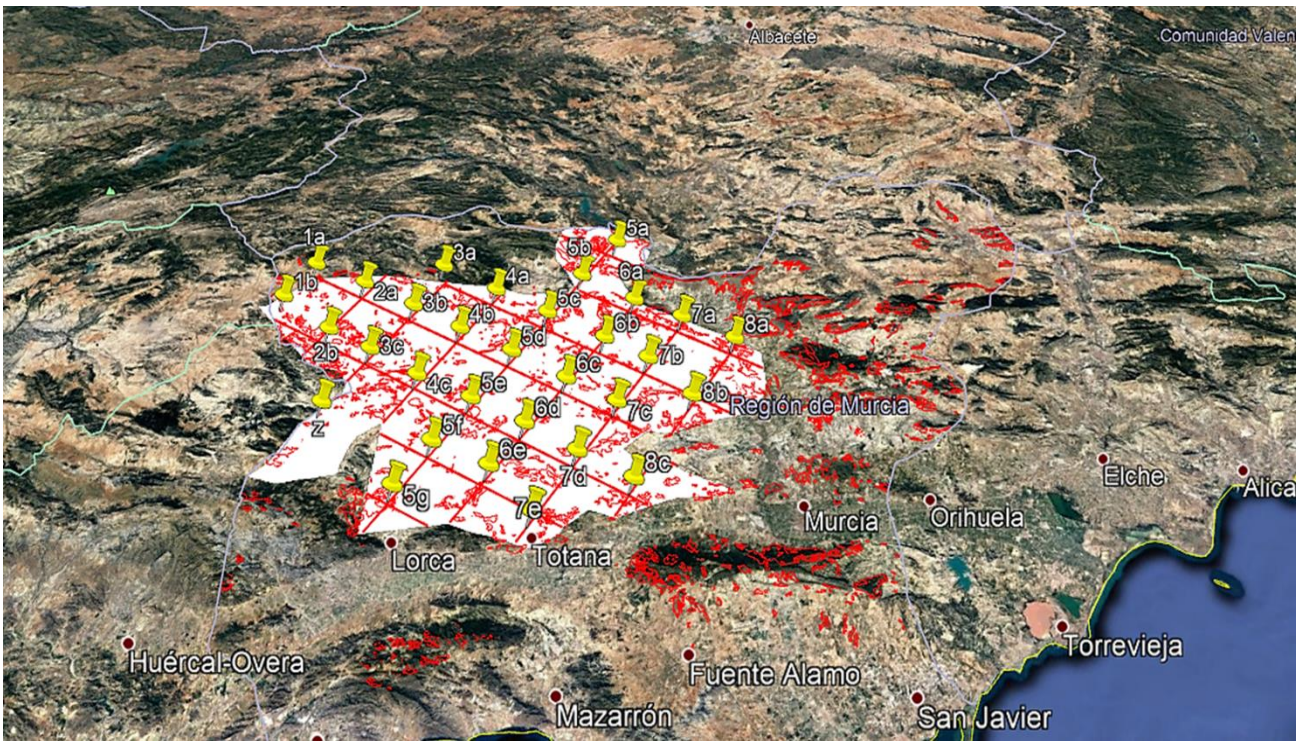

Figure 2. Samples points' with the potential area of the host plans presence (Honrubia et al. 2014).

2.1 Measuring host plant density

Nearest neighbour square approach" (Clark *et al.*1954) was used to estimate the density of host plants. First, by arrange the points in the ground, which can be randomly or systematically arranged on a transect line. Then, locate the nearest host plant to that point and measure the distance to the first point (X) and locate the second sample, which is nearest to the first one (Z).

In our study, we assumed that the car is the main point in our sample area. Therefore, we collected randomly the nearest sample of host plant and measuring the distance between them (X). For the second sample, we collected the nearest neighbour to (X) and once we collected it we measured the distance between them. From the obtained data, we are able to estimate the density with the following formula:

$D = ((\sqrt{2}) * n)/ (x^2) (z^2)]1/2$

Where D is density, n is the number of points located on the ground, x is the distance from nearest host plant to the car, z is the distance from nearest host plant to the nearest neighbour host plant.

2.2. DNA extraction

For DNA extraction, the soil samples were sieved at 500 um in order to remove root contaminant. Soil sieved and samples were flash frozen in N_2 liquid in 2ml micro centrifuge tubes and stored at -80°C. Secondary root sample was collected, flash frozen in N_2 liquid in 2ml micro centrifuge tubes and stored at -80°C.

Table 1: Coding, species and coordinates of the assigned samples

Name	Date	1st Coordinate	2nd Coordinate	Species	Ecosystem
7c	16/04/2018			*H. almeriense*	
7c	16/04/2018	38.011939	-1.522408	*F. thymifolia*	Shrubland
7c	16/04/2018			*F. thymifolia*	
6c	16/04/2018			*H. viscarium*	
6c	16/04/2018	38.014182	-1.632188	*H. almeriense*	Agriculture
6c	16/04/2018			*H. viscarium*	

Name	Date	1st Coordinate	2nd Coordinate	Species	Ecosystem
5d	16/04/2018			-	
5d	16/04/2018	38.036949	-1.759109	-	Pine Forest
5d	16/04/2018			-	
5c	16/04/2018			*H. almeriense*	
5c	16/04/2018	38.109283	-1.734894	*F. thymifolia*	Shrubland
5c	16/04/2018			*F. thymifolia*	
5c	16/04/2018			*H. almeriense*	
6b	16/04/2018			*H. almeriense*	
6b	16/04/2018	38.109357	-1.622473	*H. almeriense*	Shrubland
6b	16/04/2018			*H. almeriense*	
6b	16/04/2018			*H. almeriense*	
7b	16/04/2018			*H. viscarium*	
7b	16/04/2018	38.109330	-1.526188	*H. viscarium*	Agriculture
7b	16/04/2018			*H. viscarium*	
8b	16/04/2018			-	
8b	16/04/2018	38.075712	-1.410111	-	Agriculture
8b	16/04/2018			-	
4a	18/04/2018			*H. violaceum*	
4a	18/04/2018	38.115088	-1.873989	*H. violaceum*	Pine Forest
4a	18/04/2018			*H. violaceum*	
3a	18/04/2018			-	
3a	18/04/2018	38.118428	-2.003761	-	Pine Forest
3a	18/04/2018			-	
3b	18/04/2018			*H. hirtum*	
3b	18/04/2018	38.018691	-1.992298	*H. hirtum*	Agriculture
3b	18/04/2018			*H. hirtum*	
2a	18/04/2018	38.026700	-2.110257	Soil sample	Wasteland
2a	18/04/2018			*H. almeriense*	
1a	18/04/2018			*H. hirtum*	
1a	18/04/2018	38.022787	-2.231024	*H. hirtum*	Oak Forest
1a	18/04/2018			*H. hirtum*	
1b	18/04/2018			*H. hirtum*	
1b	18/04/2018	37.948565	-2.222483	*H. hirtum*	Oak Forest
1b	18/04/2018			*H. hirtum*	

Name	Date	1st Coordinate	2nd Coordinate	Species	Ecosystem
2b	18/04/2018			*H. hirtum*	
2b	18/04/2018	37.920013	-2.094276	*H. hirtum*	Agriculture
2b	18/04/2018			*H. hirtum*	
3c	18/04/2018			*H. hirtum*	
3c	18/04/2018	37.921075	-1.993930	*H. hirtum*	Pine Forest
3c	18/04/2018			*H. hirtum*	
4b	18/04/2018	38.020164	-1.879560	*H. violaceum*	Pine Forest
8c	23/04/2018			*H. almeriense*	
8c	23/04/2018	37.918828	-1.409769	*H. almeriense*	Wasteland
8c	23/04/2018			*F. thymifolia*	
7d	23/04/2018	37.902934	-1.494783	*F. thymifolia*	Pine Forest
6d	23/04/2018	37.914075	-1.606078	*F. thymifolia*	Agriculture
6d	23/04/2018			*F. thymifolia*	
6e	23/04/2018			*H. almeriense*	
6e	23/04/2018	37.828652	-1.643087	*H. almeriense*	Shrubland
6e	23/04/2018			*H. almeriense*	
5e	23/04/2018			*H. hirtum*	
5e	23/04/2018	37.914539	-1.758361	*H. hirtum*	Agriculture
5e	23/04/2018			*H. ledifolium*	
4c	23/04/2018	37.912581	-1.877406	-	Wasteland
Z	23/04/2018			*F. thymifolia*	
Z	23/04/2018	37.803993	-1.985435	*F. thymifolia*	Shrubland
Z	23/04/2018			*F. thymifolia*	
5g	23/04/2018	37.734683	-1.764125	*H. almeriense*	Shrubland
7e	23/04/2018	37.797333	-1.535222	*H. almeriense*	Pine Forest
8b	25/04/2018			*F. thymifolia*	
8b	25/04/2018	38.115004	-1.418013	*F. thymifolia*	Shrubland
8b	25/04/2018			*F. thymifolia*	
8a	25/04/2018	38.205428	-1.407224	soil sample	Shrubland
7a	25/04/2018			*F. thymifolia*	
7a	25/04/2018	38.206344	-1.520147	*F. thymifolia*	Shrubland
7a	25/04/2018			*F. thymifolia*	

Name	Date	1st Coordinate	2nd Coordinate	Species	Ecosystem
6a	25/04/2018	38.202550	-1.623414	*H. violaceum*	Pine Forest
6a	25/04/2018			*H. siriacum*	
6a	25/04/2018			*F. thymifolia*	
6a	25/04/2018			*F. thymifolia*	
5b	25/04/2018	38.209683	-1.749726	*H. violaceum*	Pine Forest
5b	25/04/2018			*H. violaceum*	
5b	25/04/2018			*H. violaceum*	
5a	25/04/2018	38.302729	-1.724574	*F. thymifolia*	Shrubland
5a	25/04/2018			*H. violaceum*	
5a	25/04/2018			*H. violaceum*	

For soil DNA extraction, 0.25 mg of soil was used by the commercial kit DNeasy® powerSoil® kit (Qiagen, Hilden), according to the manufacture instructions. Extracted DNA were tested using NanoDrop ™ 2000/2000c spectrophotometer to quantify and assess the purity of DNA.

About the root DNA extraction, it was isolated according to the C-TAB protocol (Chang *et al*. 1993) as a preliminary step. Afterward, for purifying the DNA, the DNeasy® PowerClean® Pro Cleanup Kit was used according the manufacture instructions. All extracted DNA samples were kept at -80°C.

Eventually, 71 DNA soil samples and 70 DNA root samples were extracted. We collected only one soil sample from one point (2A) because on the way to that point there were plenty of *Helinthemum* plants.

2.3. Quantitative real-time PCR

A real-time PCR using SYBR® Green I technique was performed. This technique depends on a dye for the quantification of double stranded DNA (Ramakers *et al.* 2003). Based on the use of the dye that emits fluorescent light when it is embedding a double strand DNA. Since, the unbound dye exhibits very little fluorescence. In other meaning, when

the amount of amplicon increases, the amount of fluorescence emitted by the dye increases as well. The cycle when the fluorescent signal exceeds certain threshold level during the exponential phase is called cycle Ct. The lower amount of initial DNA, the higher Ct is recorded.

The research group has previously determined the detection limit in a Ct of 34. Values higher than 34 cycles were as a non detected. The primers set used 'Tclaveryifor and Tclaveryirev', amplifies a 130bp fragment within the ITS-2 region of *T. claveryi*. The group of Mycology-Mycorrhiza-Plant Biotechnology have previously checked the primers as species-specific (unpublished data) to *T. claveryi*. To make the qPCR, we used per sample: 7 µl of SYBR, 0.105 µl of Primer Mix, 6.65 µl H_2O, and 0.28 µl of Template (extracted DNA from soil/Root, standards, and Autoclaved water). In all qPCR a Non Template Control were run.

Standard curves for mycelium quantification of *T. claveryi* by real-time PCR were generated using known amounts of mycelium from active growing colonies of *T. claveryi*. Target mycelium growing on a cellophane sheet on MMN medium (Marx 1969), were added it to 0.25 g of previously autoclaved soil. Serial dilutions of DNA extractions were measured by qPCR. Ct values of each dilution were plotted against the logarithm of the corresponding amount of mycelium to generate standard curves.

2.4. Collecting the environmental data

In order to find out the correlation between the existence or absence of the *T. claveryi* with environmental factor, environmental data were collected for the last 2 years (2017, 2016) from official websites; for Annual precipitation indices, it was collected from the European drought observatory (EDO; http://edo.jrc.ec.europa.eu). The temperature indices, elevation, and soil characteristics were collected from ISRIC world soil information (https://soilgrids.org). All data were extracted from these websites using the corresponding coordinates of each collected sample. Soil types were considered to know how much they affect the existence of *T. claveryi* mycelium. Afterwards, the obtained environmental date, for the elevation, temperature and soil type were converted to a categorical date, in order to be able to use it in the different

statistical analysis. Likewise, the result of qPCR Ct value range (Table 2). However, quantitative data for the same environmental variables were kept and used in other statistical analysis.

2.5. Inverse distance weight interpolation (IDW):

The data from sampling points were loaded into the software QGIS (QGIS, 2011) in shapefile format, the data was changed to the Spanish reference system (ETRS89 Zone 30), which allows us to perform operations with them. With the support of a map of the Region of Murcia were checked that the stitches were placed correctly.

The raster layer was then generated, for which the co-kriking interpolation method was used by Inverse distance weight (IDW), that is a geostatistical technique used for interpolation (mapping and contouring) which was used for estimating the host plants distributions and densities at the studied area. IDW is an exact interpolator and it is the most common form in GIS system (Lloyd 2010). It predicts within the range of the input values. Thus, the minima or maxima of not sampled points will not be predicted by IDW (Watson 1992). Its principle, using a weighted moving average, was used in order to predict the value at the locations where no data was available, by using a weighted average of the surrounding observed samples. By utilizing the sampled points coordinates, merged with the coordinates were recorded from high-density host plants while on the way. The technique defines a spatial continuity of the density in the area. Besides that, extrapolate the density and estimate it for the non-sampled area.

Finally, with the layers obtained and with the support of the different software tools, the potential distribution map was generated.

Table 2. Illustrating the different categories for using it in the statistical analysis

Temperature/ °C	Categories	Elevation/m	Categories	Soil type	Categories
Less than 10.3	1	less than 1	0	Fluvent	1
More than 10.3 and less than 12.5	2	More than 1 less than 450.8	1	Xeroll	2
More than 12.5 less than 14.8	3	More than 450 less than 900	2		
More than 14.8 less than 17	4	More than 900 less than 1350	3		
More than 17 less than 19.2	5	More than 1305 less than 1800	4		

2.6. Statistical analysis

The statistical analysis was performed using R 3.4.3 and RStudio1.1.383. ANOVA analysis was used to check the differences between the diverse ecosystems, soil types, and the extent of the host plants by least significant difference ($P \leq 0.05$). Kruskal-Wallis test was used to verify the results obtained from ANOVA. Pearson rank correlation coefficient was calculated to determine the relation between the environmental factors on the existence of *T. claveryi* mycelia in soils and roots of the host plants.

3. Results:

3.1 Data description

Host plants were found in 87% of the sampling points, where 55.6% were plants from *Helianthemum* genus, and 22.2% were from *Fumana* genus. The host plant density ranged between 0.005 and 1226 plants/100m^2, while the mean density was 72.47plants/100m^2. *Helianthemum* plants showed a mean density higher (101.64 plants/100m^2) than *Fumana* plants (31.25 plants/100m^2).

T. claveryi were found in 34% of the soil sampled point and in 24% of the root sampled points. The amount of mycelium ranged between 0.91 and 1.67 µg/g in root sampled points (Table 3) and between 0.04 and 0.52 µg/g in soil samples (Table 4). 70% of the host plants analysed showed detectable amount of mycelium in soil and/or roots.

Table 3. qPCR positive points in root samples

Code	1st Coordinate	2nd Coordinate	Mean Ct root	Log (average mycelium µg/g root)
7d	38.011939	-1.522408	32.67	1.32
6d	38.014182	-1.632188	33.02	1.40
5c	38.109283	-1.734894	33.31	1.25

Code	1st Coordinate	2nd Coordinate	Mean Ct root	Log (average mycelium µg/g root)
6c	38.109357	-1.622473	33.63	1.10
7c	38.10933	-1.526188	33.26	1.23
4c	38.115088	-1.873989	33.60	1.02
3d	38.018691	-1.992298	33.02	1.07
1d	38.022787	-2.231024	29.11	1.67
4d	38.020164	-1.87956	29.84	1.49
8d	37.918828	-1.409769	33.86	0.98
7e	37.902934	-1.494783	33.94	0.91
6f	37.828652	-1.643087	33.09	1.09
5g	37.734683	-1.764125	28.04	1.61
8c	38.115004	-1.418013	32.82	1.06
7b	38.206344	-1.520147	33.30	0.99
6b	38.20255	-1.623414	33.46	0.99

Table 4. qPCR positive points in soil samples

Code	1st Coordinate	2nd Coordinate	Mean Ct soil	log (average mycelium µg/g soil)
7c	38.10933	-1.526188	32.57	0.41
4c	38.115088	-1.873989	32.46	0.15
3d	38.018691	-1.992298	33.05	0.04
2d	38.0267	-2.110257	32.82	0.11
6f	37.828652	-1.643087	31.69	0.45

Code	1st Coordinate	2nd Coordinate	Mean Ct soil	log (average mycelium µg/g soil)
5e	37.914539	-1.758361	33.24	0.52
5g	37.734683	-1.764125	31.85	0.46
7f	37.797333	-1.535222	32.15	0.30
8c	38.115004	-1.418013	32.04	0.29
8b	38.205428	-1.407224	32.05	0.18
7b	38.206344	-1.520147	32.44	0.21
5a	38.302729	-1.724574	32.34	0.18

3.2 Effect of soil types on *T.claveryi* mycelium

The effect of different types of soils, fluvents or xerolls, on the mycelium of *T. claveryi* from host plants roots was checked. We found a significant ($p<0.05$) higher amount of mycelium in root plants from fluvents soils, than root from xerolls soils (Figure 4). Moreover, host plants are more frequently found in fluvents soils (48.4%) than on xeroll (35.5%).

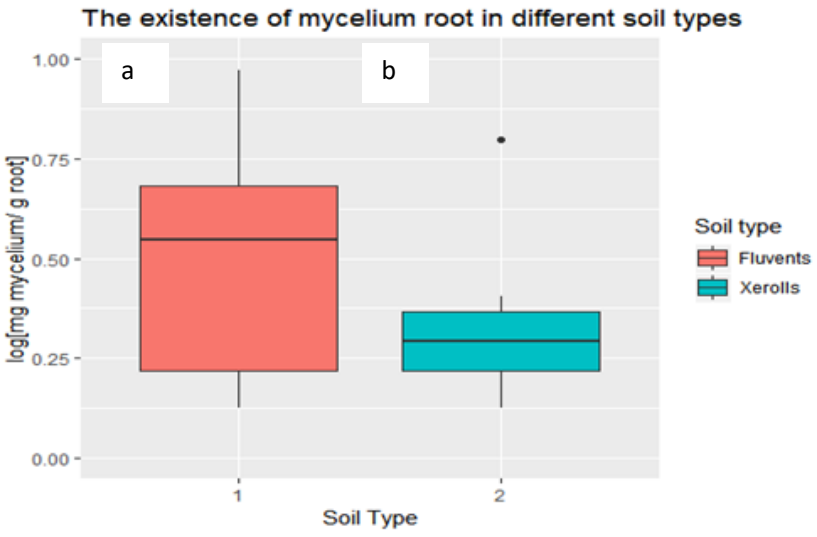

Figure 4. Relationship between soil type and the amount of mycelium (log [µg mycelium/g root]). Different letters mean significant differences ($P<0.05$) by ANOVA analysis.

3.3. Effect of altitude on *T.claveryi* mycelium

The amount of mycelium in soil was slightly correlated with the altitude of the sampling point. A significantly inverse correlation (Figure 5; p=0.032, r=-0.45) was observed where more fungal mycelium was found in soil at altitudes of less than 450 m a.s.l. (caterogy 1).

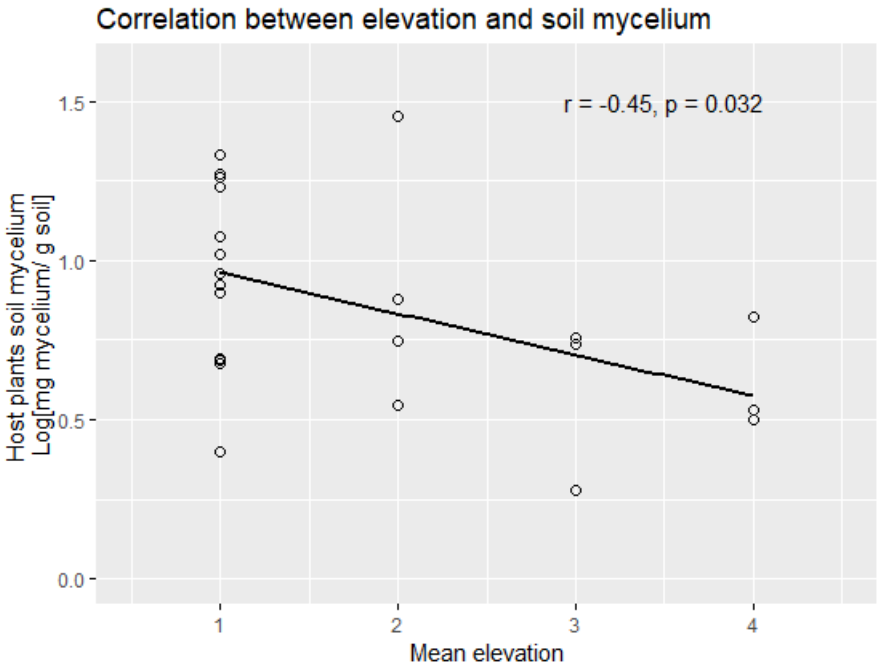

Figure 5. Effect of mean elevation on the soil mycelium quantity (log [µg mycelium/g soil]).

3.4. Effect of rainfall and host plant on *T.claveryi* mycelium

We found a quite different behaviour of *T. claveryi* mycelium regard to annual precipitation depending of the host plant. The two host plant genera found in this study was *Helianthemum* and *Fumana*. The distributions of these two genera seem to be conditioned by annual precipitation since *Helianthemum* plants were found in localizations with annual precipitation ranged from 100 to 250 mm/year and the mycelium quantity in soil was increasing with more precipitation (p=0.028, r=0.81). However, *Fumana* plants were found in localizations with lower annual precipitation (maximum 40 mm/year) and the mycelium quantity in root has a negative

correlation (p=0.021, r=0.88) with the rainfall amount. Thus, we observed a critical threshold of 100 mm annual rainfall

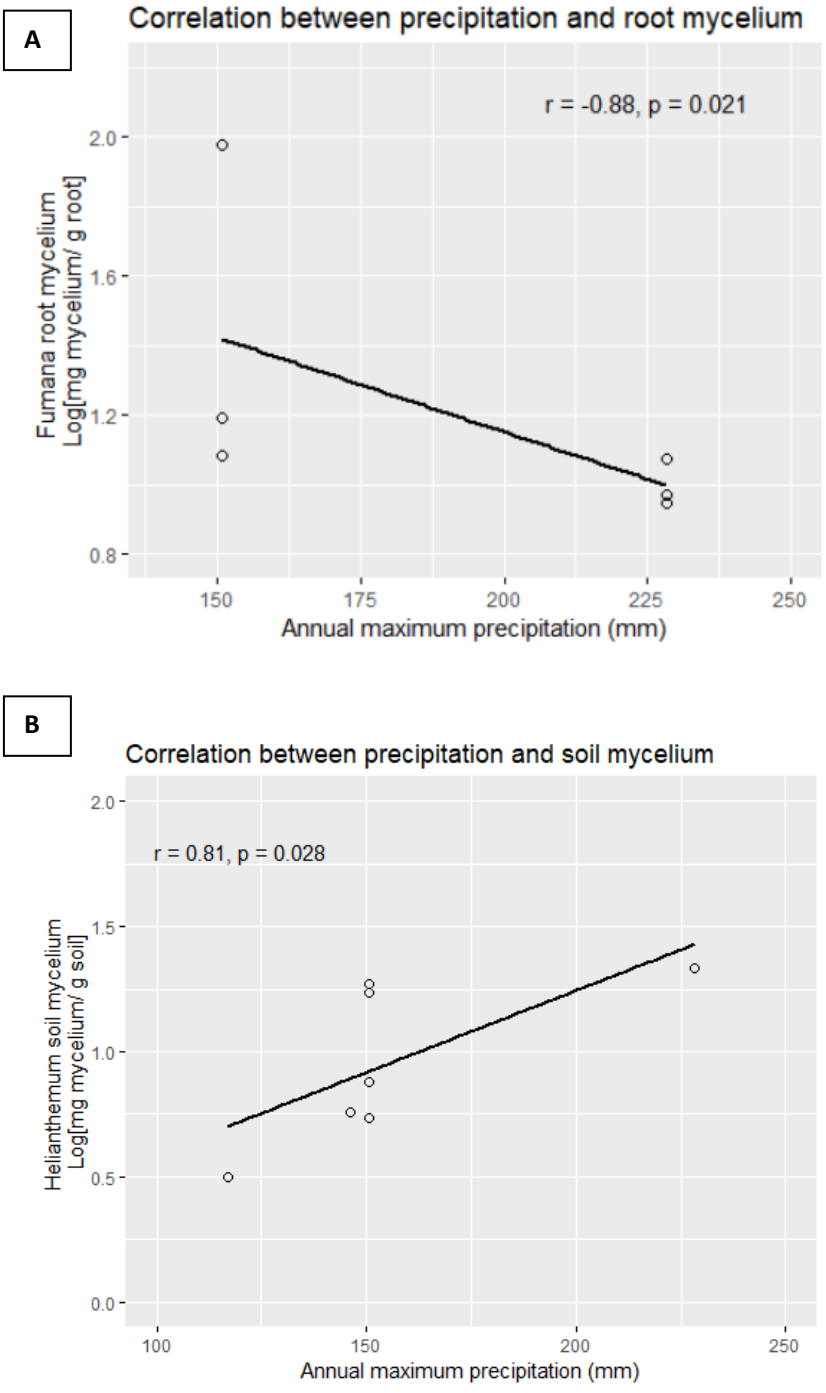

Figure 6. Effect of annual precipitation on root mycelium quantity in *Fumana* plants (A) and on soil mycelium quantity in *Helianthemum* plants (B). (log [µg mycelium/g soil]).

3.5. Effect of ecosystem on *T.claveryi* mycelium

In terms of the correlation with the ecosystem, there was a significant correlation between the density of the host plants and the different ecosystem. Agricultural lands and pine forest have lower densities of the host plants than the oak forest and wasteland (Figure 7). Whereas, the high density was determined at the shrubland ecosystem.

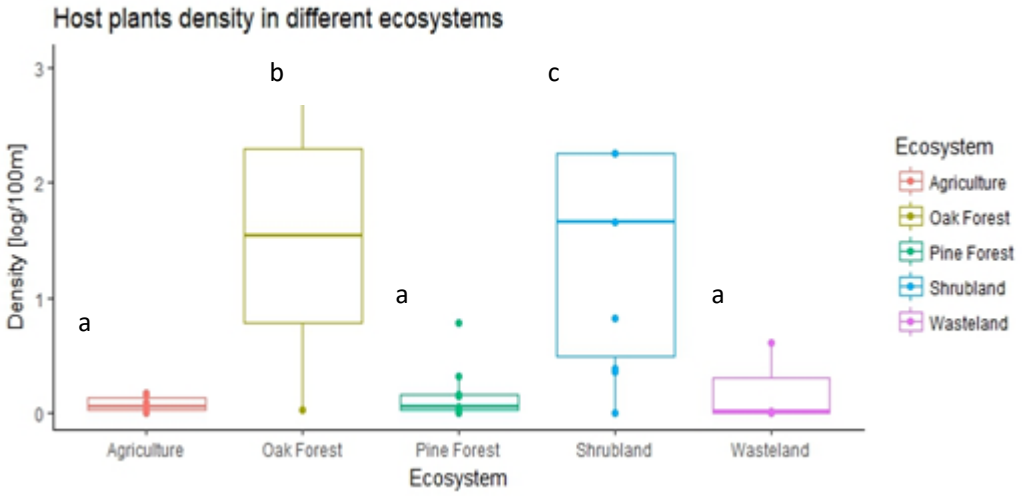

Figure 7 Effect of ecosystem on root host plant density. Different letters mean significant differences (P<0.05) by ANOVA analysis.

3.6. Potential map soil building for *T. claveryi* ascocarps production

The map illustrating the density mycelium existence (map background colour) varied from high density (red colour), medium density (yellow) and no existence of mycelium (white colour) (Figure 8). Then, ecosystem types were added accordingly to the collected sampled points. The high and medium density of mycelium was concentrated in the shrubland areas. Whereas the low densities were appearing in the oak forest and agricultural land. Lastly, there was no existence in most of the other ecosystems (pine forest areas and wasteland).

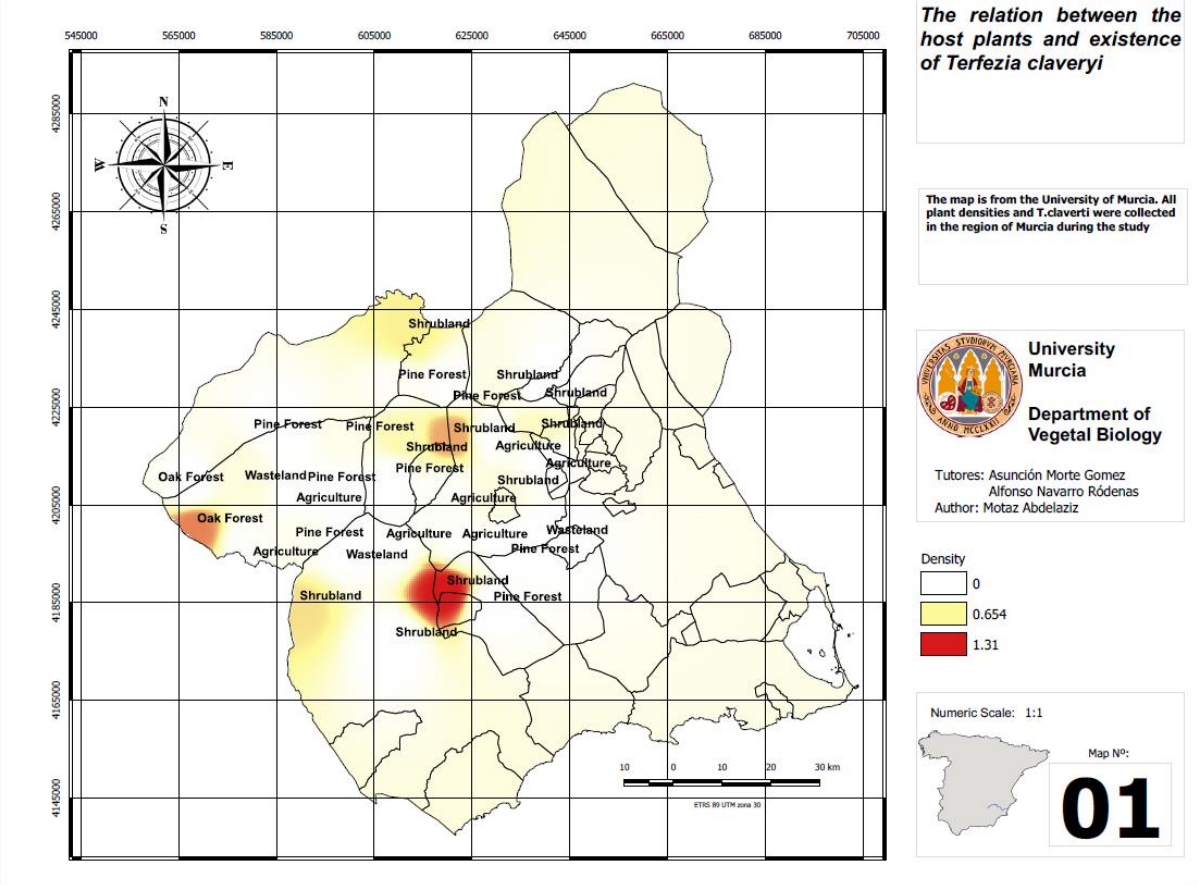

Figure 8 Potential map of desert truffle production. Density was calculate as host plant density multiplied by mycelium quantity in soil in each sample point.

4. Discussion

This study is the first of its type in the Region of Murcia. The increasing demand for this natural resource during the last years makes important to understand the *T. claveryi* distribution in the Region Murcia in order to be able to create a potential map of desert truffle resource and try to determine the environmental factor that could be limiting that distribution.

To date, very little is known about environmental factors that are directly related to fructifications of desert truffles, with the exception of some hunches gathered from truffle collectors. In general, truffles appear more frequently during March-April, and according to desert truffle hunters, rain (97.8%), soil type (62.2%) and host plant affect the desert truffle production. Around 80 % of the pickers think that winter

showers are an important factor that enable the truffle to reach a good size. However, spring showers or spring temperatures were important for 9.1% and 25% of the interweaved hunters, respectively (Mehmet, 2017). Bradai et al. (2015) has found the natural production of desert truffle is highly related to the cumulated precipitation of October–December, where the rain falling after the dry period (summer) determines the development of truffles Mandeel & Al-Laith, 2007; Bradai et al., 2014). Morte et al. (2012) observed a statistical correlation, according to Pearson's test, between the amount of precipitations during autumn (September, October, and November) of a year and the *T. claveryi* truffle production in spring of the following year.

However, at this study we should be careful and distinguish between some annual environmental factors that could be different between one year to other. And determine the annual production fluctuations such as in autumn rainfall, (Morte et al. 2012; Bradai et al 2014, 2015) or spring showers and temperatures (Mehmet, 2017), from those explaining environmental factor that remains constant such as host plant presence or soil type.

In this way, we should distinguish between factors that affect the production and factors that affect the presence and intensity of *T. claveryi*.

Among all the environmental factors analyzed in this work, the most affecting one on mycelium amount is the type of soil. Fluvent soil seems to be more optimal for *T. claveryi* grow than the Xerolls soils. The Xerolls soil is the most common encountered Mediterranean soil. It is Mollisols, which is like mineral soils with relatively thick, dark-colored, rich in humus, and good stable structure (Gómez-Miguel and Badía 2016). Also, it is xeric soil moisture with little moisture retention and excessively drained. Thus, soil is moist for brief periods following precipitation. On the other hand, Fluvent soils contain more than 2% soil organic carbon (SOC) up to -125 mm from the soil surface, which implies that it is a fertile soil thanks to its thickness and the natural fertility related to the soil organic material mineralization (Gómez-Miguel and Badía 2016). Fluvent texture is finer than loamy. It is very fine sand and less than 35% rock fragment, 11%–12% $CaCO_3$ with availability of nutrients such as iron and

phosphorous and pH from neutral to alkaline. The fluvent soil characteristics math with suitable soil described for desert truffles (Bonifacio & Morte, 2014).

Regard to the plant density, the environmental factor that affects the most is the ecosystem. Agricultural land and pine forest have lower densities of the host plants than the oak forest and wasteland. Whereas, the highest density was determined at the shrubland ecosystem. Due to the high density of the trees and the competition on the sun light, water and nutrients, it could be the main reason for the low density of the host plants. Nevertheless, it still exists in the forest ecosystem. Moreover, the shrub ecosystem has more surface exposed to sunlight and less competition on water and nutrients, so that it is more convenient to host plants for spreading, than the other ecosystems.

For these study whatever potential host plants, either annual or perennial, where considers. Two genera have been found in this work (*Helianthemum* and *Fumana*). Desert truffle gatherers have been expressed at interviews (Mehmet, 2017) that host plant is an important factor to be taken into account. In this work, we found how *T. claveryi* seems to present certain preference for species of these two genera in function of the water availability of the area. Thus, in areas with annual precipitation below 100 mm the host plants preferred was *Fumana* sp and in areas with annual precipitation between 150 and 300 mm was preferably with *Helianthemum* sp. We could stablish a precipitation threshold between 100-150 mm for host plant preference. Morte et al (2008) observed a critical point of 150 mm of rainfall in the dry years for desert truffle production under *Helianthemum* plants.

5. Conclusion

1) It is more common to find *T. claveryi* in Fluvent than the Xerolls soil.

2) Annual precipitation threshold around 100-150 mm determines the preference of host plants by *T. claveryi*, where low precipitation would favour *Fumana* sp against *Helianthemum* sp.

3) Surrounding ecosystem present a high influence in determining the host plant density and, consequently, the desert truffle potential. Shrubland ecosystems seem to present the highest potential of desert truffle production.

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