UNIVERSITY OF MOLISE

Department of Biosciences and Territory

DOCTORAL THESIS

Analysis and characterisation of the microbial communities associated with truffles (Tuber spp.)

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To my family,

pillar of my existence.

To young researchers,

to their passion, dedication and hard work.

ABSTRACT

Truffles are a polyphyletic group of fungi whose fruiting bodies sequester their spores and develop underground. Fungi of the genus *Tuber*, the so-called "true truffles", are ectomycorrhizal ascomycetes of the *Pezizales* order that undertake a complex life cycle, during which the fungal mycelium establishes symbiotic associations with the roots of several trees and shrubs.

Some of the more than 180 *Tuber* species currently known (including *T. aestivum* Vittad., *T. borchii* Vittad., *T. magnatum* Picco, and *T. melanosporum* Vittad.) are highly sought after on the food market due to their unique organoleptic properties, with a huge commercial value.

It is known that truffles harbour complex microbial communities of bacteria, yeasts, guest filamentous fungi, and viruses, with whom they interact both in the mycorrhizosphere and in the ascocarp. However, many aspects related to the diversity and the potential role of truffle-associated microorganisms, as well as the effects of the interactions among microbial communities on the biology of truffles are still poorly understood. Accordingly, the main purpose of this work was to analyse and characterise the bacterial communities associated with two of the most commercially relevant truffle species: the summer black truffle *T. aestivum* and the prized white truffle *T. magnatum*.

Analyses were carried out on *Tuber* ascomata from Molise region (Central-Southern Italy), one of the most important Italian areas suited to truffle collection (about 40% of the national production). Nevertheless, to date, Molise truffle has received very little attention from a scientific perspective and, consequently, it is not adequately valorised and preserved. Thus, the research activities illustrated in the present thesis lay the foundation to fill the lack of scientific data on the Molise truffles, representing an essential starting point for a further and more in-depth characterisation of this resource of utmost importance for the local economy.

In detail, in a first study, the microbial communities associated with six *T. aestivum* ascomata and six soil samples collected in the municipality of Vastogirardi (Isernia province) were examined using the

16S rRNA gene amplicon high-throughput sequencing. Consistently with previous researches, the main phyla retrieved in the investigated ascocarps were *Proteobacteria* and *Actinobacteria*, with the genus *Bradyrhizobium* particularly represented. Nonetheless, considerable differences between soil and truffle microbiota and an unexpected heterogeneity within the truffle bacterial communities in terms of composition, relative abundance of the main taxa, and α-diversity values were observed.

The other two reported researches focused on *T. magnatum* populations from different areas of Molise region. Overall, twenty-one white truffles were characterised from a morphological, genetic, and microbiological point of view. In particular, morphological investigations concerned the thickness of the peridium, a parameter for which no comprehensive information was available, whereas genetic and microbiological analyses focused on the Sequence-Characterised Amplified Region SCAR A21-inf (a single locus marker) and the gleba bacterial communities, respectively. A considerable variability between and within the examined *T. magnatum* groups emerged, confirming an interesting heterogeneity of Molise truffle populations that makes them ideal for further in-depth studies.

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1. INTRODUCTION

1.1 Mycorrhizal symbioses: ectomycorrhizas and endomycorrhizas.

Plant roots are an ideal niche for soil fungi, which live in the rhizosphere (the narrow portion of soil surrounding living roots) as saprotrophs or as mycorrhizal symbionts, associated with photosynthetic plants (Bonfante and Anca, 2009).

Mycorrhizal fungi represent a heterogeneous group that includes species belonging to different taxa. They can be divided into two main categories: 1) aseptate endophytes, such as *Glomeromycota*, plant-colonising fungi with syncytial hyphae lacking in transversal walls (septa), and 2) septate *Ascomycota* and *Basidiomycota*. Ascomycetes are characterised by microscopic sexual reproduction structures named asci, single cells containing non-motile spores (ascospores), whereas the sexual reproduction of basidiomycetes occurs in the so-called basidia, club-shaped cells bearing external spores (Bonfante and Genre, 2010).

Although they can spend part of their life cycle as free-living organisms, mycorrhizal fungi always need to associate with the roots of higher plants, such as forest trees, wild grasses, and several crops. Mycorrhizal fungi are widely distributed in natural and agricultural environments (alpine and boreal zones, tropical forests, grasslands, and croplands). About 6000 species of fungi in the *Glomeromycotina*, *Ascomycotina*, and *Basidiomycotina* subdivisions and more than 90% of all plant species are involved in mycorrhiza formation (Bonfante and Anca, 2009; Bonfante and Genre, 2010; Smith and Read, 2008).

The term "mycorrhiza" derives from the Greek "*mykos*" (fungus) and "*rhiza*" (root) and indicates the symbiotic association between fungi and plant roots, from which both partners benefit. Indeed, mycorrhizal fungi play a key role in plant ecosystems: they improve the nutrient status of the host plant, providing minerals and increasing water absorption from the soil through the specific activity of their mycelium, and confer resistance to stress and disease. Moreover, they develop the so-called wood-wide web, an extensive hyphal network in the soil that connects different plants, allowing an

efficient horizontal transfer of nutrients. Likewise, the fungus needs the host plant for its growth and reproduction (Bonfante and Anca, 2009; Bonfante and Genre, 2010; Helgason *et al*., 1998; Mello and Balestrini, 2018; Simard *et al*., 1997; Smith and Read, 2008).

Based on the taxonomic position of plant and fungal partners and anatomical aspects, mycorrhizas are commonly divided into two main categories: ectomycorrhizas and endomycorrhizas, depending on whether the fungus colonises the intercellular spaces or develops inside root cells (Figure 1). In particular, ectomycorrhizas (ECMs) are symbiotic associations between higher plants (trees and shrubs) and fungi belonging to *Ascomycota* and *Basidiomycota* phyla, whose hyphae never penetrate the lumen of root cells, but remain extracellular, determining important changes to root morphogenesis. A thick hyphal mantle tightly wraps the root tip, while the so-called Hartig net, a sort of hyphal labyrinth, develops around epidermal and, in some cases, cortical cells, separating them without inducing substantial modifications (Bonfante, 2001; Bonfante and Anca, 2009; Daba *et al*., 2019; Figures 1 and 2a).

On the other hand, in the case of endomycorrhizas, fungal hyphae penetrate inside living cells of root epidermis and cortex to establish an intracellular symbiosis. Endomycorrhizas can be further distinguished into ericoid, orchid, and arbuscular mycorrhizas (AMs). In the ericoid mycorrhizas, which are restricted to *Ericales* order, the fungus develops inside epidermal cells of the root, forming coils that give rise to independent infection units, whereas in the orchid mycorrhizas, which are limited to the *Orchidaceae* family, fungal coils develop mainly in the inner layers of the root (Bonfante and Anca, 2009; Figures 2b and 2c).

Arbuscular mycorrhizas are widespread endomycorrhizal associations deriving from co-evolution events between plant and fungal partners, and involve several plant taxa and fungi of the phylum *Glomeromycota* (Bonfante and Genre, 2008, 2010). Unlike ectomycorrhizal fungi, AM fungi generally do not colonise the root tip. Hyphae develop from a spore and produce a hyphopodium or appressorium (the flattened thickened tip of a hyphal branch by which the fungus attaches to and penetrates its host) on the root epidermis/surface. The colonisation within the root proceeds both

intercellularly and intracellularly, and leads to the formation of characteristic branched structures called arbuscules (little fungal trees, hence the name of arbuscular mycorrhizas). They develop inside the inner cortical cells and are the main site of nutrient exchange between plant and fungal partners (Bonfante and Anca, 2009; Bonfante and Genre, 2010; Harrison, 2005; Figures 1 and 2d).

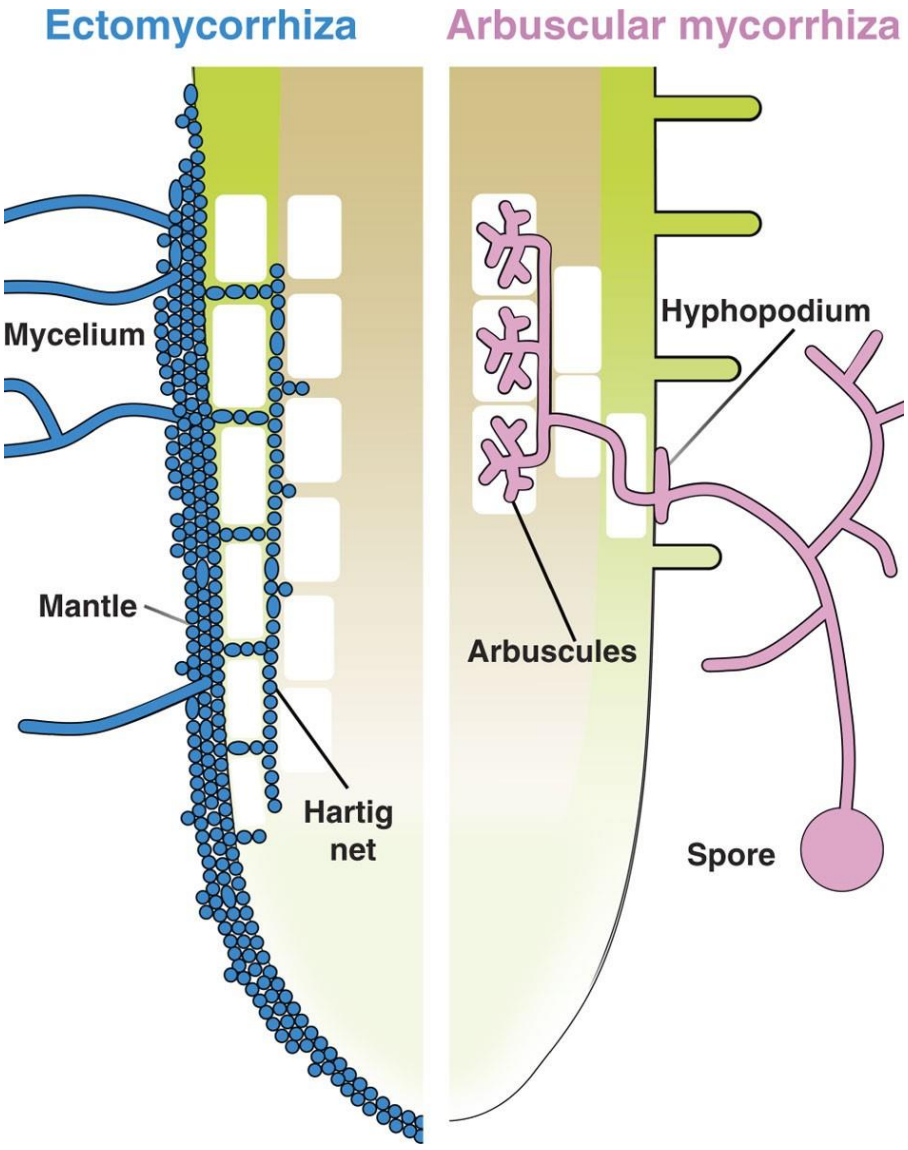

Figure 1. Illustration of root colonisation structures in mycorrhizal symbioses. In the ectomycorrhizas (blue, on the left), the fungus surrounds the root tip with a thick hyphal mantle and the Hartig net develops around the epidermal cells (green area). In the arbuscular mycorrhizas (pink, on the right), hyphae, which develop from a spore and produce a hyphopodium on the root surface, colonise the root cells both intracellularly and intercellularly, until they form the so-called arbuscules within the inner cortical cells (brown area). From Bonfante and Genre, 2010.

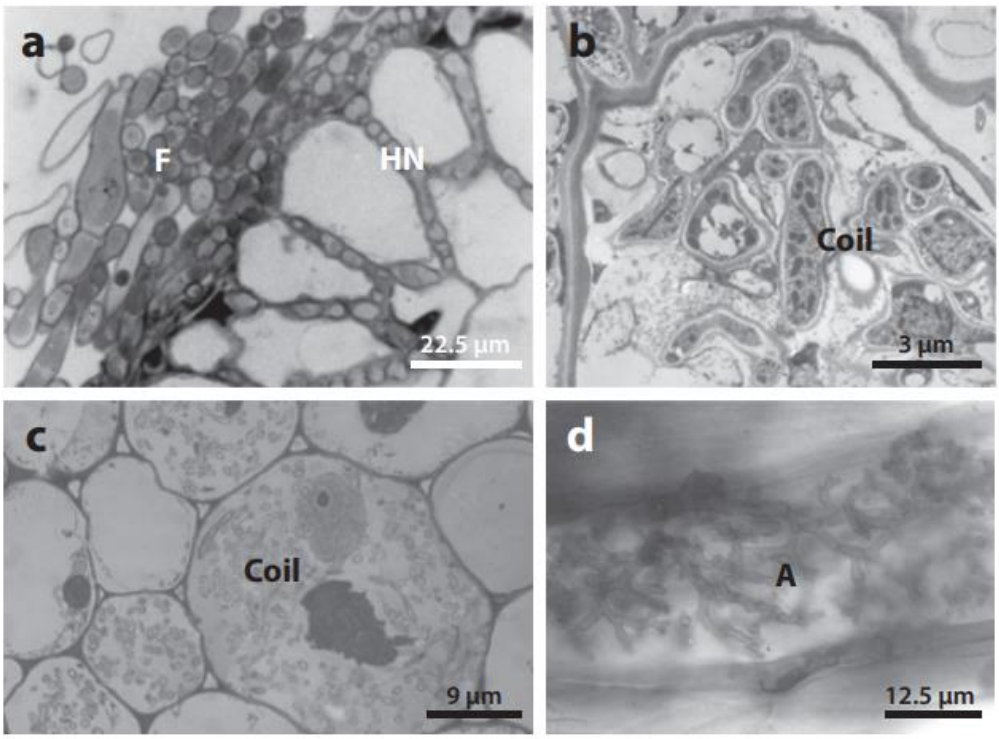

Figure 2. Micrographs representing the different types of mycorrhizas and the main colonisation structures. **a)** The fungal mantle (F) and the Hartig net (HN) in *Tilia* ectomycorrhiza. **b)** A coil produced by an ericoid fungus in a hair root of *Calluna vulgaris*. **c)** A coil produced by an endophytic fungus inside an orchid root of *Cephalanthera*. **d)** An arbuscule (A) of the AM fungus *Glomus versiforme* in the cortical cell of a leek root. From Bonfante and Anca, 2009.

The establishment of the mycorrhizal associations between fungi and plant roots is also influenced by the microorganisms of the rhizosphere and, in particular, by bacteria, which are believed to represent the third partner of the symbiosis. Garbaye (1994) was the first to introduce the concept of "mycorrhization helper bacteria" (MHB) to indicate those bacteria that promote mycorrhizal development. Over time, more and more knowledge has been acquired, leading to define a complex scenario of possible interrelations. Interactions between bacteria and fungi are more widespread than expected and play a key role in ecosystems. Therefore, understanding bacteria-fungi interactions in the mycorrhizosphere, the portion of soil influenced by both roots and mycorrhizal fungi, is essential for describing the soil-plant interface (Artursson *et al*., 2006; Bonfante and Anca, 2009; De Boer *et al*., 2005; Leveau and Preston, 2008).

Physical contact among the partners and the release of active molecules are crucial for the establishment of both plant-fungus and mycorrhiza-bacteria networks. Generally, communication between microorganisms and plants in the rhizosphere takes place through the exchange of chemical volatile compounds and signal solutes (Daba *et al*., 2019). Indeed, plant roots and mycorrhizal fungi liberate diffusible factors, such as strigolactones, Myc factors, volatiles, and auxin-like molecules, which are reciprocally perceived by the symbiotic partners (Figure 3). Moreover, bacteria thriving in the rhizosphere release compounds that can be beneficial for (this is the case of the MHB) or detrimental to mycorrhization process. As illustrated in Figure 3, bacteria can be loosely or tightly associated with mycorrhizal fungi; they can colonise the surface of extra-radical hyphae or, in the case of AM fungi, live as endobacteria in the cytoplasm of fungal cells. Some bacterial species are also associated with mycorrhizal roots and sporocarps (i.e. *Ascomycota* and *Basidiomycota* fruiting bodies), demonstrating that bacteria interact with symbiotic fungi during all stages of their life cycle (Bonfante and Anca, 2009).

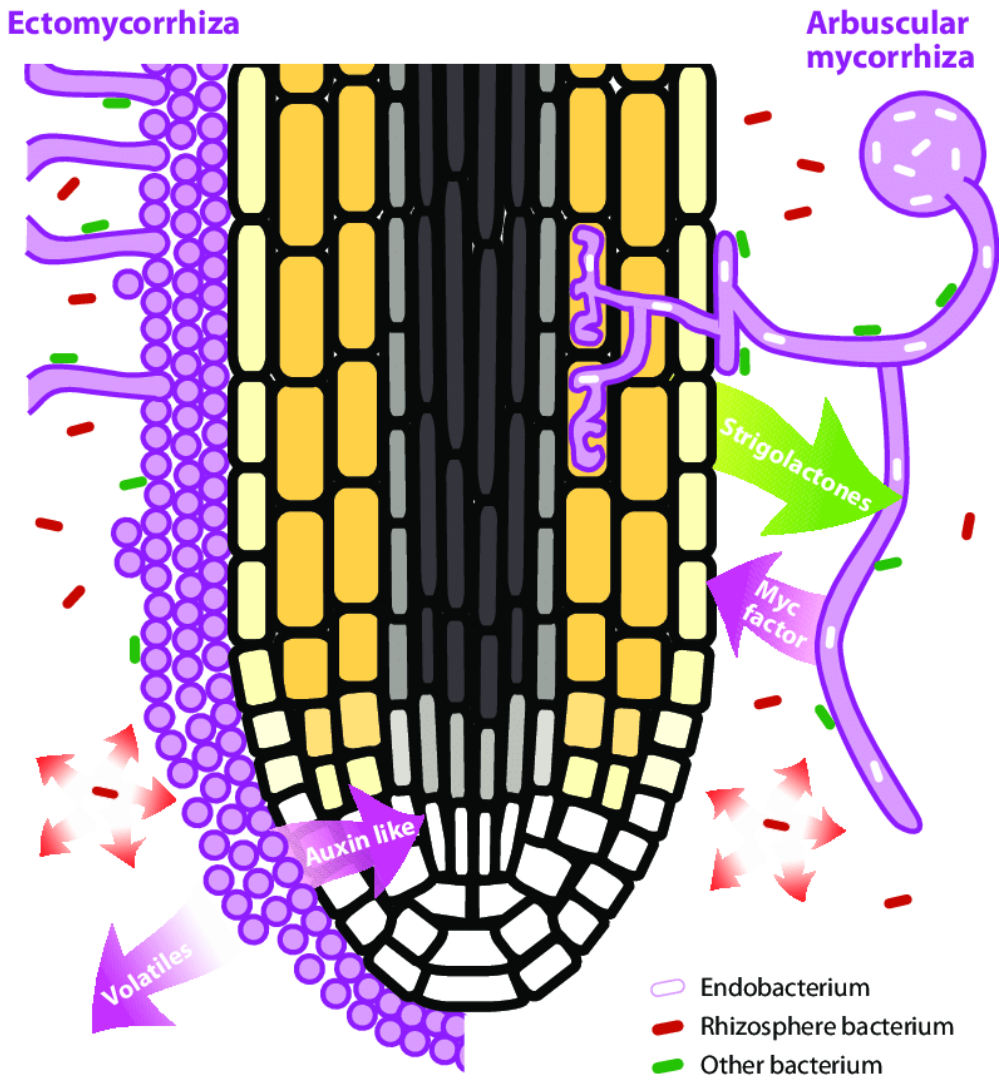

Figure 3. Representation of some interactions that take place in the rhizosphere among plants, mycorrhizal fungi, and bacteria. Endobacteria (white) are restricted to arbuscular mycorrhizal fungi (on the right) and develop from the spores toward the intra-radical mycelium. Rhizosphere bacteria (red) release diffusible factors that may be beneficial for or detrimental to mycorrhization. Other bacteria (green) establish physical contact with the mycorrhizal fungus and may have positive effects or possess mycophagic activity. Diffusible factors, such as strigolactones, Myc factors, volatiles, and auxin-like molecules released by roots and mycorrhizal fungi are represented by arrows. From Bonfante and Anca, 2009.

1.2 The truffles (*Tuber* spp.): a valuable example of ectomycorrhizal fungi. Between legend, history and curiosities.

Truffle is known since ancient times. It has always been considered a mysterious product as it develops underground, giving rise to numerous legends. The term "truffle" seems to derive from the Latin "*terrae tufer*" (with the word "*tufer*" used instead of "tuber") that means ground excrescence.

It appears that the Babylonians already knew the truffle in 3000 BC and there is evidence of its presence also in the Sumerian diet. The ancient Greeks and Romans prized truffles as an Epicurean delight (Mello *et al*., 2006). Most likely, the Romans learned their culinary use from the Etruscans. Even in Roman times, truffles had high prices due to their rarity and difficulties in finding them. However, it is plausible that the "*terrae tufer*" of the Romans was not the truffle that we collect and market today (*Tuber* spp.), but the so-called desert truffle *Terfezia arenaria*, a species currently found in Puglia and Sardinia with little commercial value, which was widely spread throughout the Roman Empire. The first recipes of truffle dishes are described in "*De re coquinaria*", a work of the Roman gastronome, cook, and writer Marcus Gavius Apicius, who lived between the first century BC and the first century AD (https://langhe.net/693/tartufo-storia-leggenda/). In his literary work *"Naturalis Historia"*, the Latin scholar Pliny the Elder (first century AD) claimed that truffle has a miraculous nature as "it is born and grows without roots". The Greek philosopher Plutarch (50 AD - after 120 AD) speculated that this precious fungus derived from the combined action of water, heat, and lightning. Likewise, the Roman poet Juvenal (55-135/140 AD) attributed the birth of the truffle to a thunderbolt thrown from Jupiter/Zeus near an oak tree. Moreover, as Jupiter/Zeus was also famous for his love affairs, truffles were considered aphrodisiac, so that the Greek physician Galeno (129-201 AD) wrote that they were very nutritious and induced the erotic pleasure.

It is said that even the Prophet Muhammed, at the time of the Islamic civilisation, recognised "truffle water" (truffle extract or juice) as therapeutic for eye diseases (Khalifa *et al*., 2019).

During the Middle Ages, truffle was indicated as devil's food and it was banished from the diet. Indeed, it was believed to be poisonous, as it grew in soils where there could be vipers, rusty tools,

and even cadavers and carcasses. However, in the Renaissance truffle was rediscovered, becoming an important protagonist of the aristocratic banquets. In the 1700s, the Piedmontese truffle was considered a delicacy in all the European courts. Truffle search represented a palace amusement: the Italian sovereigns Vittorio Amedeo II and Carlo Emanuele III delighted in organising real "truffle hunting trips". Guests and foreign ambassadors were invited to join. Probably, the practice of using an elegant animal like the dog to search for these precious fungi, instead of pigs that were employed mainly in France, comes from here (http://www.georgofili.info/contenuti/il-tartufo-tra-storia-e-leggenda/2127; https://langhe.net/693/tartufo-storia-leggenda/; https://www.tartufaialtotevere.com/). The nature of truffle has long been debated: some considered it a plant, others a ground excrescence. The first paper entirely devoted to truffles, "*Opusculum de tuberibus*", appeared in 1564 (Ciccarelli, 1564; Mello *et al*., 2006). In the same century, for the first time, the Italian botanist, physician, and anatomist Andrea Cesalpino included truffles among fungi. In the 1831, Carlo Vittadini, who is considered the father of the hydnology (the science that studies truffles), published the work "*Monographia Tuberacearum*", in which he described 51 new truffle species (Bertolini, 2015; Vittadini, 1831).

Truffle has become a cult food from 1826 (Brillat-Savarin, 1826) to the present day, to the point that the creator of the molecular gastronomy, Hervé This, defined truffles as "diamonds" and "the best food" (Mello *et al*., 2006). In fact, truffles (especially the precious white truffle *Tuber magnatum* Picco) are among the most expensive and finest foods in the world. Unique organoleptic properties confer them the status of luxury food, appreciated and marketed worldwide, and often served in the most prestigious restaurants (Li *et al*., 2017; Riccioni *et al*., 2019; Splivallo *et al*., 2011; Vahdatzadeh *et al*., 2019; Vita *et al*., 2015). Truffle prices are exorbitantly high, up to thousands of euros per kilogram (Patel *et al*., 2017). Just think that in 2007 *Tuber magnatum* reached up to 7,000 euros per kilogram (Riccioni *et al*., 2016), whereas in November 2017, a trio of white truffles weighing less than one kilogram was sold at a charity auction in Italy for over 70,000 euros (Daba *et al*., 2019).

Multiple factors contribute to the high commercial value of truffles. First of all, the inability of production to meet the market demand, since the truffle trade is constantly expanding, with some species (above all *Tuber magnatum*, *Tuber melanosporum*, and *Tuber aestivum*) highly sought after on the international market (Bach *et al*., 2021; Daba *et al*., 2019). Other factors are: the lack of automated/mechanised collection methods, given that to locate truffles underground it is necessary to rely on properly trained dogs; the limited seasonal availability (a few months a year); a short shelf life (1-2 weeks); the lack of adequate preservation methods to keep the aroma intact (Daba *et al*., 2019; Vahdatzadeh *et al*., 2019).

1.3 The "true truffles": *Tuber* genus.

Truffles are a polyphyletic group of fungi with members within *Ascomycota*, *Basidiomycota*, and *Zygomycota* phyla, whose fruiting bodies sequester their spores and develop underground (hypogeous fungi) (Smith and Bonito, 2013). Fungi of the genus *Tuber*, the so-called "true truffles", are ectomycorrhizal ascomycetes that undertake a complex life cycle, during which the fungal mycelium establishes symbiotic associations with the roots of several vascular plant species (e.g. oak, pine, poplar, willow, and hazel), and shrubs (Bonito *et al*., 2013; Iotti *et al*., 2016; Mello *et al*., 2006, 2017). As previously described for mycorrhizal interactions as a whole (paragraph 1), ectomycorrhizas are beneficial to both partners: fungi use the carbon compounds photosynthesized by the host plants, providing in return nutrients, water, and protection against biotic and abiotic stresses (Martin *et al*., 2016). Moreover, truffles need a living host plant to complete their life cycle: indeed, unless they establish ectomycorrhizas, *Tuber* species do not form fruiting bodies (Dada *et al*., 2019; Mello and Balestrini, 2018; Paolocci *et al*., 2006; Riccioni *et al*., 2019).

While in the traditional classification the "true truffles" (hereinafter referred to simply as "truffles") were included in the order *Tuberales*, together with all hypogeous ascomycetes, they are currently placed in the order *Pezizales*, which comprises both hypogeous and epigeous fungi, with either saprotrophic or symbiotic lifestyles, but all phylogenetically related (Mello *et al*., 2006). The

available data suggest that fungi of the genus *Tuber* (*Tuberaceae* family) evolved from an epigeous (aboveground) ancestor. Molecular dating traces the *Tuberaceae* divergence (from a common angiosperm-associated ectomycorrhizal ancestor) back to the late Jurassic (about 156 million years ago), with subsequent radiations during the Cretaceous and Paleogene (Bonito *et al*., 2013).

It is estimated that *Tuber* genus includes more than 180 species worldwide, which are widely distributed in the temperate zones of the Northern hemisphere, with three main regions of genetic differentiation: Europe, North America, and South East Asia (Benucci and Bonito, 2016; Bonito *et al*., 2010, 2013; Vita *et al*., 2015; Ye *et al*., 2018).

Most of the species (including *T. excavatum*, *T. maculatum*, and *T. rufum*) are not edible, whereas some others are eatable and highly sought after on the food market, with a huge commercial value.

The Italian law n° 752 of December 16, 1985 (framework legislation in matter of collection, cultivation, and sale of fresh and preserved truffles intended for consumption) identifies nine *Tuber* species intended for consumption, banning the trade in any other genus and species: *T. magnatum* Picco, *T. melanosporum* Vittad., *T. brumale* var. *moschatum* De Ferry, *T. aestivum* Vittad., *T. uncinatum* Chatin, *T. brumale* Vittad., *T. borchii* Vittad., *T. macrosporum* Vittad., and *T. mesentericum* Vittad. (please see Article 2 of law n° 752 of December 16, 1985 at https://www.gazzettaufficiale.it/eli/id/1985/12/21/085U0752/sg#). Among them, the most economically important species, which have aroused and continue to arouse special interest, are *T. magnatum*, *T. melanosporum*, *T. aestivum*, and *T. borchii* that will be described more in-depth in the following paragraphs (Iotti *et al*., 2016).

The ability to colonise the roots of a relatively wide variety of host plants, as well as the need for calcareous soils, with pH values between 7 and 8, are ecological features shared by the different truffle species, with the exception of *T. borchii* that tolerates moderately acidic soils. Nevertheless, there are important differences in the geographical distribution of the main *Tuber* species (Mello *et al*., 2006). *Tuber borchii* is found throughout Europe, whereas *Tuber melanosporum* (the Périgord black truffle) grows spontaneously only in Italy, France, and Spain, although it has been introduced

in several countries around the world (Chen *et al*., 2016; Riccioni *et al*., 2008, 2019). The Italian white truffle *Tuber magnatum* has a restricted geographic spread, which results in limited availability. It has long been considered endemic to Italy, even if, over the last few decades, the presence of this prized species has been reported in several Mediterranean countries and, in particular, in different areas of the South-East Europe (Istria and Balkans). Moreover, it was recently found in France and Switzerland (Belfiori *et al*., 2020; Marjanović *et al*., 2015; Riccioni *et al*., 2016; Tabouret, 2011). On the other side, *Tuber aestivum*, better known as the Burgundy truffle, shows a wider ecological range. Indeed, it is widespread in nearly all European countries, as well as in Iran, North Africa, and Turkey (Büntgen *et al*., 2017; Riccioni *et al*., 2019; Türkoğlu *et al*., 2015).

Thanks to the improvement of truffle cultivation techniques, some of the most economically important truffles (e.g. *T. melanosporum*, *T. aestivum*, and *T. borchii*) have been introduced in many areas of the world where these species do not grow spontaneously: Argentina, Australia, Chile, China, New Zealand, North America, South Africa, as well as some European countries (Berch and Bonito, 2016; Hall and Haslam, 2012; Hall *et al*., 2017; Iotti *et al*., 2016; Reyna and Garcia-Barreda, 2014; Zambonelli *et al*., 2015). In particular, *T. aestivum* is successfully cultivated, even outside its natural distribution range, due to its ability to adapt to a wide spectrum of host plants, climatic and pedological conditions (Chevalier and Sourzat, 2012; Molinier *et al*., 2016; Morcillo *et al*., 2015; Riccioni *et al*., 2019; Stobbe *et al*., 2013 a).

1.4 Morphological and ecological characteristics of the most economically important *Tuber* species.

1.4.1 *Tuber aestivum* Vittad.

The summer truffle *Tuber aestivum* Vittad., also known as Burgundy truffle, is one of the most commercially important truffles, with prices ranging from 160 to 300 euros per kilogram (Rossbach *et al*., 2019). It was officially described as a new *Tuber* species by the Italian mycologist Carlo Vittadini in 1831 (Vittadini, 1831). The adjective "*aestivum*" (from the Latin "summer") refers to the

maturity season of the fruiting bodies, whereas “Vittad.” became the official denomination since Vittadini, having characterised *T. aestivum* accurately, added his own name to that of the species (Molinier *et al*., 2016). Actually, the ripening period of *T. aestivum* ascomata is not limited to the summer months, but it is much longer, extending from late spring to winter (Angelini *et al*., 2016). Chefs and gourmets consider the late season fruiting bodies of higher quality and value compared to the early season truffles. However, up to now, there is no scientific evidence proving any qualitative differences between *T. aestivum* fruiting bodies harvested in summer or in winter (Molinier *et al*., 2016).

Ascocarps appear compact, with a globose or irregularly lobed shape, often with a not very pronounced concavity at the base. They vary in size from 1-3 to 10-14 cm in diameter, with a weight ranging from less than 1 gram up to more than 1 kilogram (Figure 4a). The peridium, brown-black in colour outside, pale ochre in proximity of the gleba, is 200-480 µm thick and is characterised by the presence of hard warts of variable dimensions and shape (pyramidal or irregularly polygonal at the base; Figure 4b). Distinct striations are visible by observing carefully the peridium. Presumably, these structures, synchronised across the individual warts, are pseudo-crystalline deposits rich in calcium, which may reflect temporal changes in the truffle growth environment (Angelini *et al*., 2016; Büntgen and Egli, 2014; Molinier *et al*., 2016).

The gleba is firm, fleshy, with a marbled aspect, crossed by numerous, thin, much ramified, white veins. Its colour can vary from white-ochre to dark-brown on the base of the maturation degree (Figure 4c).

Asci have a globular/sub-globular shape (55-75 x 80-100 µm) and can contain a number of spores ranging from 1 to 6. Ascospores are sub-globose to ellipsoidal in shape, about 18-22 x 25-30 µm in size, brown, brownish-red, or ochre-coloured. Moreover, *T. aestivum* spores are characteristically reticulate/alveolate, with large, irregular alveoli often with internal crests (Figures 4e and 4f; Angelini *et al*., 2016; Molinier *et al*., 2016).

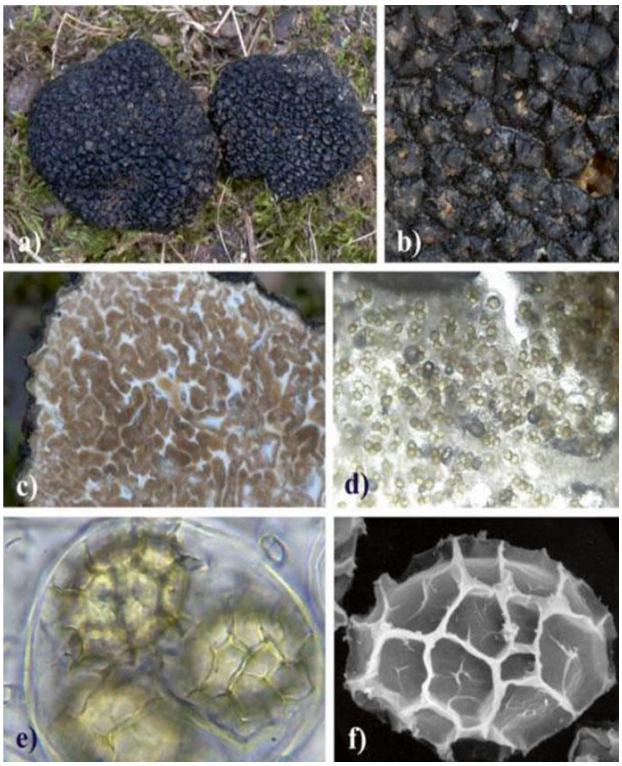

Figure 4. Morphological characteristics of *Tuber aestivum* Vittad. **(a)** Fruiting bodies; **(b)** detail of ascocarp surface (peridium) with clearly visible warts; **(c)** gleba section showing the presence of numerous white veins; **(d)** light microscope images of a gleba section with several asci containing ascospores and **(e)** of an ascus with three spores; **(f)** scanning electron microscope (SEM) image of a spore with medium sized alveoli. From Angelini *et al*., 2016.

Central Europe temperate regions represent the geographical core of Burgundy truffle distribution, although its natural range goes from Northern Africa (Morocco) to Southern Sweden and from Portugal to the Caucasian region. Unique ecological features contribute to the wide habitat range of *T. aestivum* (Stobbe *et al*., 2012, 2013 a).

It fructifies mainly in calcareous, drained and rocky soils, with pH values between 7 and 8 (Figure 5), even if it has also been exceptionally retrieved in sites characterised by the absence of active carbonate and with a pH of 5.9 (Gógán *et al*., 2012; Robin *et al*., 2016).

Tuber aestivum ascocarps are generally found within mixed and broad-leaved woods, in conifer plantations, but also under isolated trees. The main symbiont plants are *Betula verrucosa*, *Carpinus betulus*, *Cedrus* spp., *Corylus avellana*, *Fagus sylvatica*, *Ostrya carpinifolia*, *Picea abies*, *Pinus* spp. (e.g. *P. nigra*, *P. pinea*, *P. sylvestris*, *P. halepensis*, *P. brutia*), *Populus* spp., *Quercus* spp. (e.g. *Q.*

pubescens, *Q. ilex*, *Q. robur*, *Q. petraea*, *Q. cerris*), *Salix* spp., and *Tilia platyphyllos* (Angelini *et al*., 2016; Garcia-Montero *et al*., 2014; Hilszczanska *et al*., 2014; Stobbe *et al*., 2013 b).

As illustrated in Figure 5, summer truffle habitats extend from an altitude close to sea level in the cold Northern regions (such as Sweden) up to 1400-1600 m above sea level in Southern areas with a warm climate (e.g. Morocco). In relation to the different altitude and climatic conditions, *T. aestivum* ascomata can be harvested in an extremely wide time interval. In the temperate regions of Germany and Switzerland, the fruiting season has two maxima, in July and November. It occurs earlier in the warmer habitats and at lower altitudes (e.g. from May to July in Greece) whereas it is postponed in cold environments and at higher altitudes. Temperature is an important parameter that influences ascocarp development; the average temperatures of the coldest and warmest months are of particular relevance, as they can determine a halt in truffle production because of frost and ice or a decrease in production due to heat-induced drought. Lastly, the mean annual precipitation recorded in *T. aestivum* habitats ranges between 400 and 1500 mm (Molinier *et al*., 2016; Stobbe *et al*., 2012, 2013 a).

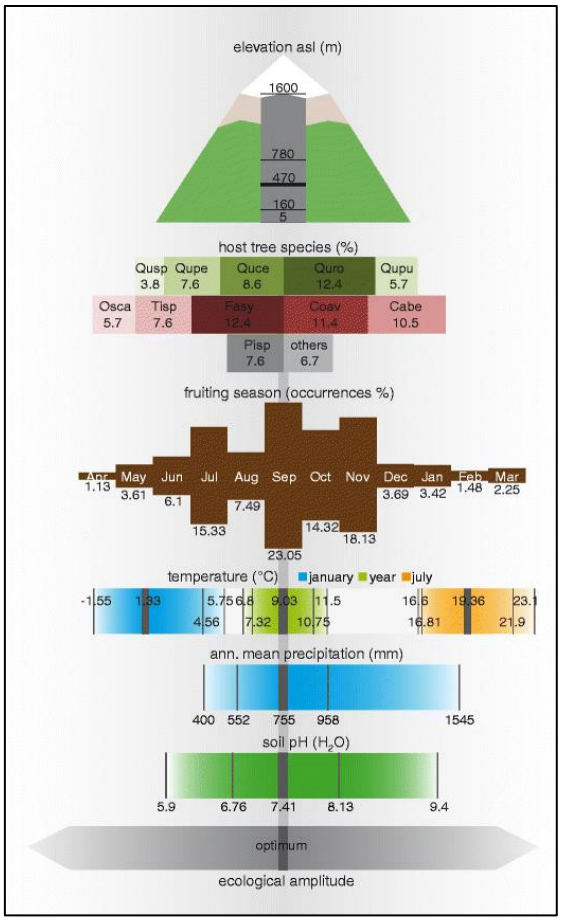

Figure 5. Main ecological features of *Tuber aestivum* (altitude, host plants, fruiting season, temperature, average annual rainfall and soil pH). The thick line in the middle of each partial graphic indicates (for each parameter) the optimum value, followed outward by the standard deviation and the minimum and maximum, respectively. The relative proportions referring to the main host plants are shown. Species abbreviations: *Qusp* = *Quercus* spp., *Qupe* = *Quercus petraea*, *Quce* = *Quercus cerris*, *Quro* = *Quercus robur*, *Qupu* = *Quercus pubescens*, *Osca* = *Ostrya carpinifolia*, *Tisp* = *Tilia* spp., *Fasy* = *Fagus sylvatica*, *Coav* = *Corylus avellana*, *Cabe* = *Carpinus betulus*, *Pisp* = *Pinus* spp. From Stobbe *et al*., 2013 a.

1.4.2 *Tuber borchii* Vittad.

Although it has a lower commercial value than *T. magnatum* and *T. melanosporum*, *Tuber borchii* Vittad. (commonly known as "whitish truffle", "bianchetto" or "marzuolo") is particularly appreciated and is consumed fresh or used in preserved products. It is widely distributed all over Europe, from Southern Finland to Sicily and from Ireland to Hungary and Poland (Gezer *et al*., 2014; Hall *et al*., 2007; Jeandroz *et al*., 2008).

Tuber borchii was cultivated for the first time in Italy in 2000 (Zambonelli *et al*., 2000) and then it has been successfully introduced also outside its natural distribution range, even in the Southern hemisphere (New Zealand), thanks to its ability to adapt to different types of soil and a wide range of climatic conditions. Indeed, fruiting bodies can develop in calcareous (pH 7-8), clayey, but also sandy and acidic soils, within broad-leaved, conifer or mixed woods, from areas located at sea level up to 1000 meters of altitude.

Tuber borchii forms ectomycorrhizas with numerous host plant species, such as oaks (*Quercus pubescens, Q. ilex, Q. cerris, Q. petraea*), hazel trees (e.g. *Corylus avellana*), poplar trees (*Populus alba*, *P. nigra, P. tremula*), lindens (*Tilia* spp.), beeches (e.g. *Fagus sylvatica*), chestnuts, alders, firs (*Abies* spp.), pine trees, (*Pinus nigra, P. pinea, P. halepensis*), cedar trees (*Cedrus* spp.), and *Cistus* spp. (Angelini *et al*., 2016; Hall *et al*., 2007; Iotti *et al*., 2010).

The maturation period goes from autumn to spring.

Tuber borchii ascocarps have a globular to lobed or irregular shape, often with a depression at the base, and are usually small, with a diameter between 2 and 7 cm (Figure 6a).

The peridium (100-500 µm thick), slightly pubescent when the fruiting bodies are immature, appears glabrous, humid and smooth at maturity, whitish to greyish-yellow and brown ochre in colour, often with reddish spots. The gleba, initially whitish/pale ochre, tends to fawn and reddish-brown with maturation. It has a smooth plectenchimatic structure and it is crossed by rather wide whitish veins, ramified from multiple points of the peridium and anastomosed (Figure 6b). Asci are mostly globular in shape, sessile or barely pedunculated, with a number of spores generally between 1 and 3 (Figure 6c). Ascospores, which are typically ellipsoidal (rarely sub-spherical), reddish-brown in colour, show an alveolate-reticulum with more or less regular polygonal alveoli (Figure 6d; Angelini *et al*., 2016).

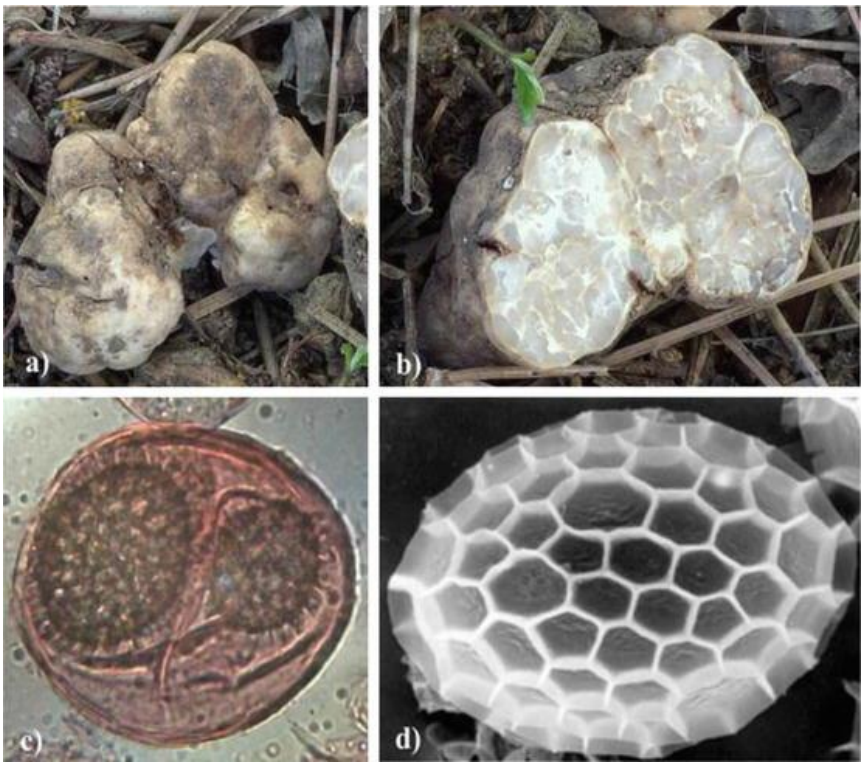

Figure 6. Morphological traits of *Tuber borchii* Vittad. **(a)** Truffle sample (ascocarp); **(b)** truffle section showing the inner part (gleba) of the fruiting body; **(c)** light microscope image of an ascus containing two spores; **(d)** scanning electron microscope (SEM) image of an ascospore with small regular alveoli. From Angelini *et al*., 2016.

1.4.3 *Tuber magnatum* Picco

The Italian/Piedmont white truffle *Tuber magnatum* Pico was officially recognised as a new *Tuber* species in 1788 by the Turinese physician Vittorio Picco (hence the suffix Pico that, according to Pacioni *et al*. (2018), would be a mistranslation of Picco).

This species is the most valued, and also the most difficult to find and cultivate (Christopolous *et al*., 2013). Indeed, compared to other truffles of economic interest, *T. magnatum* presents a narrower geographical distribution, since it grows spontaneously only in some European countries (Belfiori *et al*., 2020; Rubini *et al*., 2005). Moreover, although the attempts to cultivate *T. magnatum* date back to the 1970s, they have often failed (Iotti *et al*., 2014). Only recently, it has been possible to collect white truffle ascocarps within a cultivated orchard in France, outside the natural geographic range of this species (Bach *et al*., 2021).

T. magnatum ascocarps are generally found, from autumn to early winter, in valley bottoms and ditches below 700-800 m of altitude, in slightly inclined and not very sunny areas. Fruiting bodies develop in calcareous marly soils, composed of sandstone, marl, marly limestone and marly clay, but not in sandy or siliceous terrains. They also require tilled, slightly moist but drained soils, with a pH between 7 and 8, poor in humus, phosphorus (P), and nitrogen (N).

Amongst the host plants, which most commonly form ectomycorrhizal symbioses with *T. magnatum* fungi, there are *Quercus* spp. (such as, *Q. robur*, *Q. petraea*, *Q. pubescens*, *Q. cerris*, and *Q. ilex*), *Populus* spp. (e.g. *P. alba*, *P. nigra*, and *P. tremula*), *Salix* spp. (*S. alba*, for instance), *Tilia* spp., *Corylus avellana*, *Ostrya carpinifolia*, *Carpinus betulus*, *Pinus pinea*, and *Abies alba* (Angelini *et al*., 2016).

Tuber magnatum ascocarps may appear globose, lobed, gibbous, or flattened, often of irregular shape and of variable size (from 1-2 cm up to 10-15 cm and beyond). They can be yellowish, pale ochre, olivaceous or greenish grey in colour, often with reddish tones and black/brownish spots on the surface. The peridium is smooth and suede-like, slightly papillose, adherent to the gleba, 50-500 µm thick (Figures 7a and 7b). The gleba, which is crossed by numerous, fine, clear, and branched veins, varies in colour from whitish, pale yellow, light ochre, to hazel/brown, often with reddish patches (Figure 7c). These different tones are related to the maturity degree of the ascocarps, the type of soil, and the symbiont species (Amicucci *et al*., 2018; Angelini *et al*., 2016; Hall *et al*., 2007).

Asci are usually globose, sessile or short stalked, and contain from 1 to 4 spores (Figure 7d). *Tuber magnatum* ascospores are globous or ovoid, yellowish/pale ochre to brownish in colour, and are typically reticulate/alveolate, with more or less regular alveoli characterised by internal crests (Figure 7e) (Angelini *et al*., 2016).

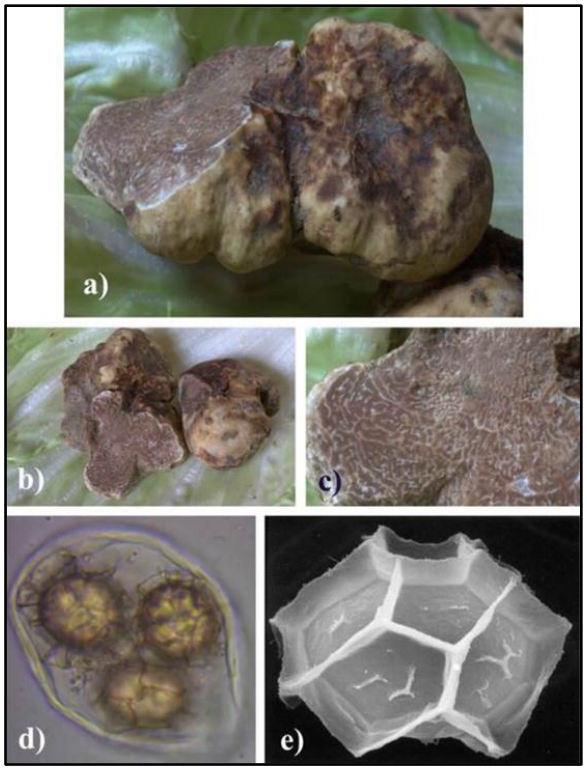

Figure 7. Morphological traits of *Tuber magnatum* Picco. **(a)** and **(b)** Italian white truffle ascocarps; **(c)** truffle section showing the inner part (gleba) of the fruiting body; **(d)** light microscope image of an ascus containing three spores; **(e)** scanning electron microscope (SEM) image of an ascospore with wide alveoli. From Angelini *et al*., 2016.

1.4.4 *Tuber melanosporum* Vittad.

The Périgord black truffle, *Tuber melanosporum* Vittad., is a well-defined species described for the first time by Carlo Vittadini (1831) in his "*Monographia Tuberacearum*". The sequencing of its genome (Martin *et al*., 2010) allowed to clarify many aspects related to the truffle biology.

Tuber melanosporum is naturally distributed in Southern Europe, primarily in France, Italy, and Spain, where it can grow in areas characterised by different climatic conditions, from the warmer Mediterranean regions (Southern Spain and Italy) to the colder continental areas, such as Alsace (North-Eastern France), from 100 to 1100 meters above sea level (Angelini *et al*., 2016). Moreover, thanks to artificial cultivation techniques, it has also been introduced in several countries where it is not endemic, such as USA, Australia, Morocco, New Zealand, South Africa, China, and Sweden (Belfiori *et al*., 2012; Chen *et al*., 2016; Jeandroz *et al*., 2008; Parladé *et al*., 2013; Rubini *et al*., 2011; Wedén *et al*., 2013).

In Europe, *Tuber melanosporum* cultivation began in the 1970s and, to date, it has achieved such success that over 90% of the Périgord black truffle currently produced in France comes from cultivated truffle grounds (Daba *et al*., 2019; Reyna and Garcia-Barreda, 2014).

Tuber melanosporum forms ectomycorrhizal symbioses with a wide range of host plants, mainly belonging to the genera *Quercus*, *Corylus*, *Populus*, *Tilia*, *Ostrya*, *Carpinus*, *Cistus*, *Pinus*, and *Cedrus* (Chen *et al*., 2016; Taschen *et al*., 2015).

Fruiting bodies, harvested from autumn to winter, develop in calcareous or in calcareous and clayey soils (clay percentage ≤ 40%), sandy or rocky, with a pH between 7.5 and 8.5, characterised by the presence of iron (Fe) and microelements, and small amounts of nitrogen (N), phosphorus (P), and potassium (K) (Angelini *et al*., 2016).

Tuber melanosporum ascocarps, generally globose, sometimes lobed and irregularly shaped, are compact and have a diameter between 2 and 10 cm (Figure 8a). The peridium, blackish-brown, often with dark red/rusty spots, is characterised by the presence of pyramidal warts, flattened or depressed at the apex, 3-6 mm wide at the base (Figure 8c). It is up to 700 µm thick and is strongly adherent to the gleba, whose colour tends to purplish-black or brownish-black with violet highlights. Numerous thin, branched, whitish, sterile veins cross the gleba (Figure 8b).

Asci, sessile or short stalked, have a globular shape and generally contain 1 to 6 spores (Figure 8d). Ascospores are of an intense brownish colour, with an elongated ellipsoidal shape, ornamented with short (2.5-3 µm) and rigid spines (Figure 8e; Angelini *et al*., 2016).

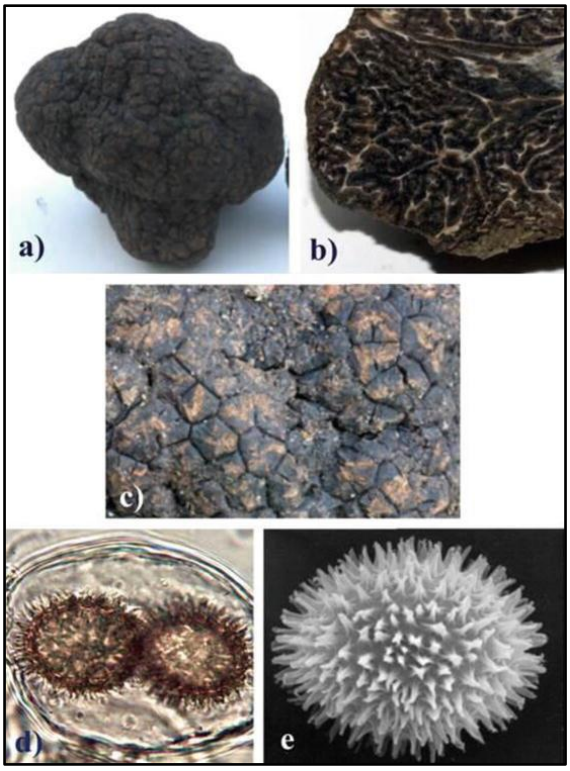

Figure 8. Morphological features of *Tuber melanosporum* Vittad. **(a)** Truffle ascocarp; **(b)** truffle section showing the gleba with sterile clear veins and fertile dark veins; **(c)** detail of the warty peridium; **(d)** light microscope image of an ascus containing two aculeate spores; **(e)** scanning electron microscope (SEM) image of an ascospore with the characteristic spines. From Angelini *et al*., 2016.

1.5 Truffle (*Tuber*) life cycle.

Tuber species undergo a complex life cycle, during which the mycelium establishes ectomycorrhizal symbioses with the roots of several trees (mainly oak, poplar, willow, and hazel) and shrubs (e.g. *Cistus*). As a final step, hyphae aggregate to form the fruiting body that contains asci and ascospores, which are produced following meiotic events (Figure 9; Mello *et al*., 2006).

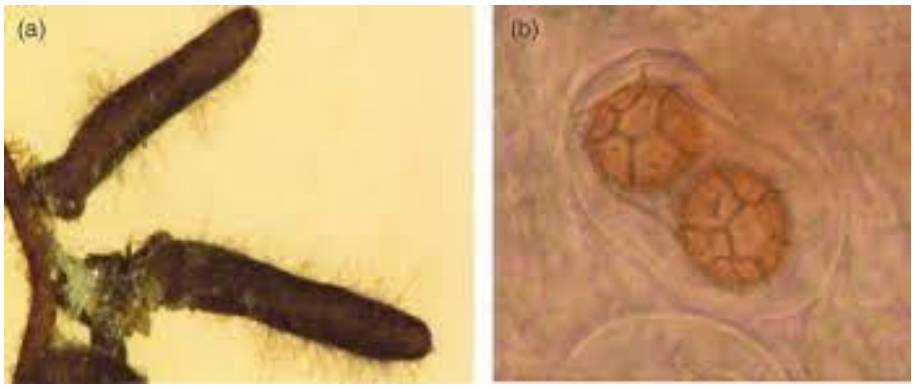

Figure 9. Mycorrhizas **(a)** and ascospores **(b)** of the white truffle *Tuber magnatum*. From Mello *et al*., 2006.

As shown in Figure 10, the life cycle of truffle takes generally place in six phases. It starts with the germination of haploid spores and the development of the haploid mycelium into the soil (steps 1 and 2). This first saprophytic phase of vegetative growth is followed by the establishment of a symbiotic association between the fungus and plant roots (phase 3) and by the formation of ectomycorrhiza (phase 4), a new organ that is functionally and morphologically distinct from the two original partners (Antony-Babu *et al*., 2014; Daba *et al*., 2019; Vita *et al*., 2015). Ectomycorrhizas are thought to give rise to antheridia and ascogones, which produce male and female gametes respectively. After plasmogamy, these structures of opposite mating type form an ascogenous heterokaryotic tissue surrounded by homokaryotic maternal tissues. The growth of these tissues leads to the formation of the ascocarp, which remains linked to the ectomycorrhizas through extrametrical hyphae until its complete maturation (Antony-Babu *et al*., 2014).

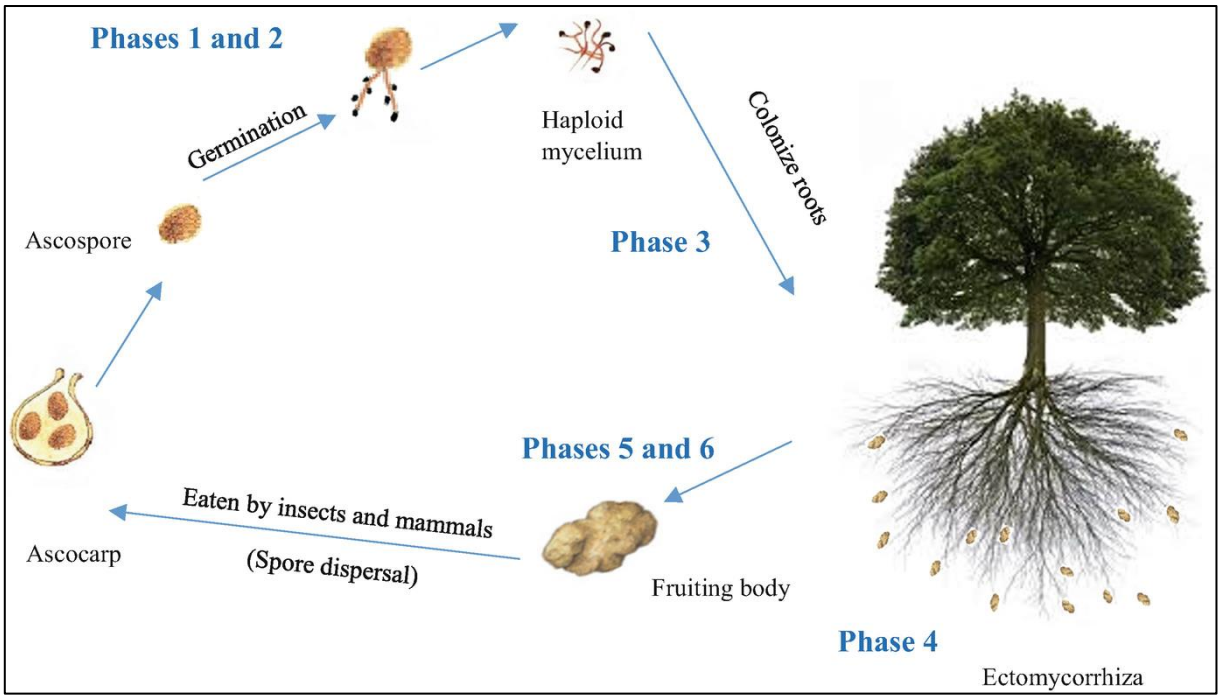

Figure 10. Main stages of *Tuber* life cycle: "saprotrophic phase" with the proliferation of the mycelium in the soil (steps 1 and 2); "ectomycorrhizal phase" that leads to the formation of ectomycorrhizas following the mutual recognition between the fungus and the host plant (steps 3 and 4); "reproductive phase" with the ascocarp formation (steps 5 and 6; Amicucci *et al*., 2018). From Daba *et al*., 2019.

Ascocarps develop starting from the so-called primordium, a clear yellowish mycelial pellet of 80-350 μm in size, covered by radiating hyphae in contact with the soil. When these hyphae disappear, the surface becomes defined and the cell walls of the external layers thicken, forming a peridium that protects the inner part (gleba). In the following months, the fruiting body grows up and sporogenesis occurs after differentiation of the ascogenous tissues. The growth of the ascocarp involves the formation of repetitive cracks in the peridium (Antony-Babu *et al*., 2014).

Therefore, in the final stage of *Tuber* life cycle (phases 5 and 6), the mycelium is organised into the fruiting body, whose role is to produce sexual fructifications (ascospores) that will be subsequently dispersed in the environment by insects and mammals (Figure 11). Then, vegetative mycelia develop from the germination of these spores, originating a new extra-radical phase and completing the truffle life cycle (Daba *et al*., 2019; Vita *et al*., 2015).

Unlike many epigeous fungi, which develop and reach maturity within a few days, truffles require up to 6 months to mature (Antony-Babu *et al*., 2014).

Figure 11. Detail of truffle life cycle. Sexual reproduction requires two compatible mycelia of opposite mating type, *MAT* (+) and *MAT* (-), from whose fusion the fruiting body (the truffle) develops. **(a)** Particular of the gleba containing the ascospores that can be *MAT* (+) or *MAT* (-). Adapted from Morcillo *et al*., 2015.

The life cycle of *Tuber* is affected by numerous biotic and abiotic factors, which can promote or inhibit ascocarp formation. Such factors include the nutritional status and the mutual communication with the host plant before and after the establishment of the ectomycorrhizal symbiosis, the soil composition/properties, the climatic and/or stress conditions (e.g. temperature, drought, rainfall, contaminants in the soil), the microbial communities of the mycorrhizosphere (especially the bacterial communities), and the mesofauna (Amicucci *et al*., 2018; Mello *et al*., 2006, 2013).

Moreover, differences in the capability of colonising the roots of the host plant were reported between strains of the same truffle species (Giomaro *et al*., 2000).

Undoubtedly, the complexity of the "truffle ecosystem" results in a considerable inter- and intraspecific diversity, which helps to understand the variability observed in the biological cycle between the various *Tuber* spp. (Amicucci *et al*., 2018).

1.6 Microbial (bacterial) communities associated with truffles.

Interactions between fungi and bacteria have long been studied in mycology (Waksman, 1927). Some bacterial species are beneficial to fungi, promoting the establishment of mycorrhizal symbiosis and the formation of fruiting bodies (Aspray *et al*., 2006; Frey-Klett *et al*., 2007, 2011), whereas other bacteria can control fungal sporulation and pathogenicity (Benucci and Bonito, 2016; Partida-Martinez and Hertweck, 2005).

Tuber species harbour complex microbial communities of bacteria, yeasts, guest filamentous fungi, and viruses, with whom they interact both in the mycorrhizosphere and in the ascocarp. Indeed, truffles are in close contact with microorganisms throughout their life cycle (spores, mycelium, ectomycorrhiza, fruiting body), to the point that bacteria can be considered the third partner of the symbiosis between *Tuber* and its host plant. It seems that bacterial communities play a central role in the complex biological processes of signalling and nutrient exchanges involving hyphae, ectomycorrhizas and ascocarps (Barbieri *et al*., 2016; Benucci and Bonito, 2016; Ratti *et al*., 2016; Splivallo *et al*., 2015a, 2019; Stielow and Menzel, 2010; Vahdatzadeh *et al*., 2015, 2019).

A combination of culture-dependent and independent methods has been used to investigate the truffle microbiota, by focusing on the species of greatest commercial interest (*Tuber aestivum*, *Tuber borchii*, *Tuber magnatum*, and *Tuber melanosporum*).

Bacteria heavily colonise both the inner tissues (gleba) and the surface (peridium) of fruit bodies, with densities ranging from millions to billions of cells per gram of truffle (dry weight) (Figure 12; Reale *et al*., 2009; Splivallo *et al*., 2015a, 2019; Vahdatzadeh *et al*., 2015, 2019).

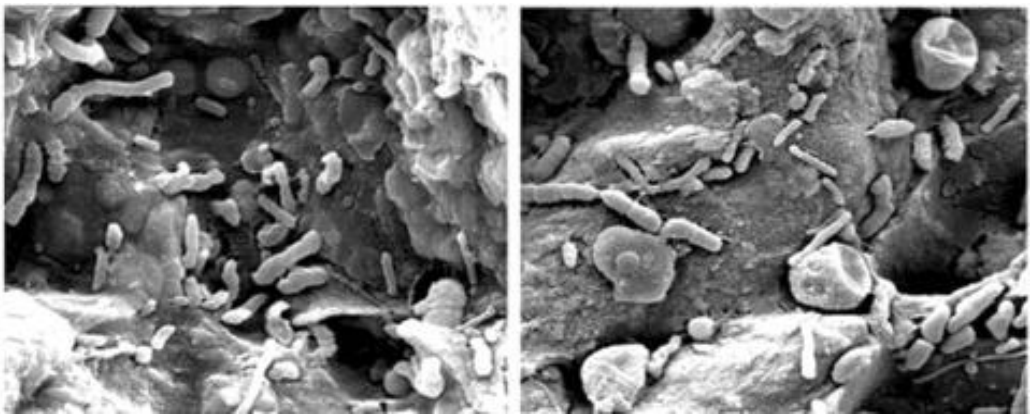

Figure 12. Scanning electron microscope (SEM) images of the external surface of fresh truffles immediately after collection, showing bacterial cells that colonise the peridium. From Reale *et al*., 2009.

Peridium and gleba seem to attract specific bacterial genera (Barbieri *et al*., 2016), which appear to be selected from the soil communities during the early stage of truffle formation (Antony-Babu *et al*., 2014; Monaco *et al*., 2020; Splivallo *et al*., 2019; Vita *et al*., 2020). Although factors responsible for the selection of these bacteria remain largely mysterious (Splivallo *et al*., 2015a), Antony-Babu and colleagues (2014) proposed the following model: soil bacteria would colonise truffle primordia before the differentiation of ascocarpic tissues occurs, when the primordium is directly in contact with soil. Subsequently, after the differentiation of the peridium, bacteria would be trapped in the gleba and partly protected from soil exchanges by the warty peridium, which on the contrary remains in contact with ground throughout the ascocarp development. Due to this compartmentalisation, the composition of the bacterial communities would mainly evolve in response to changes in the physiology of the maturing ascocarp (Antony-Babu *et al*., 2014; Vahdatzadeh *et al*., 2015). In addition to natural variations, the harvest of truffles could induce changes in the associated bacterial communities, because of modifications in physicochemical parameters, such as temperature and CO_2 level (Rivera *et al*., 2010 b; Vahdatzadeh *et al*., 2015).

The composition of *Tuber* bacterial communities is also influenced by the life cycle stage of the fungus. Indeed, significant variations in the microbial community structure between soil, ectomycorrhizas and truffle fruiting bodies have been reported (Vahdatzadeh *et al*., 2015).

Comparative analysis of the bacterial communities associated with fruiting bodies and ectomycorrhizas (ECMs) of *T. melanosporum* also showed considerable differences. The *Actinobacteria* phylum was dominant in the ECMs but poorly represented in the ascocarps and vice versa for the *α-Proteobacteria* class, suggesting that the fungus could provide two different habitats to bacteria (Figure 13; Antony-Babu *et al*., 2014; Vahdatzadeh *et al*., 2015). Moreover, an enrichment in several genera of *Actinobacteria* has also been observed within *T. melanosporum* orchards in specific zones called brûlés, which are particularly rich in truffle mycelia (Mello *et al*., 2013; Suz *et al*., 2008).

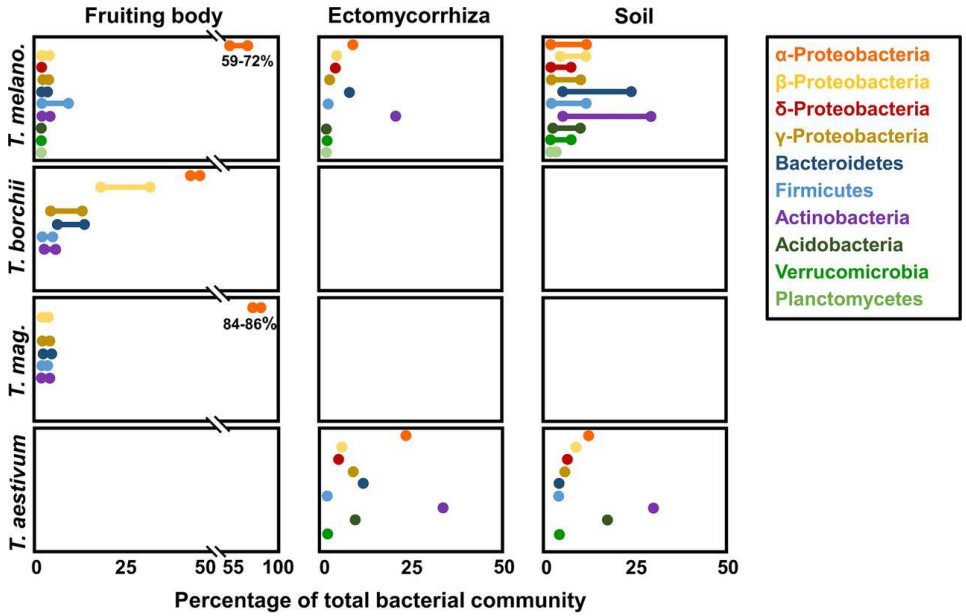

Figure 13. Microbial communities associated with truffle fruiting bodies (on the left), ectomycorrhizas (in the middle), and soil (on the right). The main bacterial phyla associated with four *Tuber* species (*T. aestivum*, *T. magnatum* [*T. mag.*], *T. borchii*, and *T. melanosporum* [*T. melano.*]) are shown. The displayed results were obtained by using culture-independent methods. The bars represent the minimum and maximum values reported in the literature whereas the points refer to a single value available in literature. From Vahdatzadeh *et al*., 2015.

Overall, the bacterial communities associated with truffle ascocarps consist mainly of *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. As shown in Figure 13, *T. borchii*, *T. magnatum* and *T. melanosporum* fruiting bodies share a dominance of α-*Proteobacteria* and relatively low

percentages of *Firmicutes* and *Actinobacteria*, whereas β-*Proteobacteria*, γ-*Proteobacteria* and *Bacteroidetes* appear more abundant in *T. borchii* than in the other truffle species (Amicucci *et al*., 2018; Barbieri *et al*., 2005, 2007; Gryndler *et al*., 2013; Monaco *et al*., 2020, 2021; Sbrana *et al*., 2002; Vahdatzadeh *et al*., 2015).

The characterisation of the bacterial communities colonising the gleba of the most commercially relevant truffles (e.g. *T. aestivum*, *T. borchii*, *T. magnatum*, and *T. melanosporum*) highlighted the presence of a "core microbiota" mainly constituted of species belonging to the *α-Proteobacteria* class, with members of the *Rhizobiaceae* and *Bradyrhizobiaceae* families representing a common denominator (Barbieri *et al*., 2005, 2016; Perlińska-Lenart *et al*., 2020; Vahdatzadeh *et al*., 2015, 2019). Notably, bacteria in the genus *Bradyrhizobium* are constantly associated with truffle ascocarps. *Bradyrhizobium* spp. were detected for the first time within *T. magnatum* fruiting bodies by Barbieri and colleagues (2010), who speculated that these bacteria could be involved in nitrogen fixation, with a potential role in the fungal growth or nutrition during ascocarp development (Barbieri *et al*., 2010; Benucci and Bonito, 2016). A high presence of *Bradyrhizobium* sequences has been reported also in *T. melanosporum* (Antony-Babu *et al*., 2014; Benucci and Bonito, 2016) and *T. aestivum* (Monaco *et al*., 2020; Splivallo *et al*., 2019) ascomata.

Therefore, it is likely that ascocarps provide a specific habitat for a "core truffle microbiota", irrespective of the species. Nevertheless, various bacterial taxa could complement and expand this common microbiota depending on the *Tuber* species and environmental factors (Perlińska-Lenart *et al*., 2020). For example, the genus *Acidovorax* (β-*Proteobacteria*) seems to be associated with *T. aestivum* fruiting bodies (Monaco *et al*., 2020; Perlińska-Lenart *et al*., 2020) but it was not found in the white truffles *T. borchii* (Barbieri *et al*., 2005) and *T. magnatum* (Niimi *et al*., 2021). Other bacterial taxa appear to be related only to samples from certain geographical areas and, thus, they could represent potential markers of truffle origin (Monaco *et al*., 2021; Niimi *et al*., 2021; Perlińska-Lenart *et al*., 2020).

Several studies based on culture-dependent methods highlighted that *Tuber* fruiting bodies also host viable populations of pseudomonads and spore-forming bacilli (*Bacillus* spp.) (Bedini *et al*., 1999; Citterio *et al*., 1995; Nuti and Sbrana, 2013; Perlińska-Lenart *et al*., 2020; Rivera *et al*., 2010 a).

Bedini *et al*. (1999) isolated from *T. borchii* ascocarps about 300 strains of *Pseudomonas* (mainly belonging to *P. fluorescens, P. corrugata*, and *P. tolaasi* species) able to produce indole-3-acetic acid (IAA), which is the most common phytohormone of the auxin class. Together with ethylene released by *Tuber* spp., indole-3-acetic acid promotes the formation of ectomycorrhizas (Daba *et al*., 2019). Furthermore, some of the *Pseudomonas* isolates were also producers of biocontrol agents, such as hydrogen cyanide (HCN), proteases, fluorescent siderophores, and 2,4-diacetylphloroglucinol (DAPG), which is active against numerous organisms, including plant pathogens (Bedini *et al*., 1999; Meyer *et al*., 2009). Ultrastructural examination of *T. borchii* ascocarps revealed the presence of *Pseudomonas* and *Bacillus* strains in the interhyphal space, with some bacterial cells adhering to the ascus wall. It has been suggested that these bacteria (positive for the degradation test of cellulose and chitin, the main components of the hyphal walls), could be involved in ascus opening, contributing to the release of the ascospores, thanks to their cellulolytic and chitinolytic activities (Amicucci *et al*., 2018; Citterio *et al*., 2001; Gazzanelli *et al*., 1999; Nuti and Sbrana, 2013).

It is noteworthy that considerable differences in the composition of the bacterial communities associated with *Tuber* ascocarps emerged depending on the analysis method (culture-dependent or independent). If on one side, the 16S rDNA sequencing generally shows a high percentage of α-*Proteobacteria*, mainly represented by *Bradyrhizobium* species, on the other side, cultivation-based approaches reveal a predominance of pseudomonads and *Bacillus* strains (Bonfante and Anca 2009; Perlińska-Lenart *et al*., 2020).

It is known that culture-dependent methods have some limitations: in fact, many bacterial species are difficult to grow due to their interdependence with other microorganisms or because of the lack of knowledge regarding their specific growth conditions and nutritional requirements. Thus, since microorganisms that can be cultured in the laboratory represent only a small fraction of the total

diversity existing in nature (Stewart, 2012), even the bacterial species isolated so far from truffles could constitute just a few percent of the natural communities factually associated with ascocarps (Perlińska-Lenart *et al*., 2020). Hence, while molecular techniques (such as metagenomic sequencing) allow to describe more accurately the bacterial communities as a whole, the full comprehension of the physiology of the truffle bacteria and their ecological role requires a cultivation approach.

More than 200 volatile organic compounds (VOCs) have been isolated from ectomycorrhizal roots, mycelia and fruiting bodies of *Tuber* species (Daba *et al*., 2019; Menotta *et al*., 2004; Zeppa *et al*., 2004). These volatiles (alcohols, esters, ketones, aldehydes, aromatic and sulphur compounds) play a central role in truffle biology. Indeed, they are "communication tools" involved in the signalling between truffle and its host plant, can act as mycorrhization signals and, because of a phytotoxic action, may have a role in the formation of the so-called "burnt", an area without herbaceous cover around *Tuber* symbiotic plants. Moreover, VOCs are responsible for truffle aroma, which beyond attracts mammals, is perceived by humans (Culleré *et al*., 2010; Liu *et al*., 2012; Menotta *et al*., 2004; Pacioni, 1991; Splivallo and Ebeler, 2015b; Splivallo *et al*., 2007, 2011, 2015a; Tarkka and Piechulla, 2007). Actually, only a small fraction (15 to 20 odorants for each species) of the hundreds of volatile compounds that constitute truffle flavour is detectable by humans (Schmidberger and Schieberle, 2017; Vahdatzadeh *et al*., 2019).

Interestingly, the microbial communities of bacteria, yeasts, and fungi associated with *Tuber* species are involved in the elaboration of the complex aroma of truffles through the production of sulphur volatile compounds (Benucci and Bonito, 2016; Buzzini *et al*., 2005; Daba *et al*., 2019; Splivallo *et al*., 2012, 2015a; Vahdatzadeh *et al*., 2015). Some of these chemicals are common to several *Tuber* species and might be of mixed truffle and microbial origin, whereas other odorants are species-specific and could derive only from microbes (e.g. 2,4-dithiapentane in *T. magnatum*, thiophene derivatives in *T. borchii*). It is likely that bacteria, the dominant group in truffle microbiota, are the most important contributors to *T. aestivum*, *T. borchii*, *T. magnatum*, and *T. melanosporum* fruiting

body aroma (Figure 14), with implications also on truffle preservation. Indeed, the progressive replacement of the commensal microbiota with food spoilage bacteria during truffle storage reflects changes in the volatile profiles and could be related to the loss of the typical flavour of fresh truffles (Vahdatzadeh *et al*., 2015, 2019).

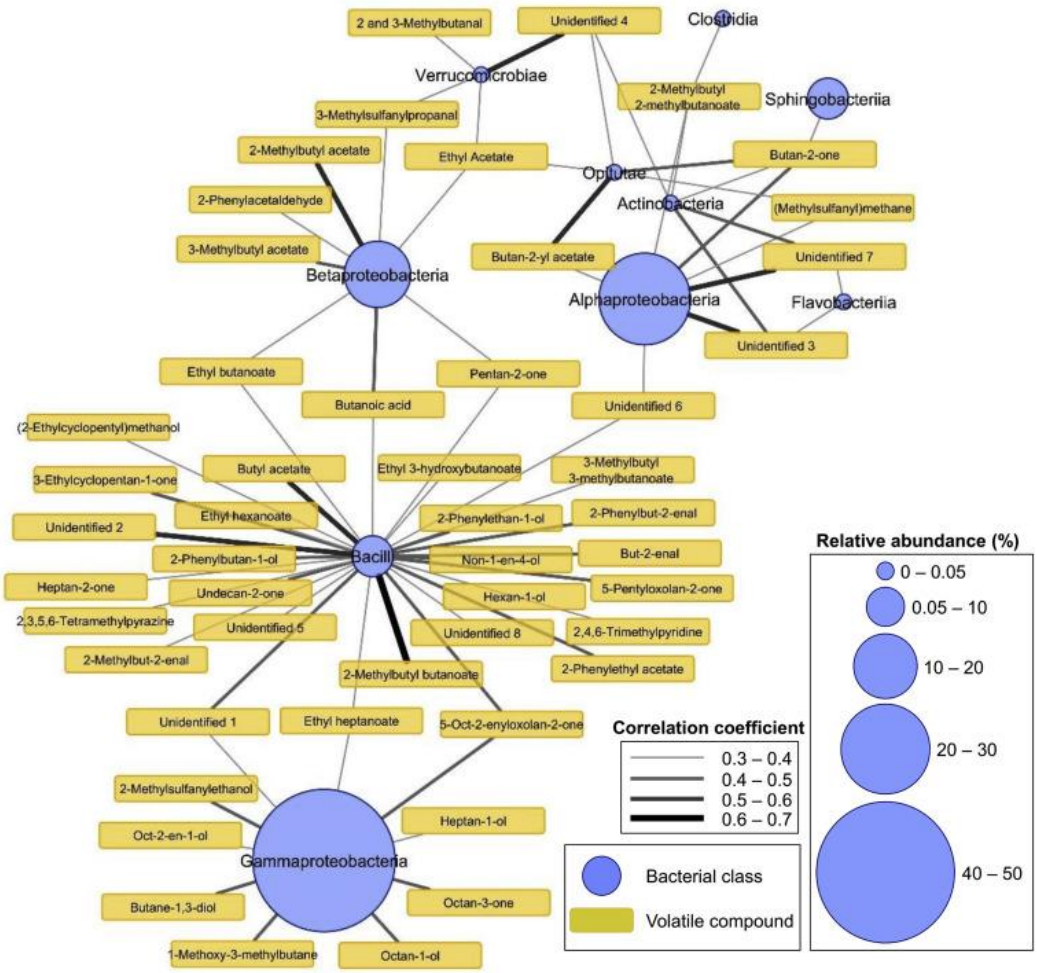

Figure 14. Correlation network between volatile compounds and bacterial classes associated with *T. aestivum* ascocarps during truffle storage. From Vahdatzadeh *et al*., 2019.

Thus, in summary, *Tuber* species provide variable microhabitats hosting complex and changeable microbial communities (Perlińska-Lenart *et al*., 2020). In exchange for water and nutrients, bacteria:

- Produce biostimulants (phytohormones, such as IAA, and specific amino acids);

- Promote the growth of fungal mycelium and ectomycorrhiza formation (MHB) (Frey-Klett *et al*., 2007);
- Participate in the development and maturation of truffle ascocarps (Amicucci *et al*., 2018; Antony-Babu *et al*., 2014; Mello *et al*., 2010);
- Contribute to the release of the ascospores thanks to their cellulolytic and chitinolytic activities and could be involved in spore germination (Gazzanelli *et al*., 1999);
- Inhibit/counteract the growth of pathogenic microorganisms and contaminating fungi through the production of antimicrobial substances;
- Contribute to truffle aroma by synthesising sulphur volatile compounds that attract mammals; in this way, bacteria take indirectly part in the dissemination of truffle spores, playing a key role in the life cycle of the fungus (Splivallo *et al*., 2015).

Despite the availability of information regarding the structure and diversity of the microbial communities associated with *Tuber* species in the different phases of the truffle life cycle, many aspects related to the role of bacteria and the effects of the interactions among microbial communities on the biology of truffle still remain to be clarified.

1.7 Molise region: land of truffles.

Molise region occupies a small territory (≈ 4,440 Km^2) in the Central-Southern Apennines (Italy). It is bordered by Abruzzo, Puglia, Lazio, and Campania, and has a population of about 300,000 inhabitants (Istat, 2021; http://dati.istat.it/Index.aspx?QueryId=18546#).

This region is one of the most important Italian areas suited to truffle collection. In fact, Molise woods are particularly rich in the prized white truffle *Tuber magnatum* Picco and summer black truffle *Tuber aestivum* Vittad., the so-called "scorzone". The best-known areas for the presence of *T. magnatum* are San Pietro Avellana and Carovilli in the high-Molise territory, Roccamandolfi (Isernia province), Bojano and San Massimo in the province of Campobasso (Annexes A and B).

Although official data related to the average annual production of truffles are not available, authoritative local associations, such as "Molise Food", indicate that Molise region contributes about 40% (of which 80% is destined for export) to the national production of this valuable resource. It is estimated that the total amount of truffles annually collected in the Molise forests is between 30 and 70 quintals for *T. magnatum* and approximately 300 quintals for *T. aestivum*, whereas the annual production of "bianchetto" (*T. borchii*) and other *Tuber* species is overall around 50 quintals (https://www.agi.it/lifestyle/news/2021-01-11/molise-diventa-patria-tartufo-10978317/).

"The National Plan of the truffle supply chain 2017-2020" (https://www.politicheagricole.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/11100) reports another interesting *datum* about the high percentage of truffle hunters operating in the region. Indeed, in relation to the resident population, it is the highest in Italy (1.46%). This could be related, on one side, to the high truffle vocation of the territory and the wide availability of collection areas, and on the other side, to the high unemployment rate in the region that makes truffle harvesting an important source of income in the rural areas.

1.8 Aims of the research.

Although it represents an important resource for the local economy, to date Molise truffle has received very little attention from a scientific point of view compared to truffles from other Italian regions (see, for example, the renowned "Alba white truffle") or Countries. As a consequence, it is not adequately valorised and preserved. Therefore, this research aimed at filling the existing gaps of knowledge, paving the way for a preliminary characterisation of Molise truffle. Furthermore, given that many aspects related to the diversity and the potential role of truffle-associated bacteria remain largely unexplored (Splivallo *et al*., 2019), particular attention has been paid to the analysis of the microbiota of two commercially relevant *Tuber* species: *Tuber aestivum* Vittad. and *Tuber magnatum* Picco.

In detail, the main purposes of this work were:

1. Examine the bacterial communities associated with *T. aestivum* fruiting bodies harvested from natural truffle grounds in Vastogirardi municipality (Isernia province). The composition of truffle microbiota was also compared with that of soil samples collected around the ascocarps, in order to get an idea of the possible differences between the two habitats.

   The obtained findings have been published in the scientific journal "*Annals of Microbiology*" (Monaco P., Toumi M., Sferra G., Tóth E., Naclerio G., Bucci A. (2020) The bacterial communities of *Tuber aestivum*: preliminary investigations in Molise region, Southern Italy. *Ann Microbiol* **70**: 37. https://doi.org/10.1186/s13213-020-01586-5) and are reported in the Chapter 2 of this doctoral thesis.

2. Characterise from a morphological, genetic, and microbiological point of view two populations of *T. magnatum* collected in two different areas of Molise region. Overall, twenty-one white truffles were considered for the analysis (nine from natural truffle orchards located between Carovilli and Vastogirardi municipalities, twelve from a wider area on the border with Abruzzo).

   The results of this research, presented in Chapter 3, led to the publication of the paper "Heterogeneity of the white truffle *Tuber magnatum* in a limited geographic area of Central-Southern Italy" in the "*Environmental Microbiology Reports*" journal (Monaco P., Bucci A., Naclerio G., Mello A. (2021) Heterogeneity of the white truffle *Tuber magnatum* in a limited geographic area of Central-Southern Italy. *Env Microbiol Rep*. https://doi.org/10.1111/1758-2229.12956).

3. Determine the peridium thickness of the 21 *T. magnatum* ascocarps included in the previous study, in order to 1) fill the lack of information in scientific literature on this parameter and 2) obtain a more in-depth morphological characterisation of the Molise white truffles as reported in the short communication "Determination of the peridium thickness of *Tuber magnatum* ascomata from Molise region" published in the "*Italian Journal of Mycology*" (Monaco P., Naclerio G., Bucci A., Mello A. (2021) Determination of the peridium thickness

of *Tuber magnatum* ascomata from Molise region. *Italian Journal of Mycology* **50**: 92-98. https://doi.org/10.6092/issn.2531-7342/13052) (Chapter 4).

2. The bacterial communities of *Tuber aestivum*: preliminary investigations in Molise region, Southern Italy.

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ORIGINAL ARTICLE Open Access

The bacterial communities of *Tuber aestivum*: preliminary investigations in Molise region, Southern Italy

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Abstract

Purpose: Truffles are colonized by a complex microbial community of bacteria, yeasts, and filamentous fungi, whose role has not yet been fully understood. The main purpose of the research was to characterize the bacterial communities associated with *Tuber aestivum* Vittad. fruiting bodies collected from natural truffle grounds in the Molise region (Southern Italy). Despite it is one of the Italian richest areas of truffles, little is known about truffles in Molise.

Methods: Six ripe fruiting bodies of *Tuber aestivum* Vittad. and six soil samples were collected in July 2018 at Villa San Michele in the municipality of Vastogirardi, Molise region. Then, soil and truffle microbial communities were analyzed through 16S rRNA gene sequencing on the Illumina MiSeq platform and bioinformatics analyses.

Results: Consistently with previous studies, the main phyla retrieved in the investigated ascocarps were *Proteobacteria* and *Actinobacteria*, with the genus *Bradyrhizobium* particularly represented. Nevertheless, significant differences between soil and truffle microbiota and an unexpected heterogeneity across truffles were observed. It is likely that a specific recruitment of bacteria from soil to ascocarps occurs during the truffle formation and that local-scale factors play an important role in determining the structure of the investigated truffle microbial communities.

Conclusion: Although further analyses (based on a larger soil and truffle sample size and aimed at defining in more detail microbial diversity, soil physical and chemical properties, microclimatic conditions, and vegetation) are required to better understand which are these factors and how they could influence the composition of truffle bacterial communities, this study represents the starting point for a deepened characterization of this economically important product.

Keywords: Italian summer black truffle, *Tuber aestivum* Vittad., Bacterial communities, Next generation sequencing

Introduction

Most of terrestrial plant roots are colonized by mycorrhizal fungi which play a key role in soils by improving plant water and mineral nutrient uptake and receiving carbon compounds in return (Mello and Balestrini 2018). Among mycorrhizal symbioses, ectomycorrhizae (ECM) are mutualistic relationships between plants (both angiosperms and gymnosperms, as well as shrubs) and fungi (almost exclusively belonging to *Basidiomycetes* and *Ascomycetes*). A well-known example of ectomycorrhizal fungi is given by truffles, a polyphyletic group of hypogeous fungi whose fruiting bodies sequester their spores and develop underground, bringing benefits to the forest ecosystems and to the host plant (Pavić et al. 2013; Mäkelä et al. 2015; Vita et al. 2015; Benucci and Bonito 2016; Mello and Balestrini 2018). Different species of truffles are edible and highly sought after due to their organoleptic properties (Vita et al. 2015; Benucci and Bonito 2016). Among these, truffles belonging to the *Tuber* genus (including more than 180

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species) are considered very precious and delicious food (Pacioni et al. 2014; Schmidberger and Schieberle 2017). *Tuber magnatum* Pico, the Italian white truffle, and *Tuber melanosporum* Vittad., the Périgord black truffle, represent the most valued species on the food market due to their excellent flavor. *Tuber* spp. are widely distributed across Europe, North America, South East Asia, and limited parts of Africa and South America (Barbieri et al. 2005; Vita et al. 2015; Benucci and Bonito 2016; Iotti et al. 2016; Ye et al. 2018).

Truffles are colonized by a complex microbial community of bacteria, yeasts, and guest filamentous fungi (Splivallo et al. 2015; Vahdatzadeh et al. 2015). Bacteria can be found in inner and outer parts of truffle with densities ranging from a million to a billion cells per gram of fruiting bodies (dry weight) (Vahdatzadeh et al. 2015). They seem to be selected from the soil microbial communities during the early stage of truffle formation and could play an important role in the development, growth, and nutrition of fruiting bodies (Splivallo et al. 2015). Moreover, they contribute to truffle aroma through the production of volatile organic compounds (VOCs) (Splivallo et al. 2015; Vahdatzadeh et al. 2015; Benucci and Bonito 2016; Ye et al. 2018). A combination of culture-dependent and independent methods has been used to extensively investigate truffle-associated bacteria (Splivallo et al. 2015; Vahdatzadeh et al. 2015; Benucci and Bonito 2016) and showed that different *Tuber* species may harbor diverse bacterial communities, mainly constituted of *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* phyla (Vahdatzadeh et al. 2015). Nevertheless, recent studies highlighted that the genus *Bradyrhizobium* is particularly abundant in several *Tuber* species (Benucci and Bonito 2016).

Many of these investigations have been focused on *T. borchii* Vittad. and *T. magnatum* Pico (Sbrana et al. 2002; Barbieri et al. 2005; Barbieri et al. 2007; Pavić et al. 2013; Splivallo et al. 2015) whereas less is known on bacterial communities of *T. aestivum* Vittad. despite its wider geographic distribution (Büntgen et al. 2017). The main purpose of this research was to characterize the bacterial communities associated with *Tuber aestivum* fruiting bodies collected from truffle grounds in Molise region (Southern Italy), by using next generation sequencing techniques (NGS). Despite it has received less attention, Molise region represents one of the Italian richest areas of truffles.

Materials and methods

Study area and samplings

Six ripe fruiting bodies of *Tuber aestivum* and six soil samples were collected in July 2018 from natural truffle grounds at Villa San Michele in the municipality of Vastogirardi, Molise region (Southern Italy, Fig. 1a). The study area is located at an altitude of about 900 m above sea level (a.s.l.) with a vegetation composed by Turkey oak (*Quercus cerris*).

Tuber aestivum ascocarps were collected at a depth of approximately 10 cm in six different sites at a distance of some meters from each other. Truffles were dug out with the help of an expert person and a hunter/truffle dog by using a spade (Fig. 1b, c, d). Then, they were individually placed in sterile polypropylene containers. In addition, soil samples were also collected using sterile spoons and sterile polypropylene tubes under the fruiting bodies, at a depth of about 10–15 cm. Truffle and soil samples were then transported in a refrigerated container to the laboratory.

Tuber aestivum ascocarps were gently brushed with a sterile soft brush, rinsed with sterile distilled water and stored at − 20 °C before proceeding with biomolecular investigations. Species identification and maturation stage assessment were performed on the basis of morphological traits by experienced mycologists (Splivallo et al. 2015; Büntgen et al. 2017).

Fig. 1 **a** Localization of the study area. **b**, **c**, **d** Truffle hunting and ascocarp collection. The white arrow indicates a truffle ascocarp collected during the sampling campaign

Biomolecular investigations

DNA extraction

DNA was extracted from truffles (1t, 2t, 3t, 4t, 5t, 6t) and soil samples (1s, 2s, 3s, 4s, 5s, 6s) in order to assess bacterial diversity and taxonomic composition using Illumina amplicon sequencing of 16S rRNA genes (sample names with matching numbers indicate truffles and soils collected from the same site within the study area).

Tuber aestivum ascocarps (inclusive of peridium and gleba) were shredded and then pulverized by the addition of liquid nitrogen. Total genomic DNA was subsequently extracted from the fruiting bodies and soils by using the E.Z.N.A.® *Plant DNA DS Mini Kit* (Ye et al. 2018) and *E.Z.N.A.® Soil DNA Kit* (Omega Bio-tek, USA) respectively and following the manufacturer's instructions.

16S rRNA gene amplicon library preparation and sequencing

Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair Probio_Uni (5′-CCTACG GGRSGCAGCAG-3′) and Probio_Rev (5′-ATTACC GCGGCTGCT-3′), which target the V3 region of the 16S rRNA gene sequence (Milani et al. 2013; Di Luccia et al. 2018). Illumina adapter overhang nucleotide sequences were added to the partial 16S rRNA gene-specific amplicons, which were further processed employing the 16S Metagenomic Sequencing Library Preparation Protocol (Part #15044223 Rev. B–Illumina). Amplifications were carried out using a Verity Thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis on a 2200 TapeStation Instrument (Agilent Technologies, USA). DNA products obtained following PCR-mediated amplification of the 16S rRNA gene sequences were purified by a magnetic purification step involving the Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) in order to remove primer dimers. DNA concentration of the amplified sequence library was determined by a fluorimetric Qubit quantification system (Life Technologies, USA). Amplicons were diluted to a concentration of 4 nM, and 5 μL quantities of each diluted DNA amplicon sample were mixed to prepare the pooled final library.

16S rRNA gene sequencing was performed using an Illumina MiSeq System (Illumina, San Diego, CA, USA) at the GenProbio srl Laboratory (Milani et al. 2013; Di Luccia et al. 2018).

Bioinformatics analysis

Following sequencing, the .fastq files were processed using a custom script based on the QIIME software suite (Caporaso et al. 2010). Paired-end read pairs were assembled to reconstruct the complete Probio_Uni/Probio_Rev amplicons.

Quality control retained those sequences with a length between 140 and 400 bp and mean sequence quality score > 20 while sequences with homopolymers > 7 bp and mismatched primers were omitted.

In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at 100% sequence homology using DADA2 (Callahan et al. 2016); OTUs not encompassing at least 2 sequences of the same sample were removed. Notably, this approach allows highly distinctive taxonomic classification at single nucleotide accuracy (Callahan et al. 2016). All reads were classified to the lowest possible taxonomic rank using QIIME2 (Caporaso et al. 2010; Bokulich et al. 2018) and a reference dataset from the SILVA database (Quast et al. 2013).

Biodiversity within a given sample (alpha-diversity) was calculated with Shannon and Chao1 indices. Similarities between samples (beta-diversity) were calculated by weighted UniFrac (Lozupone and Knight 2005). The range of similarities is calculated between values 0 and 1. PCoA representations of beta-diversity were performed using QIIME2 (Caporaso et al. 2010; Bokulich et al. 2018).

To identify the core taxa retrieved in the soil and truffle habitats, microbial genera were analyzed for their presence or absence in each sample and the related Venn diagram drawn by Venny 2.1 (Oliveros 2007-2015).

For differential abundance analysis between soil and truffle microbial communities at phylum level, a negative-binomial-based approach in tandem with paired Wald test, as available in DESeq2 version 1.24.0 (Love et al. 2014) in R environment (R core team 2019), was performed.

In order to structure soil, truffle, or soil-truffle microbiomes, co-occurrence patterns were determined by applying *rcorr* function from Hmisc package in R (Harrell Jr et al. 2019) and calculating correlations among species abundances by Pearson method. Correlation coefficient significance was also assessed by using the same R function.

Networks were composed by selecting highly significant correlations ($P < 0.01$) between species abundances. Specifically, networks were derived from abundances of species identified in the soil or in the truffle habitat and also from a dataset composed by the species identified in the soil and truffle habitats together. The analysis and visualization of the networks and the related statistical analysis were performed by Cytoscape (Shannon et al. 2003) and R.

Network structure was assessed by measuring the node degree of each node that is the number of partners each node has. Node degree is related to sparsity, a

property that relies on the number of connections observed in the network (Busiello et al. 2017). In a "complete network," each node is connected with all other nodes; thus, the average number of node degree is equal to the number of nodes minus 1. A lower mean node degree is a sign of a sparser network. The larger is the network the higher is the sparsity (Busiello et al. 2017). The density, as the number of observed edges respect all possible ones, shows how dense are the connections per node. In biological networks, density has been demonstrated to be lower than 0.1 (Leclerc 2008). This observation indicates that the structure of a biological network fits with a general sparse connected graph which gives evolutionary advantages in terms of resilience to possible network damages (Leclerc 2008; Pavlopoulos et al. 2011).

To have a stronger evidence about network structure, the centrality of the microbial populations present in the combined habitat network was measured in order to define the relative importance of soil species with respect to the truffle ones. To achieve this goal, node degree distributions of soil-truffle network were analyzed by considering first the totality of the nodes present, then only the nodes related to species identified in the soil habitat, and finally only the species identified in the truffle habitat. The frequency distribution of node degree provides information about the structure of a network (sparsity), and it is often used to compare the nature of networks (Liu et al. 2011; Nacher and Akutsu 2013; Suweis et al. 2013; Grilli et al. 2017). In order to achieve this goal, Welch's unequal variance *t* test (Welch 1947) on node degree distributions was applied.

Nodes relevant to the structure of the microbiomes (keystone nodes) were identified according to their node degree and betweenness centrality that is a measure of the shortest paths that pass through the node (Freeman 1977).

To test the robustness of the network and its ability to be resilient to species loss, an approach based on the progressive removal of species (nodes) according to their relevance in the network structure (Iyer et al. 2013; Ruiz et al. 2017), both considering node degree and betweenness centrality, was applied. The area under the curve (AUC) was calculated according to Gini's formula.

Results

NGS results allowed to obtain detailed information about the composition of microbial communities in soil samples and associated with summer black truffle (*Tuber aestivum*) ascocarps collected from six different truffle grounds in the Molise region (Fig. 1).

The analyses highlighted a high percentage (≈ 75%) of shared taxa at genus level (511). On the other hand, 114 (≈ 17%) and 55 (≈ 8%) genera were unique in soil and truffle, respectively (Venn diagram in Additional file 1). Actually, even though it seems that truffle could host peculiar bacterial communities including taxa absent in soil, those genera retrieved only in the truffle group were not found in all the analyzed six ascocarps and were often present at very low relative abundances.

Overall, important differences were found between soil and truffle groups even though a significant heterogeneity of ascocarp microbiota has also been detected. Principal Coordinate Analysis (PCoA) based on weighted UniFrac index revealed that the truffle microbial communities varied from those of the soil (Fig. 2). The rarefaction curves obtained by using Shannon and Chao1 indices for each sample highlighted a greater microbial diversity in soil samples compared to the *Tuber aestivum* fruiting bodies and differences among samples of the truffle group in terms of α-diversity (Additional file 2).

Differential abundance analysis at phylum level revealed the presence of 14 categories with significantly different relative abundances between soil and truffle (Fig. 3). Among these, the phyla *Tectomicrobia*, *Nitrospirae*, *Fibrobacteres*, *Planctomycetes*, *Gemmatimonadetes*, *Chloroflexi*, and *Acidobacteria* were more abundant in soil whereas *Proteobacteria*, *Saccharibacteria*, *Firmicutes*, *Cyanobacteria*, *Fusobacteria*, *Tenericutes*, and other unclassified

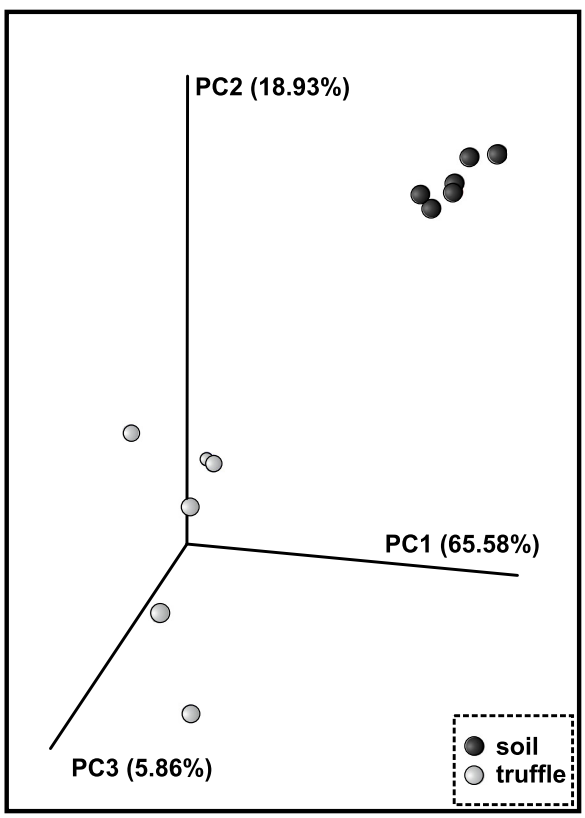

Fig. 2 Principal Coordinate Analysis (PCoA). Plot was generated using weighted UniFrac distance matrix. Soil (black spheres) and truffle (gray spheres) samples are shown

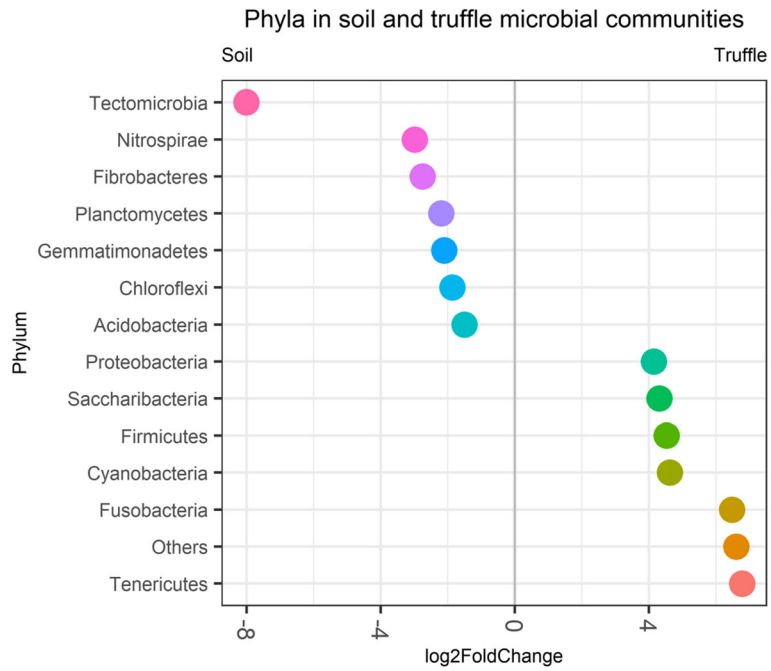

Fig. 3 Differentially abundant phyla in soil (on the left) and truffle (on the right) microbial communities

members of the *Bacteria* domain were found at significantly higher relative abundances in truffle (Fig. 3).

When analyzing the bacterial community composition on the whole, the main phyla retrieved in the analyzed *Tuber aestivum* fruiting bodies were *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* (Fig. 4). *Proteobacteria* ranked first in all truffle samples, with a relative abundance between 57.6 and 95.9%. The phylum *Actinobacteria* showed percentages ranging from 2.2 to 25.1%, with *Nocardioides* being one of the main bacterial genera present in the ascocarps. *Bacteroidetes* were found in one of the truffle samples with a relative abundance of 25.9%, while in the other fruiting bodies they ranged between 0.8 and 4.7%. *Firmicutes* occurred in percentages ranging from 0.2 to 12.1%.

Among *Proteobacteria*, the main families were represented by *Bradyrhizobiaceae*, *Rhizobiaceae*, *Comamonadaceae*, and *Hyphomicrobiaceae*, with the genera *Bradyrhizobium*, *Rhizobium*, and *Devosia* present in all the analyzed summer black truffles at relatively high percentages (up to 40.9%, 6.5%, and 14.5%, respectively). The family *Comamonadaceae* showed variable values ranging from 1.9 to 73.8%, with the genera *Acidovorax* and *Polaromonas* poorly represented in most of fruiting bodies but with a relative abundance of 67.6% and 4.0% in sample 3t, respectively (Fig. 4).

In soil samples, the main phyla were *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, and *Bacteroidetes* (Fig. 4). *Proteobacteria* occurred at percentages ranging from 34.8 to 38.7%, with the genera *Skermanella* (2.1–6.0%), *Bradyrhizobium* (1.5–2.4%), and *Sphingomonas* (1.0–4.8%) being common to all the samples. *Actinobacteria* showed a relative abundance between 24.5 and 36.7%, with the genera *Rubrobacter* and *Microlunatus* found at percentages ranging from 1.3 to 2.7% and from 1.7 to 2.5%, respectively. An unclassified bacterium of the *Acidobacteria* phylum (9.2–16.2%) ranked first in the soil sample group with a relative abundance between 5.7 and 11.2%. *Chloroflexi*, poorly represented in truffle samples, showed percentages from 5.1 to 7.9% whereas *Bacteroidetes* ranged from 2.4 to 5.0% (Fig. 4).

In order to have an insight on the relevance and impact of cross-habitat associations between species, three microbial networks were generated for soil and truffle communities both separately and taken together, based on correlation between species abundances (Fig. 5). The topology was investigated in order to identify the most significative features of each network and keystone species relevant for their structures and organizations.

The network obtained for the soil habitat (Fig. 5a) was characterized by 747 nodes, each representing a species, and 8326 connections among them. The nodes were organized in a large connected component (clique) made of 99.19% (741) of the nodes and three dyads (0.81%). The mean number of partners for each node of this network (mean node degree) was equal to 22.29 whereas network density was 0.0149.

The network obtained for truffle habitat (Fig. 5b) contained 683 nodes and 28,057 connections, and it was characterized by a mean node degree of 82.16 and a

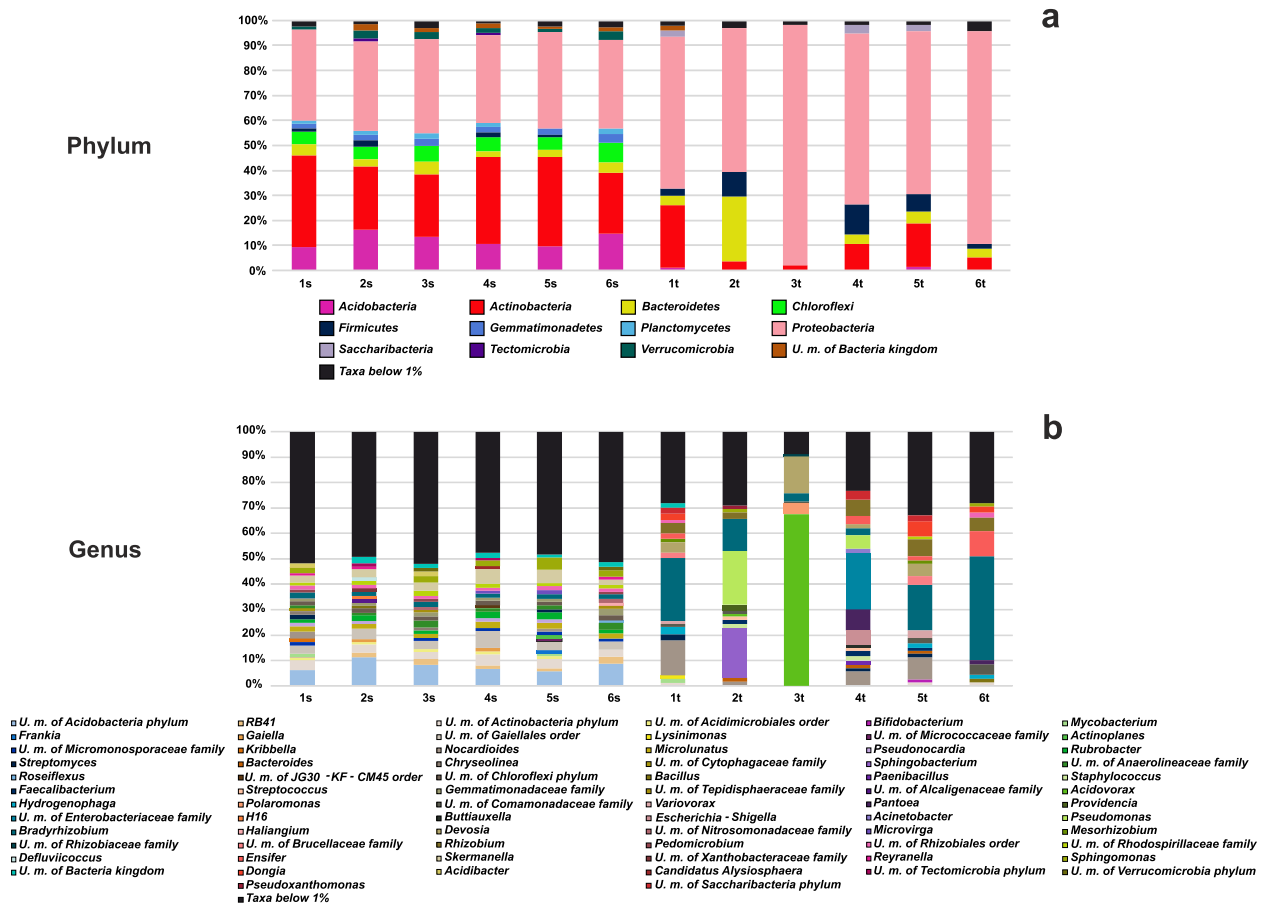

Fig. 4 Soil (1s, 2s, 3s, 4s, 5s, 6s) and truffle (1t, 2t, 3t, 4t, 5t, 6t) microbial composition. Relative abundance at the phylum (**a**) and genus (**b**) levels. U.m. refers to unclassified microorganisms

network density of 0.0601. The largest component of the network, the clique, was made of 649 nodes which represented the 95.02% of the total nodes. A component of 28 nodes (4.01%) was also present, and there were three dyads (0.97%).

The network inferred by soil and truffle microbial communities combined together (Fig. 5c) was characterized by 1439 nodes organized in a large connected component (clique) comprising 99.72% of the nodes and two dyads (0.38%). This network was also characterized by 53,721 connections, of which 8326 (15.51%) were between species identified in soil habitat, 28,057 (52.27%) were between species from truffle habitat, and 17,299 (32.22%) were across the habitats (a partner from soil habitat, the other from the truffle habitat). The average node degree of the soil-truffle network was 74.59, and the density was 0.026. A high number of significant cross-habitat correlations was found, suggesting that the species from the two habitats interacted or were similarly influenced by the environmental conditions.

Subsequently, the centrality of the microbial populations present in the combined habitat network was measured in order to define the relative importance of soil species with respect to the truffle ones. As shown in Fig. 5, panel d, the number of network connections between soil species was strongly and significantly lower with respect to the connections found between truffle species (Welch t test p value $< 10^{-14}$) within the soil-truffle network. It is likely that those microorganisms recruited in the truffle from the soil must have a closer and stricter linkage to optimize the colonization of that specific ecological niche.

Based on betweenness centrality scores, the top five keystone OTUs in soil communities included unclassified members of the *Sedimentibacter*, *Nitrospira*, *Gemmatirosa*, and *Dyadobacter* genera, and *Acidiferrobacteraceae* family (Fig. 6a). For truffles, *Kosakonia cowanii*, *Massilia* sp. UMI-21, unclassified members belonging to the genera *Piscinibacter* and *Blastococcus*, and to the *Polyangiaceae* family represented the more relevant bacterial groups (Fig. 6b). On the other hand, the higher stability observed in the network showing soil and truffle microbial communities together seemed to rely on the presence and abundance of OTUs (such as *Pseudomonas brassicacearum*

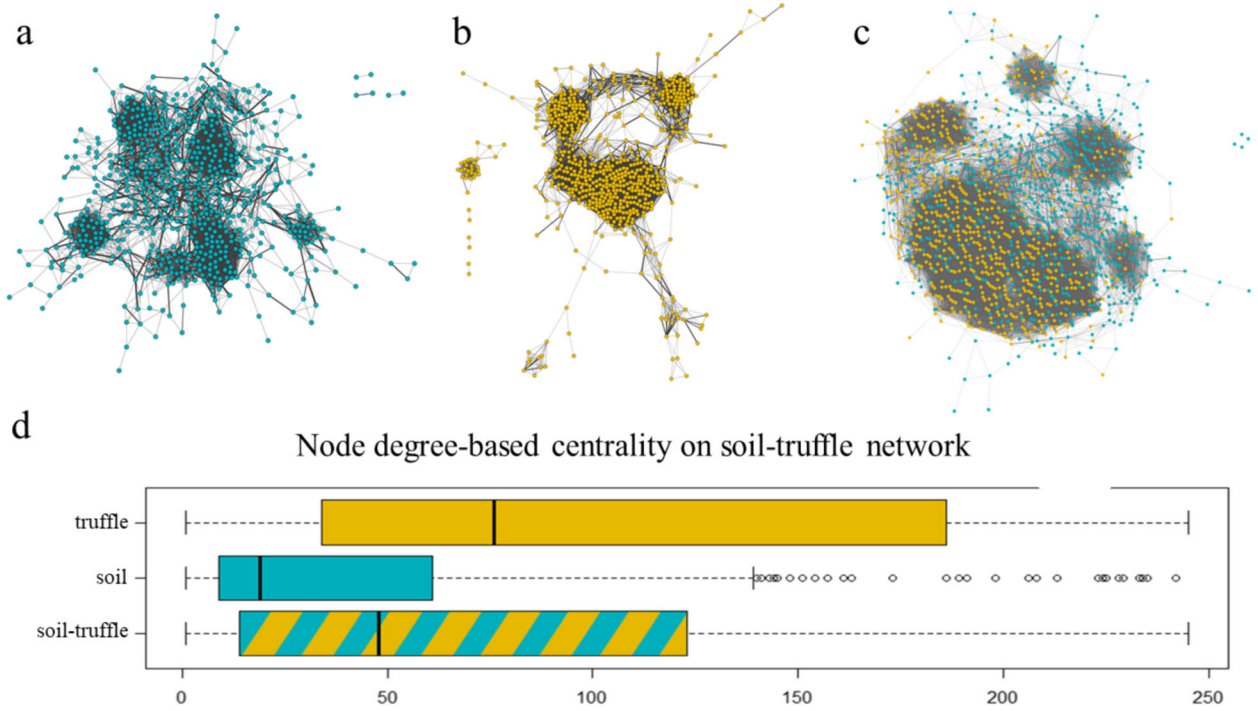

Fig. 5 Networks representing microbial communities from soil habitat (**a**), from truffle habitat (**b**), and from combined soil and truffle habitats (**c**). Each node represents a species, and node color is related to the habitat the species belongs to (blue for soil habitat and yellow for truffle one). Edges (lines) between nodes represent a significant correlation between the abundances of the species connected by. Edge width is proportional to the significance of correlation between species. Networks are plotted by organic layout in Cytoscape. **d** Species identified in the truffle habitat have a higher connectivity (measured by node degree centrality) than species identified in the soil habitat (respectively yellow and blue boxplots). The network derived from abundances combined from species identified in soil and truffle habitats has a connectivity lower respect the only truffle component and higher respect the only soil component (striped boxplot)

subsp. *brassicacearum*, *Paenibacillus polymyxa*, *beta proteobacterium* DC2b-18, and unclassified members of the *Sedimentibacter* genus and *Saccharibacteria* phylum) defining both the environments, demonstrating a more complex inter-relationship of microbial taxa (Fig. 6c) which were more closely related to one or to the other habitat. In addition, this higher stability was also shown by the analysis of the influence of species loss on the network connectivity through the measure of the "attack robustness" (Fig. 7). This analysis clearly showed that the size of the largest component of soil microbial community network decreased faster compared to truffle or soil-truffle communities whose response to robustness attack differed only of a light skew. Among all, the soil-truffle robustness attack curve showed the highest AUC values (Fig. 7). This suggested also a higher structural similarity between the truffle and soil-truffle microbial communities that is coherent with a specific recruitment of the truffle microbiota from the soil.

Discussion and conclusions

Several methods (cultivation-dependent and molecular approaches) have been employed to reveal microbial community composition and responses to environmental changes in various environments and in different contexts (Bucci et al. 2011, 2014, 2015a, 2015b; Crescenzo et al. 2017; Di Luccia et al. 2018; Petrella et al. 2018; Pietrangelo et al. 2018). In fact, an understanding of the temporal and spatial structures, functions, interactions, and population dynamics of microbial communities is critical for many aspects of life, including scientific discovery, biotechnological development, sustainable agriculture, environmental protection, and human health (Bucci et al. 2017).

In the present study, we used NGS technology to investigate microbial communities associated with summer black truffle ascocarps collected in Molise region (Southern Italy), one of the richest Italian areas of this product. Despite its economically important value, *T. aestivum* from Molise has received less attention from a scientific point of view compared to truffles from other Italian regions. Thus, the research aimed at filling the gap in the current knowledge by analyzing bacterial communities associated with fruiting bodies as an initial step for a further and deepened characterization.

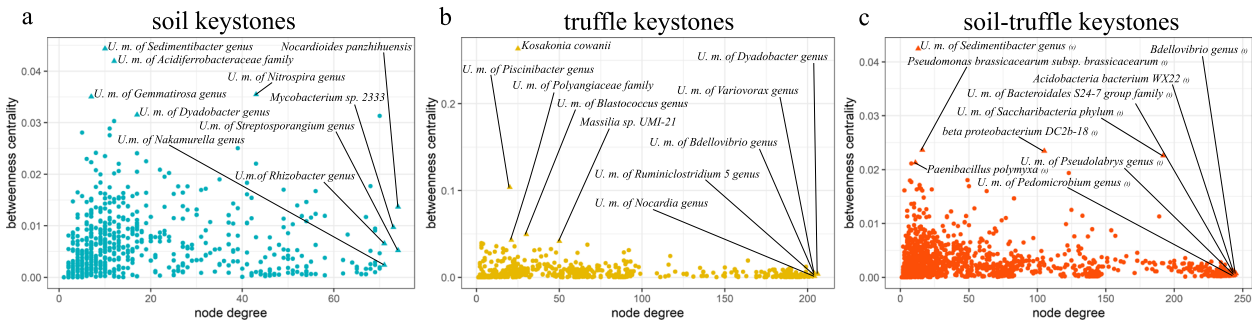

Fig. 6 Keystone species obtained from the network analysis. Nodes represent species that are shown agreeing to their centrality metrics (triangles indicate the top 5 keystone species based on node degree and betweenness centrality). Each node of a plot represents a species in the related network (**a** for soil, **b** for truffle, and **c** for soil-truffle). Nodes are colored according to the network they belong to (blue for soil, yellow for truffle, and red for soil-truffle). Keystone species names are showed for some of the most relevant nodes of each network. In the soil-truffle network the apex beside of species name indicates the habitat in which the species was detected: (s) for soil and (t) for truffle

The main bacterial phyla retrieved in the Molise truffle were the same found in the fruiting bodies of *Tuber* spp. of different geographic origin (Vahdatzadeh et al. 2015; Benucci and Bonito 2016; Ye et al. 2018). In fact, the analyzed communities were dominated by *Proteobacteria* and *Actinobacteria*, with the genus *Bradyrhizobium* particularly represented (Gryndler et al. 2013; Benucci and Bonito 2016; Ye et al. 2018).

As expected, summer black truffle microbiota was significantly different from that of soil which showed a higher α-diversity although a high number of shared taxa at genus level. Some bacterial genera, such as *Bradyrhizobium* and *Devosia*, were detected with relatively higher abundance values in most of the fruiting bodies compared to the soil, demonstrating that most likely a recruitment of bacteria from soil to ascocarps occurs during the truffle formation, in agreement with previous researches (Antony-Babu et al. 2013; Splivallo et al. 2015).

The presence of genera comprising nitrogen-fixing bacteria (such as *Bradyrhizobium* and *Rhizobium*), as already reported for other *Tuber* species (Le Tacon et al. 2016), is relevant: in fact, the ability to modify nutrient availability during their biological cycle could be of particular importance during the development of fruiting bodies which need nutrients in order to complete the maturation process independently of the host plant (Barbieri et al. 2010).

The network analysis performed to elucidate the interactions between microbial taxa showed a higher robustness of the system when microbial communities were examined as a whole rather than individually. To this greater stability contributed bacterial taxa more strictly related both to one and to the other habitat, indicating

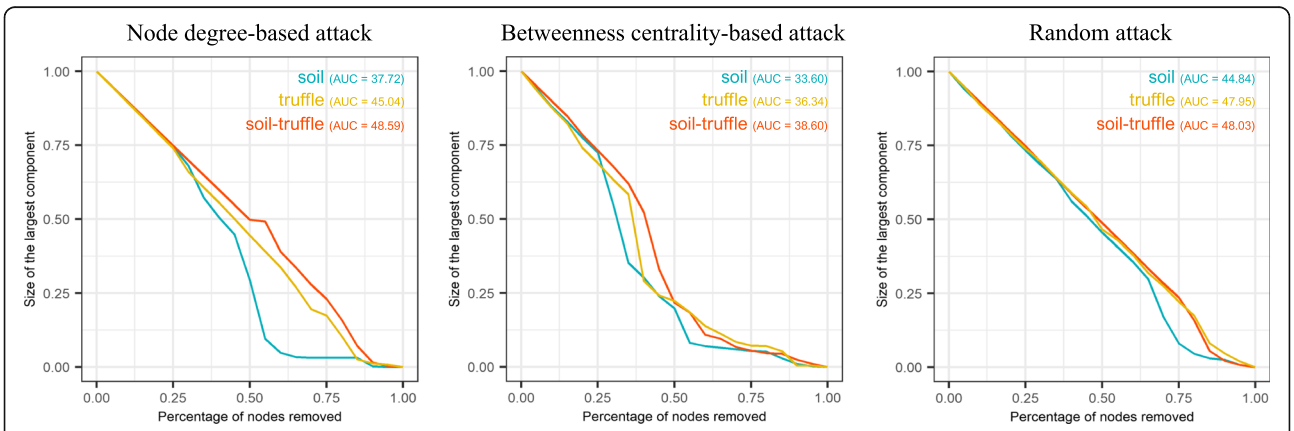

Fig. 7 Attack robustness strategy to measure network resilience. Node removal was performed in agreement with their centrality, considering both node degree (**a**) and betweenness centrality (**b**) or by a random selection (**c**). The robustness was measured as percentage of nodes remaining in the main connected component of the network (clique). Here we plotted the results of this analysis performed on each of the three networks as a line (blue for soil, yellow for truffle, and red for soil-truffle). On the X-axis are reported the percentages of nodes removed, while on the Y-axis the percentage of the nodes remaining in the clique. AUC indicates the area under the curve calculated according to Gini's formula

complex connections among species that could somehow determine and maintain the equilibrium of this peculiar ecosystem.

Nevertheless, a surprising and remarkable heterogeneity across truffle samples, in terms of microbial community composition and relative abundance of the main taxa, was also observed. Since the study area is small and the host plants belong to the same species, we suppose that local-specific factors could play an important role in determining the structure of the investigated truffle microbial communities. Further analyses are, thus, required to better understand which these factors are and how they could influence the composition of microbial communities. In our opinion, the full comprehension of the role of bacteria in mycorrhization process and their contribution to the development of specific traits in truffles as well as of the factors that drive the establishment of specific microbial communities in the ascocarps, could have a significant impact on truffle industry, mainly at regional scale: for the detection and identification of specific quality marks in the foodstuff (product promotion) and also for the improvement of cultivation techniques in artificial grounds.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13213-020-01586-5.

Additional file 1. Venn diagram of bacterial communities with shared and unique genera between soil and truffle samples

Additional file 2. Rarefaction curves of soil (black lines) and truffle (gray lines) samples. Alpha-diversity plots obtained by using the Shannon (left) and the Chao1 (right) indices

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Authors' contributions

PM: Conceptualization, Formal analysis, Investigation, Validation, Visualization, Writing-original draft, Writing-review & editing. MT: Investigation, Writing-original draft. GS: Formal analysis, Writing-review & editing. ET: Writing-original draft, Writing-review & editing. GN: Conceptualization, Writing-original draft, Writing-review & editing. AB: Conceptualization, Supervision, Writing-original draft, Writing-review & editing. The authors read and approved the final manuscript.

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Competing interest

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

N/A.

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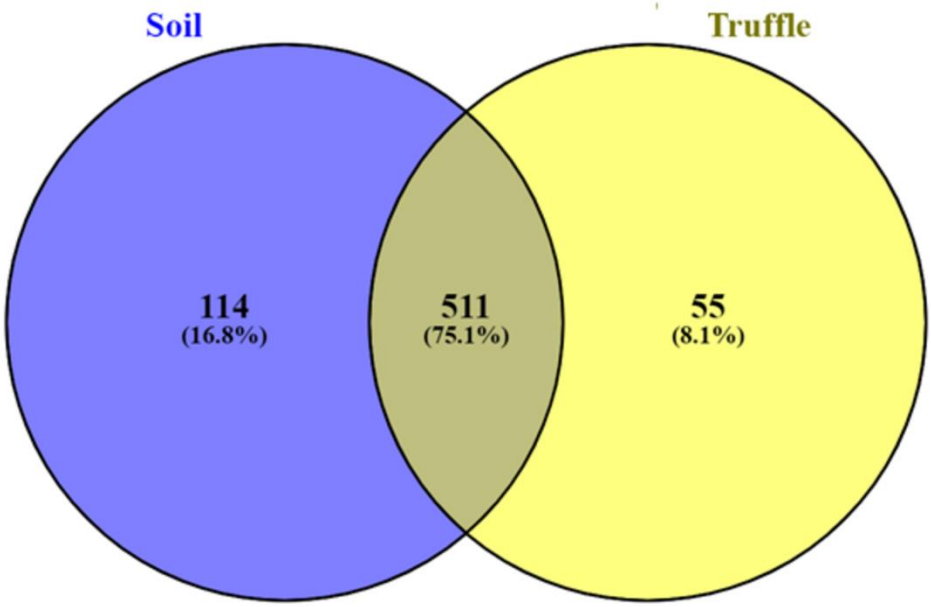
Publisher's Note

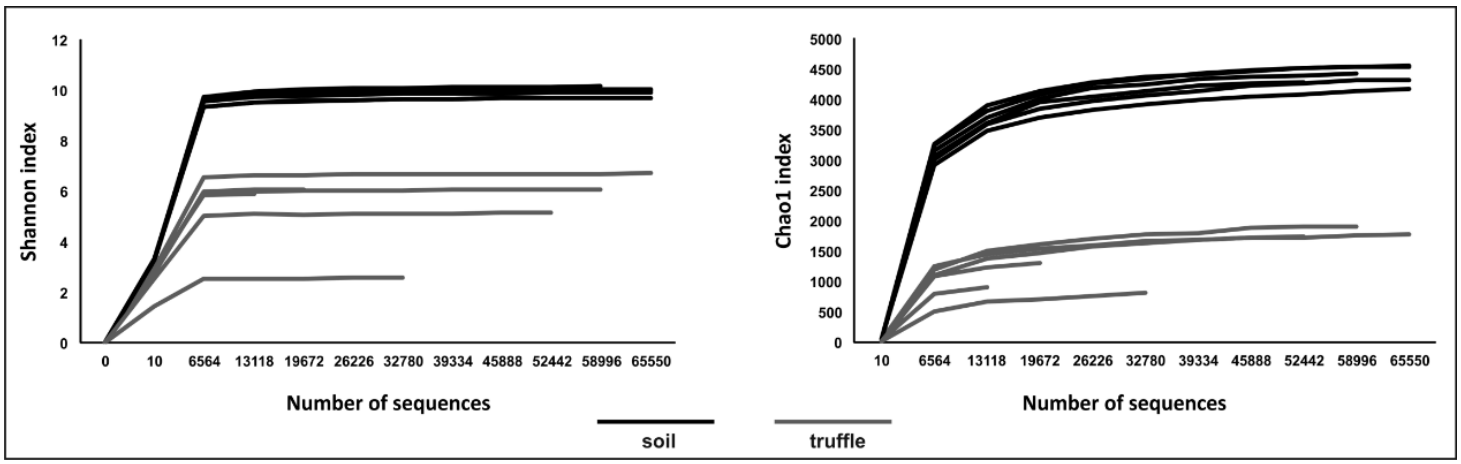
Supplementary information

Additional file 1

Venn diagrams at genus level from soil and truffle samples



Additional file 2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6
4
2
0
0
10
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13118
19672
26226
32780
39334
45888
52442
58996
65550
Number of sequences
Chao1 index
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
10
6564
13118
19672
26226
32780
39334
45888
52442
58996
65550
Number of sequences
soil
truffle

3. Heterogeneity of the white truffle *Tuber magnatum* in a limited geographic area of Central-Southern Italy.

Heterogeneity of the white truffle *Tuber magnatum* in a limited geographic area of Central-Southern Italy

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Summary

Molise region (Central-Southern Italy) is one of the Italian richest areas of truffles and contributes significantly to the national production of the precious *Tuber magnatum*. Nevertheless, Molise truffle has received little scientific attention. Accordingly, in the present study, two *T. magnatum* populations collected in two different sites of Molise region were characterised from a morphological, genetic and microbiological point of view. A considerable variability between and within the two analysed groups emerged, suggesting an interesting heterogeneity of Molise white truffle populations. Ascocarps of the two groups significantly differed in size and maturation degree, although no linear correlation between weight and maturity was found. Genetic investigations focused on the Sequence-Characterised Amplified Region SCAR A21-inf. Three haplotypes, randomly distributed within the two truffle groups regardless of their collection sites, were detected. The 16S rRNA gene amplicon high-throughput sequencing provided an overview of the composition of the ascocarp-associated bacterial communities. A predominance of α-*Proteobacteria* was observed, with *Bradyrhizobium* among the main genera. However, some truffles showed unusual microbial profiles, with *Pedobacter*, *Polaromonas* and other bacterial genera as dominant taxa.

4. Determination of the peridium thickness of *Tuber magnatum* ascomata from Molise region.

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Short note

Determination of the peridium thickness of *Tuber magnatum* ascomata from Molise region

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Abstract
Several studies focused on *Tuber magnatum*, the most valuable truffle species, with a limited geographical distribution. However, no comprehensive information on the thickness of its peridium (the external surface of fruiting bodies) is available. Accordingly, to fill this lack of information and in order to provide a more in-depth morphological characterisation of white truffle populations from Molise region (one of the Italian richest areas of truffles), the peridium thickness of twenty-one *T. magnatum* ascomata collected from two different study sites was measured by light microscope observations. A considerable variability within the analysed populations emerged, with values ranging from 271.25 µm (minimum) to 1231.25 µm (maximum), and an average peridium thickness of 622.33 µm. Interestingly, significant differences were observed between the two groups, with truffles harvested in an inner area of the region showing a peridium significantly thinner than those collected on the border with Abruzzo region. No linear correlation between peridium thickness and other morphological parameters (ascoma weight and maturity) emerged. It is likely that the differences observed between the two *T. magnatum* populations could be related to factors such as soil characteristics, site-specific features, genetic traits, as well as truffle collection period, which should be properly investigated.

Keywords
white truffle; fruiting body; external surface; morphological traits; light microscope; Central-Southern Italy

Introduction
Tuber magnatum Picco is an ectomycorrhizal ascomycete of the *Pezizales* order that, in symbiosis with several trees and shrubs, produces edible hypogeous fruiting bodies, the so-called Italian white truffles, one of the most expensive foods in the world (thousands of euros per kilogram; Laruccia et al., 2020). In addition to the valuable culinary properties, the exorbitant prices of *T. magnatum* ascomata reflect their low availability on the market, which is related to both the difficulties in cultivation and the limited distribution range (Mello et al., 2006; Christopolous et al., 2013; Iotti et al., 2014; Riccioni et al., 2016). Indeed, this prized truffle species, for a long time considered endemic to Italy, grows spontaneously only in a few European countries (Rubini et al., 2005; Belfiori et al., 2020).

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Molise region (Central-Southern Italy) is one of the most productive area of the Italian peninsula. Indeed, it is estimated that the total amount of *T. magnatum* truffles annually collected in the Molise forests is between 30 and 70 quintals (https://www.agi.it/lifestyle/news/2021-01-11/molise-diventa-patria-tartufo-10978317/). However, although the prized white truffle has been extensively characterised from many points of view, Molise truffles have received very little scientific attention (Monaco et al., 2020, 2021).

Several studies focused on *T. magnatum* phylogeography and genetic characteristics (Mello et al., 2005; Rubini et al., 2005; Belfiori et al., 2020; Monaco et al., 2021), associated microbial communities (Barbieri et al., 2007, 2010; Amicucci et al., 2018; Monaco et al., 2021; Niimi et al., 2021), volatile organic compounds (Vita et al., 2015; Niimi et al., 2021), molecular and biochemical traits (Vita et al., 2020), ecological features (Marjanović et al., 2015), and cultivation techniques (Bach et al., 2021).

Nevertheless, to date, very few data relating to the thickness of the peridium (the external surface of truffle fruiting bodies) are available.

The peridium takes on different features depending on *Tuber* species. In the black truffles *Tuber aestivum* Vittad. and *Tuber melanosporum* Vittad., it is brown-black and characterised by the presence of hard pyramidal warts; in *Tuber borchii* Vittad. it appears at first whitish, then greyish-yellow and finally brown ochre in colour, quite pubescent when immature and, at maturity, glabrous, humid, and smooth, often with darker or lighter reddish spots, whereas *T. magnatum* peridium is smooth, suede-like, and adherent to the gleba (the inner part of the ascoma) (Hall et al., 2007; Angelini et al., 2016).

This short note reports the preliminary results of peridium thickness measurements of two white truffle populations (*T. magnatum*) harvested within natural truffle grounds in Molise region, with the aim to 1) fill the lack of information on this parameter and 2) provide a more in-depth morphological characterisation of the Molise white truffles.

Materials and Methods

Sampling

In this study, twenty-one ascomata belonging to two distinct *T. magnatum* populations were considered. They were collected in two different areas of Molise region (Fig. 1). In particular, nine ascomata (samples from 1 to 9, group 1) were harvested in November 2019 in the study site 1, between Carovilli and Vastogirardi municipalities (Isernia province), an area highly suited to truffle collection (Paolanti et al., 2014; https://www.agi.it/lifestyle/news/2021-01-11/molise-diventa-patria-tartufo-10978317/). Other twelve samples (indicated with progressive numbers from 10 to 21; group 2) were collected in January 2020 in the study site 2, a more extensive area on the border with Abruzzo region. Truffles were found, with the help of trained dogs and expert truffle hunters, in natural orchards located within mixed coppice woods, with a vegetation composed mainly of hazel trees (*Corylus avellana* L.), Turkey oaks (*Quercus cerris* L.), beeches (*Fagus sylvatica* L.), and cornels (*Cornus sanguinea* L.). Then, they were placed in polypropylene containers and transported to the laboratory under refrigerated conditions (Monaco et al., 2021).

Dried samples of each specimen were deposited in the herbarium at the University of Molise, Italy.

Determination of peridium thickness

Tuber magnatum ascomata were gently brushed and washed with distilled water to remove soil residues. In order to determine the thickness of the peridium, fruiting bodies were divided in half and cut lengthwise with a steel blade to obtain thin sections. For each truffle, five sections were considered by taking the peridium in several points of the ascoma. In addition to the peridium, sections also included a small portion of gleba to facilitate the subsequent determination of the peridium thickness by microscopic observation (Fig. 2). The slices so obtained were placed on a microscope slide and covered/wetted with distilled water before applying a coverslip. The thickness of the peridium was

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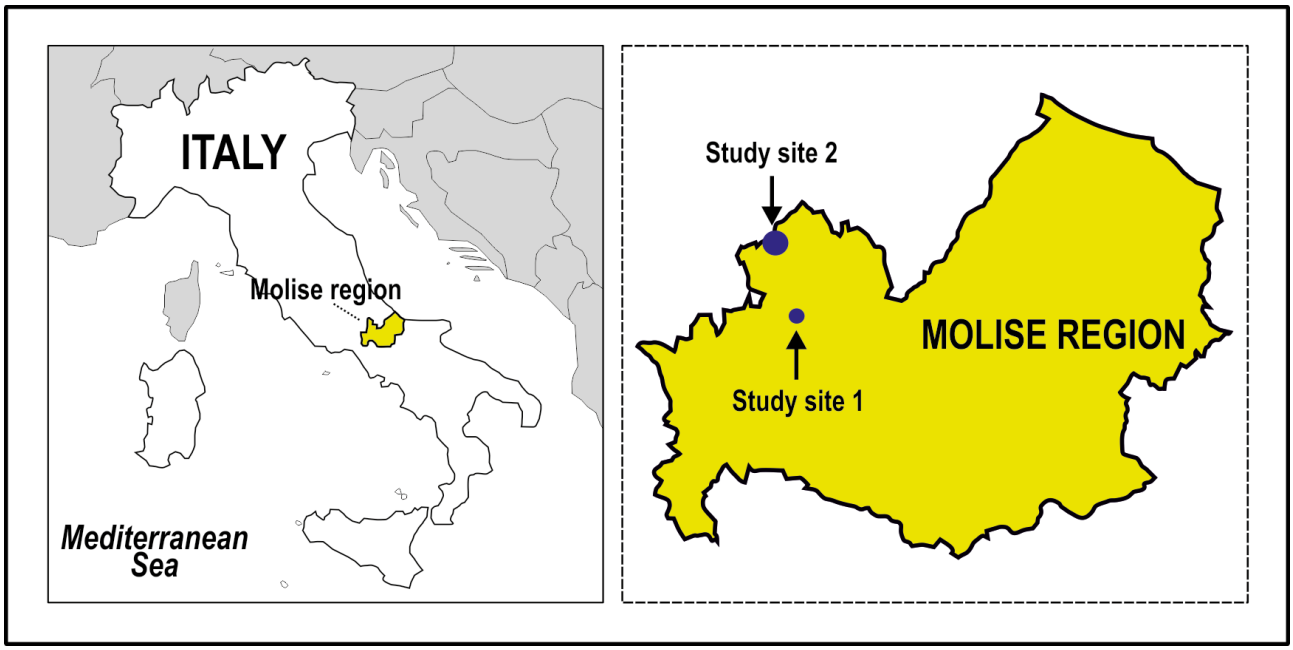

Fig. 1 - Study area. The two collection sites are shown on the map: the study site 1 is located between Carovilli and Vastogirardi municipalities whereas the study site 2 covers a more extensive area on the border with Abruzzo region.

determined with the aid of a light microscope equipped with a ruler (Leica Microsystems, 10× objective). For each fruiting body, it was measured in twenty-five different points (5 for each section), positioning the microscope ruler perpendicularly to the section to be analysed (Fig. 2). The average thickness of the peridium was then determined by calculating the arithmetic mean of the twenty-five measured values.

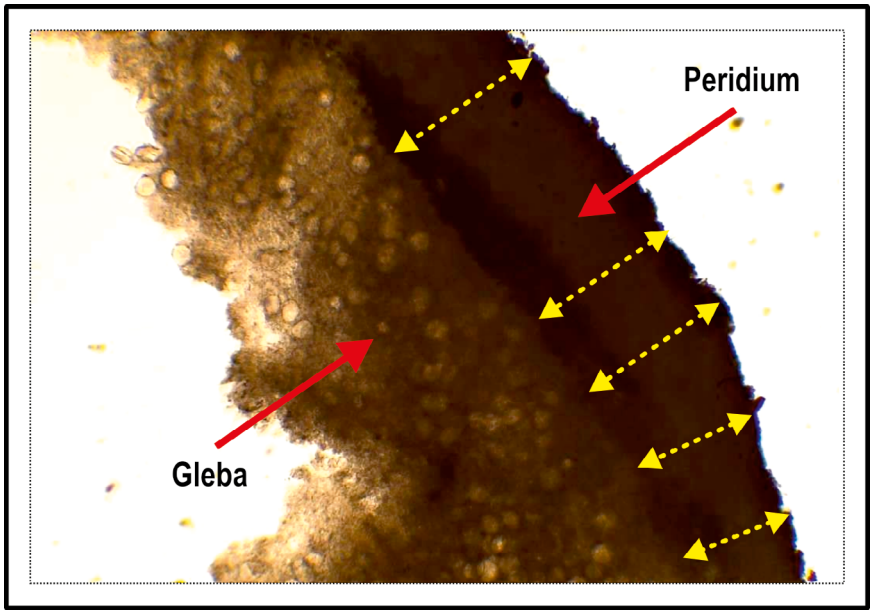

Fig. 2 - Example of peridium thickness determination by microscope observation of a thin truffle section also comprising a small portion of gleba. As shown by the yellow arrows, for each section the thickness of the peridium was measured in five points to obtain a total of 25 values for each ascoma.

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In order to assess whether there were statistically significant differences in the peridium thickness of the two investigated *T. magnatum* populations, Student's t-test was performed. Moreover, an analysis with the Pearson's correlation coefficient was conducted in order to verify the possible linear correlation between peridium thickness and ascoma weight and maturity [these two morphological data are reported in Monaco et al. (2021) for the same samples here analysed].

Results and discussion

Even if several works exist on the prized white truffle, to date, no comprehensive information on the thickness of fruiting body peridium is available. Indeed, there is a lack of systematic studies aimed at analysing this aspect. Some of the few data found in scientific literature have been reported by Zambonelli et al. (2000), who examined this characteristic for 5 samples of *T. magnatum* from Emilia Romagna region, and by Angelini and colleagues (2016), as part of a more extensive work mainly focused on the isolation and identification of allelochemicals from *Tuber* ascomata. Consequently, these Authors, having different goals, did not investigate the peridium thickness in relation to other parameters (such as, for example, fruiting body size or maturity, truffle collection period, etc.).

Accordingly, to fill this lack of information and in order to provide a more in-depth morphological characterisation of the Molise white truffles, the peridium thickness of the twenty-one ascomata collected from two different areas of the region, between November 2019 and January 2020, was accurately measured by light microscope observations.

The two examined *T. magnatum* populations were previously included in a wider research, in which they were analysed from a morphological, genetic, and microbiological point of view, highlighting a surprising heterogeneity between and within the investigated groups (Monaco et al., 2021). Although, in agreement with prior investigations (Büntgen et al., 2017; Garcia-Barreda et al., 2021), no strong linear correlation between fruiting body weight and maturity was found, truffles of the first group were, overall, of greater dimensions and riper compared to those of the second group (Monaco et al., 2021).

Measurements of the fruiting body peridium carried out in this study further confirmed the heterogeneity detected since, as shown in Table 1, a considerable variability within the analysed populations emerged, with values ranging between 271.25 µm (minimum) and 1231.25 µm (maximum).

Overall, the peridium thickness of the examined Molise truffles was on average 622.33 µm. This mean value differs from those recorded by Zambonelli et al. (2000), for samples collected in Northern Italy, and by Angelini et al. (2016).

Interestingly, significant differences were observed between the two investigated populations (Student's t-test, $p < 0.01$): indeed, truffles harvested between Carovilli and Vastogirardi municipalities (group 1) showed a peridium significantly thinner than those of the group 2, with average values of 452.42 and 749.76 µm, respectively.

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Table 1 - Average peridium thickness of the twenty-one white truffles (*T. magnatum*) from Molise region.

SAMPLE CODE	GROUP	AVERAGE PERIDIUM THICKNESS* (µm)
1	1	298.75
2	1	271.25
3	1	287.68
4	1	521.36
5	1	487.00
6	1	667.50
7	1	482.22
8	1	533.50
9	1	522.50
10	2	1231.25
11	2	813.75
12	2	985.16
13	2	444.29
14	2	798.75
15	2	653.13
16	2	757.50
17	2	394.17
18	2	637.67
19	2	854.46
20	2	615.91
21	2	811.03

*The average peridium thickness of each fruiting body was given by the arithmetic mean of the twenty-five measured values

The analysis with Pearson's correlation coefficient revealed that there is no linear correlation between truffle weight and peridium thickness (r = - 0.21) and only a slight/moderate negative relationship between fruiting body maturity and the thickness of the peridium (r = - 0.50).

In our opinion, it is likely that the differences observed in the peridium thickness between the two analysed *T. magnatum* populations, similarly to the differences previously reported for truffle weight and maturity (Monaco et al., 2021), could be related to the diverse collection period of the ascomata (fruiting bodies of the first group were collected in November whereas those of the second group in January). Indeed, it is known that the white truffles of January can be morphologically different from *T. magnatum* collected between October and December, because of the ripening in winter period, under frost and ice (http://www.artopoltrepo.it/Documenti/manualetartufi.pdf). Therefore, we might assume that the thicker peridium of the fruiting bodies harvested in January 2020 (group 2) could represent a kind of adaptation to unfavourable environmental conditions. However, since no comparison data are available, further analyses will be required to shed light on an effective correlation between peridium thickness and environmental/climatic conditions. On the other hand, the remarkable variability detected within the examined *T. magnatum* populations could be explained taking into account other factors that should be properly investigated, such as genetic traits, site-specific features, soil characteristics, and microclimatic conditions.

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Conclusion

In the future, it would be interesting to carry out further analyses including a larger number of ascomata collected in different areas of the region and in other Italian regions, also to verify the existence of a possible link between the peridium thickness and *T. magnatum* provenance. Indeed, the potential use of the peridium thickness as an effective parameter to track the geographical origin of truffles could have a significant impact on the economy related to this product (mainly at regional scale) from different perspectives (truffle traceability, promotion and conservation), and represents a relevant aspect to be properly investigated in future researches.

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5. CONCLUSIONS

The results obtained after the three-year PhD course contributed to broaden the current knowledge on microbial communities associated with some of the most commercially relevant truffle species (the prized white truffle *Tuber magnatum* Picco and the summer black truffle *Tuber aestivum* Vittad.). Moreover, the research activities illustrated in the present thesis laid the foundation to fill the lack of scientific data on the Molise truffles and represented an essential starting point for a further and more in-depth characterisation of this resource of utmost importance for the local economy. Indeed, although the regional territory is particularly rich of these precious hypogeous fungi, Molise truffles have received less attention compared to *Tuber* species from other Italian regions or Countries and, consequently, they have not been properly valorised.

An unexpected variability (from different points of view) was observed among the examined *T. aestivum* and *T. magnatum* truffles, and a remarkable heterogeneity of the Molise truffle populations was detected.

- With reference to the Molise summer black truffles (Monaco *et al*., 2020), microbiological investigations revealed considerable differences between soil and *T. aestivum* fruiting body microbial communities, despite a high percentage (≈ 75%) of shared taxa at genus level. In addition, while the microbial communities found in the soil samples were very similar to each other, the bacterial communities associated with summer black truffle ascocarps presented a greater heterogeneity in terms of composition, relative abundance of the main taxa and α-diversity. It is likely that the differences observed within the truffle group may be due to site-specific factors that could play an important role in determining the structure of the fruiting body microbiota. The main phyla retrieved in the analysed *T. aestivum* ascocarps were *Proteobacteria, Actinobacteria, Bacteroidetes,* and *Firmicutes,* with *Bradyrhizobium, Rhizobium, Devosia,* and *Nocardioides* among the most represented genera.

The network analysis performed to elucidate the relevance and impact of cross-habitat interactions between microbial species allowed to formulate interesting hypotheses. A greater robustness of the system emerged when the soil and truffle microbial communities were examined as a whole rather than individually. To this higher stability contributed bacterial taxa more strictly related both to one and to the other habitat, indicating complex connections among species that could somehow determine and maintain the equilibrium of this peculiar ecosystem. The greater stability of the soil-truffle system was also highlighted by analysing the influence of species loss on the network connectivity through the measure of the "attack robustness". This analysis clearly showed that the size of the largest component of soil microbial community network decreased faster compared to truffle or soil-truffle bacterial community networks, whose response to robustness attack differed only slightly, suggesting also a higher structural similarity between truffle and soil-truffle communities. This could be consistent with the specific recruitment of ascocarp-associated bacteria from the microbial communities of the soil during the early stages of truffle formation (Antony-Babu *et al*., 2014). The high number of significant correlations between soil species and truffle species suggested that soil and truffle bacteria could interact, influence each other and/or be similarly affected by environmental conditions. Furthermore, the significantly higher number of connections between species found in *T. aestivum* ascocarps with respect to the connections between soil species, within the soil-truffle network, might hint that ascocarp-associated bacteria must have a closer and stronger linkage to optimise the colonisation of the specific and complex ecological niche "truffle".

- The results obtained from the analysis of *Tuber magnatum* fruiting bodies collected from two different study sites in the Molise region (Monaco *et al*., 2021) confirmed an interesting heterogeneity not only from a microbiological point of view, but also in terms of morphological and genetic traits, making these populations ideal for further in-depth studies.

Overall, the bacterial communities retrieved in the investigated white truffles were made up almost exclusively of *Proteobacteria*, mainly belonging to the *α-Proteobacteria* class, followed by *Bacteroidetes* and, to a lesser extent, *Firmicutes*. It should be noted that the phylum *Actinobacteria*, among the most represented in the examined *T. aestivum* ascocarps (Monaco *et al*., 2020), was not found in *T. magnatum* fruiting bodies, proving that the composition of the truffle microbiota can vary depending on the *Tuber* species (Vahdatzadeh *et al*., 2015). Nevertheless, as for *T. aestivum*, most of the analysed white truffle ascomata (nearly 70%) harboured microbial communities largely dominated by bacteria of the genus *Bradyrhizobium*. It is thought that *Bradyrhizobium* species could be implicated in the nutrition of the fruiting bodies due to their nitrogen-fixing activity (Amicucci *et al*., 2018; Barbieri *et al*., 2010; Splivallo *et al*., 2019) and could be responsible for truffle aroma through the production of sulphur volatile compounds (Splivallo *et al*., 2015, 2019).

Interestingly, some of the examined ascocarps within both the Molise white truffle populations showed "unusual microbial profiles", with *Flavobacterium*, *Mesorhizobium*, *Pedobacter*, *Phyllobacterium*, and *Polaromonas* as dominant taxa.

A greater heterogeneity in terms of microbiota composition, a higher α-diversity, as well as a greater dispersion were observed within the truffle group collected on the border with Abruzzo region (study site 2) compared to truffles harvested between Carovilli and Vastogirardi municipalities (study site 1). Since several biotic and abiotic factors may influence the bacterial community structure, the higher microbial variability found among the ascocarps from study site 2 could be related to the greater extension of the sampled area and to site-specific features such as soil properties and microclimatic conditions, which should be properly deepened. On the other hand, the different maturation degree of the ascocarps and the three allelic variants at SCAR A21-inf locus found within the two *T. magnatum* populations did not seem to influence the microbiota composition of the analysed ascomata (Monaco *et al*., 2021).

A less fragmentation (especially at genus taxonomic level) in the structure of the microbial communities associated with *T. magnatum* fruiting bodies compared to *T. aestivum* ascocarps was detected (Monaco *et al*., 2020, 2021), and could be somehow related to the stricter and peculiar conditions required by *Tuber magnatum* to grow.

The choice to consider the Sequence-Characterised Amplified Region SCAR A21-inf as tool to investigate the genetic variability within the Molise white truffle populations was related to the researches carried out by Mello *et al*. (2005), who proved that the SCAR region is polymorphic in *T. magnatum* by identifying two SNPs that generate three haplotypes (I, II, and III). These Authors found that, out of 62 analysed ascocarps of different geographical origin (collected in Italy and Croatia), the haplotype III was present only in two samples from Molise region (Mello *et al*., 2005). As mentioned, the results obtained in this study confirmed the presence of the three allelic variants at A21-inf locus. They were randomly distributed within the two examined *T. magnatum* populations: the haplotype III was the most frequent (found in more than 50% of the ascocarps), followed by haplotypes II and I. Thus, although more in-depth analyses are required to better understand the genetic structure of these populations, it is likely that the haplotype III could represent a peculiar trait of some Molise white truffles.

As explained in Chapters 3 and 4, a remarkable morphological heterogeneity among the examined *T. magnatum* ascomata emerged, with significant differences between the two populations in terms of fruiting body size/weight, maturity and peridium thickness. Indeed, truffles collected in the study site 1 were of greater dimensions, riper, and with a peridium significantly thinner compared to those from study site 2. The analysis with Pearson's correlation coefficient revealed no strong linear correlation between ascocarp weight and maturity. Similarly, no significant correlation between peridium thickness and fruiting body size or maturity was found.

Differences in weight and maturation level observed between the two *T. magnatum* populations could be related to the different harvesting period of the ascocarps (truffles of the first group were collected in November whereas those of the second group in January). In fact, it is known that the white truffles of January can be morphologically different from *T. magnatum* collected between October and December; they are usually smaller and partially immature, with asci often lacking in spores, because of the ripening in winter period, under frost and ice (http://www.artopoltrepo.it/Documenti/manua letartufi.pdf). Therefore, for the same reason, it is likely that the differences detected in the thickness of the peridium between the two analysed white truffle populations could depend on the diverse fruiting body collection period. In addition, other factors, such as site-specific features, soil characteristics, microclimatic conditions, and genetic traits, which should be properly analysed, could contribute to determine the variability found within the two groups.

In conclusion, despite the limited number of examined truffle populations and aware that more in-depth investigations will be required, the results discussed in this doctoral thesis allowed to extend the scientific knowledge on the Molise truffle, a resource of great importance for the regional economy, but for a long time scientifically neglected, providing an interesting overview of two of the most common species, *T. aestivum* and *T. magnatum*, with particular reference to the ascocarp-associated microbiota composition.

The full comprehension of the role of bacteria in truffle life cycle and the factors that drive the establishment of specific microbial communities in the ascocarps could also contribute to the improvement of truffle cultivation techniques, with important repercussions in the field of truffle farming.

Thinking in terms of future perspectives it would be interesting to carry out further analyses including a larger number of samples with the aim to identify possible features (such as unique bacterial taxa that might contribute to the development of distinctive traits in truffles, as well as peculiar

morphological and genetic characteristics) useful to define specific markers of truffle origin. This could have a significant impact on truffle industry, mainly at regional scale, in order to ensure the traceability, conservation, and promotion of this valuable resource.

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Annex A

UNIVERSITÀ DEGLI STUDI DEL MOLISE
DIPARTIMENTO S.T.A.T.
Contrada Fonte Lappone - PESCHE (IS)

Carta della potenzialità tartuficola in scala 1:100.000 della provincia di Campobasso (Molise)

RELAZIONE PROV. CAMPOBASSO

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ALLEGATI:

CARTOGRAFIE IN SCALA 1:100.000

- ✓ Carta della vocazionalità tartuficola per *Tuber magnatum* della provincia di Campobasso
- ✓ Carta della vocazionalità tartuficola per *Tuber melanosporum* della provincia di Campobasso
- ✓ Carta della vocazionalità tartuficola per *Tuber aestivum d*ella provincia di Campobasso
- ✓ Carta della vocazionalità tartuficola per *Tuber borchii* i della provincia di Campobasso

CD:

- ✓ File vettoriali delle cartografie (georiferimento UTM - Fuso 33 - Datum WGS 1984)
- ✓ Tabelle di valutazione
- ✓ Relazione

PREMESSA

L’oggetto dell’incarico assegnato a choros sas di Massimo Paolanti e C è inserito nell’ambito della convenzione che il Dipartimento di Scienze e tecnologie per l’ambiente ed il territorio dell’Università degli studi del Molise ha stabilito con la Regione Molise, che si riferisce all’ *Individuazione preliminare delle Zone Geografiche di Raccolta (ZGR) dei tartufi nelle Province di Campobasso e Isernia* (Allegato A della Convenzione).

La relazione si riferisce ai risultati ed i prodotti relativi alla Provincia di Campobasso.

Nel 2008 nell’ambito del progetto di ricerca “progettazione e prime azioni per la valorizzazione della vivaistica regionale”è stata svolta un’indagine finalizzata all’ecologia dei tartufi molisani”, il cui risultato è stata l’elaborazione di una cartografia della *Vocazionalità* in scala 1:250.000, riferita alle principali specie di tartufo (*Tuber magnatum, Tuber melanosporum, Tuber aestivum, Tuber aestivum var. uncinatum, Tuber borchii*)

In questa seconda fase l’obiettivo è l’elaborazione, a livello provinciale, delle carte delle potenzialità per lo sviluppo di tartufaie spontanee delle predette specie tartufigene in scala 1:100.000.

Molte sono le esperienze condotte a livello di dettaglio che hanno indagato le caratteristiche ecologiche di tartufaie naturali delle diverse specie di tartufo, da queste indagini esce un quadro di relazione complesse che legano i tartufi a suoli, fisiografia, substrati, vegetazione, gestione e clima. Alcuni di questi parametri sono generalizzabili a scale meno dettagliate altri non lo sono, ed ovviamente è necessario tenere anche conto degli strati geografici disponibili.

Ovviamente sono state consultate le esperienze svolte in Italia per la mappatura di aree potenzialmente idonee a scala regionale (Abruzzo, Piemonte, Marche, Basilicata e Lombardia)

La conoscenza di siti ove sia stata accertata la presenza di tartufi allo stato spontaneo, può essere considerata un indice dell’esistenza di condizioni pedo-climatiche idonee. Tuttavia, l’assenza di tartufi in una certa area, o meglio l’assenza di segnalazioni, non è considerata un motivo sufficiente per l’esclusione della stessa dal novero di quelle potenzialmente idonee.

Le cartografie in via di elaborazione è un prodotto nel quale aree potenzialmente idonee allo sviluppo di tartufaie naturali comprendono siti idonei a tale simbionte, ma anche siti non idonei, non consentendo la scala di discriminare ulteriormente. Ricordiamo che le specie di tartufo eduli, ed in particolare il tartufo bianco pregiato (*Tuber magnatum*), sono molto selettive per quanto riguarda le caratteristiche ambientali, con forti esigenze per il loro diffondersi e soprattutto per il loro fruttificare.

La potenzialità allo sviluppo di tartufaie spontanee viene desunta dall’analisi delle caratteristiche territoriali favorevoli ricavate da ricerche condotte a livello di dettaglio. L’ecologia delle tartufaie naturali, analizzate a scala di precisione evidenza un quadro di relazioni complesse che legano i tartufi alle caratteristiche dei suoli, alla morfologia, ai substrati geologici, alla vegetazione, alla gestione agricola e forestale ed al clima.

È ipotizzabile in questa fase identificare aree con potenzialità già all’attualità ed altre con potenzialmente idonee nel caso di un cambiamento dell’uso attuale del suolo. Ovviamente in questo caso le ipotesi debbono tener conto di scenari ipotizzabili all’attualità e quindi vengono esclusi dinamiche a carico di superfici artificiali, le “*aree nude*”, i corsi ed i corpi d’acqua e le aree boscate.

METODOLOGIA

Le attività che hanno coniugato le conoscenze acquisite sull’ecologia delle varie specie di tartufo da una parte e le caratteristiche territoriali dall’altra, sono ormai molte. Dall’analisi di queste relazioni, peraltro complesse, diversi sono stati i tentativi di formalizzare sotto forma di cartografia la distribuzione di ambiti potenzialmente idonei allo viluppo dei tartufi.

In tal senso si possono riportare le esperienze già maturate in Abruzzo, Basilicata, Marche, Lombardia e Piemonte.

Molto interessanti sono anche i risultati che sono messi a disposizione dagli enti di ricerca con l’elaborazione di modelli sia a scala regionale che di semidettaglio.

La maggior parte di queste esperienze alla scala regionale hanno elaborato griglie valutative che hanno applicato ad alcuni caratteri territoriali considerati particolarmente significativi per lo sviluppo dei tartufi. La tecnica generalmente utilizzata è quella dell’intersezione definendo in primo luogo le aree che esprimono condizioni considerate ostative almeno per uno dei caratteri considerati e quindi *non idonee*.

La questione principale è quindi disporre di strati informativi utili o perché informano direttamente sulle caratteristiche territoriali ecologiche considerate determinanti o perchè abbiano una correlazione più o meno stretta con gli stessi.

La disponibilità di dati sulla diffusione dei tartufi in Molise, utilizzabili ai fini della costruzione di una banca dati geografica sono scarsi, così come sono scarse sul territorio Molisano le esperienze utili ai fini di una spazializzazione cartografica.

Ai nostri fini da un punto di vista operativo sono state consultate in maniera specifica le esperienze delle regioni, con particolare attenzione a quanto elaborato nella regione Abruzzo, regione confinante a cui sono relazionabili molti ambiti territoriali del Molise.

Importante notare che in Abruzzo è presente una cartografia dei tipi forestali ed una cartografia geologica regionale ed in entrambi i casi il gruppo di lavoro è lo stesso che ha elaborato le cartografie presenti in Molise. Bisogna comunque segnalare come in Abruzzo sia disponibile una cartografia pedologica regionale ed una banca dati pedologica che archivia alcune migliaia di osservazioni pedologiche, e che nei progetti collegati alla predisposizione della cartografia sia stata effettuata anche una campagna di rilevamento pedologico in circa 200 siti.

In Abruzzo inoltre è stata predisposta una banca dati in cui sono stati censiti oltre mille siti di tartufaie spontanee: (429 siti con specie prevalente di tartufo bianco pregiato e 226 nero pregiato, 305 scorzone, 35 estivo e 97 di bianchetto). Studiando la distribuzione territoriale delle tartufaie in un territorio che presenta analogie con quello della regione Molise ha permesso di comprendere le relazioni che esistono tra alcune caratteristiche territoriali e lo sviluppo di tartufaie spontanee delle specie in oggetto, e più precisamente, dal punto di vista delle elaborazioni cartografiche, come utilizzare le banche territoriali disponibili nella regione Molise.

Le elaborazioni sono state effettuate in ambiente GIS (Arc GIS 9.3), elaborando ed archiviando i dati in un geodatabase.
Lavorare con un geodatabase aumenta l’efficacia delle nostre elaborazioni permettendo di verificare in maniera relativamente semplice ipotesi differenti.

Gli strati elaborati finali sono coerenti con i limiti regionali forniti da UNIMOL e topologicamente corretti

GLI STRATI INFORMATIVI DISPONIBILI

Nella fase preliminare.sono stati acquisiti gli strati informativi utili ai fini del presente progetto.

- Carta geologica del Molise in scala 1:100.000. (Vezzani Ghisetti, 2004)
- Uso del suolo e vegetazione:
  - ✓ Cartografia dei tipi forestali (fonte Unimol)
  - ✓ CORINE land cover 2006 (Fonte ISPRA)
  - ✓ Carta dei Tipo forestali e dell’uso del suolo (fonte UNIMOL in preparazione)
- Suoli
  - ✓ Cartografia Pedologica di semidettaglio (ERSA Molise scala 1:50.000)
- Carta del Fitoclima (GIS Natura 2005)
- DEM
- Altri dati
  - ✓ Strati topografici IGMI
  - ✓ Idrografia
  - ✓ Ortofoto digitali
  - ✓ Informazioni sulla distribuzione delle tartufaie spontanee (fonte dati Unimol)

Oltre a questi strati informativi è stata raccolta e consultata una corposa bibliografia, il cui elenco riportiamo in appendice.

Tutti gli strati informativi sono stati trattati e georiferiti in UTM fuso 33 datum WGS 1984
Solo alcuni di questi dati sono stati utilizzati per le elaborazioni che sono alla base della costituzione della valutazione di idoneità potenziale.

Carta geologica del Molise

Il documento con informazioni geologiche più attendibili è sicuramente la recente carta geologica del Molise. (A. Festa, F. Ghisetti & L. Vezzani CARTA GEOLOGICA DEL MOLISE (Scala 1:100.000). 2004-). Il documento oltre che un’attendibilità certificata che altri dati disponibili, prevalentemente di incerta fonte, non hanno, ha i seguenti pregi:

- ✓ È recente e tiene conto quindi di tutte le conoscenze anche locali disponibili
- ✓ Non necessità di un attività di correlazione fra fogli differenti
- ✓ Ha una legenda e delle note illustrative che permettono di interpretare comportamenti e caratteristiche, meglio di quanto non possano consentire altre informazione che spesso forniscono solo indicazioni generiche che al più si concretizzano in una definizione sintetica

È doveroso però sempre ricordare che, per il meccanismo proprio delle cartografia geologica che occorre sempre compiere una certa approssimazione per dedurre dalle formazioni descritte le caratteristiche dei substrati pedogenetici.

Essendo indisponibile il dato vettoriale è stato necessario elaborarlo appositamente partendo dal documento cartaceo. Si tratta di un oltre 3.100 poligoni articolati in circa 102 unità cartografiche.

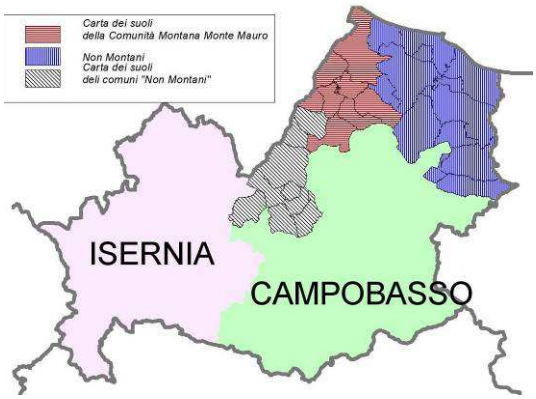
Le informazioni sui suoli

Le informazioni sui suoi disponibili che sono state consultate sono le seguenti:

- Cartografia pedologica ERSAL Molise
- dati pedologici presenti presso UNIMOL
  - ✓ PedoMol_WGS32.shp
  - ✓ Pedo_wgs32_diss2.shp
  - ✓ carta_pedologica_TN_wgs33.shp

È bene precisare che i dati pedologici presenti presso UNIMOL, sono banche dati geografiche di cui non sono disponibili dati sulle fonti e metodologie, che rendono disponibile solo un set limitato di dati da cui non è possibile valutazioni su caratteristiche e qualità dei suoli, come quelle

necessarie per le valutazioni sulle varie specie di tartufo considerate



Cartografia pedologica ERSAL Molise

La regione Molise non dispone di uno banca dati dei suoli uniforme per tutta la regione

Le informazioni sui suoli disponibili per la regione Molise, sono relative a cartografie pedologiche di semidettaglio che coprono circa il 28% della regione, con una notevole disformità tra le due province (42% CB e 2% IS).
Come prima fase di questo lavoro i dati presenti su supporto cartaceo sono stati acquisiti in formato digitale (Shape file ESRI)

Esiste presso ERSAM Molise anche un prodotto relativo ad un cartografia preliminare dei sottosistemi di terre, che non è però disponibile e non risulta comunque collega ad una banca dati pedologica.

Si tratta di dati molto utili da utilizzarsi per valutazioni di semidettaglio e per la costruzione di un approfondimento del modello di valutazione, sempre ricordando che solo una parte del territorio è coperta. La copertura di informazioni disponibili relative ai suoli risultano essere per la provincia di Campobasso non avendo copertura completa del territorio sono state utilizzate in particolare per la definizione delle chiavi di interpretazione, sopratutti per le relazioni substrati pedopaesaggi..

Uso del suolo e vegetazione

Sono disponibili due fonti dati vettoriali, una relative ai tipi forestali che interessa il territorio forestale della regione e la banca dati *CORINE land cover* aggiornata all’anno 2006 elaborata per l’Italia da ISPRA.
La Cartografia dei tipi forestali (fonte UNIMOL) e contiene 31066 poligoni, ha un dettaglio nominale in scala 1:10.000 e ad ogni poligono sono associati attributi relativi alla fisionomia, struttura e copertura.
DISTAT UNIMOL ha attualmente in elaborazione una cartografia a copertura completa che integra la carta dei tipo forestali per quanto riguarda le superfici artificiali, le aree agricole e le acque.

Per la provincia di Campobasso non è ancora disponibile tale strato, il cui dettaglio permetterebbe di discriminare in maniera molto più efficace il territorio rispetto all’utilizzo della banca data Corine Land Cover 1:100.000.

Analisi bioclimatica

Per quanto riguarda il clima, l’unico dato spazializzato utile per la valutazione all’idoneità è quello reso disponibile da GIS Natura (Il Fitoclima d’Italia. Ministero dell’Ambiente e della Tutela del Territorio. Consiglio Nazionale delle Ricerche, Istituto di Ecologia e Idrologia Forestale). Questo suddivide il territorio Italiano in classi fitoclimatiche italiane derivate dall’integrazione di parametri e indici climatici con le caratteristiche geobotaniche del territorio.

Uno studio condotto da Ciaschetti et alii (*Ciaschetti G., Marchetti F., Di Lena B., De Laurentiis G., Cimini G, Spinelli D. Caratterizzazione climatica delle aree a vocazione tartuficola della regione Abruzzo*), ha evidenziato che seppure in un'ampia valenza ecologica per *Tuber melanosporum* e *Tuber magnatum*. *Tuber melanosporum* mostra una chiara preferenza per l'orizzonte **Supratemperato inferiore** (Rivas- Martinez et al., 2002), mentre *Tuber magnatum* è diffuso prevalentemente nel **Mesotemperato inferiore** (Rivas- Martinez et al., l.c.).

DEM (Digitale Elevation Model)

Dal modello digitale del terreno, sono stati ricavate diverse elaborazioni. Tra queste è stato creata uno strato poligonale con intervalli di quota di 100 metri. È stata svolta una prova applicativa

legando l’idoneità allo sviluppo potenziale dei tartufi a fasce di quota, scelte in base ad indicazioni bibliografiche ed in relazione alle esperienze analoghe effettuate recentemente in Abruzzo. Analogamente possono essere fatte elaborazione sull’acclività.

Altri dati

Strati topografici IGMI

Sono stati acquisiti, georiferiti e mosaicati le tavolette IGMI 1:25.000 ed i fogli in scala 1:100.000, utili in fase di verifica dei risultati.

Idrografia

Il reticolo idrografico ha una forte correlazione con la distribuzione delle tartufaie di *Tuber magnatum*, ed quindi uno strato informativo da ritenersi utile in sede di verifica dei risultati ottenuti e per le fasi di interpretazione a video. È stato creato un file poligonale che identifica un bufere di 100 metri attorno agli elementi censiti dal reticolo idrografico regionale in scala 1:25.000.

Ortofoto digitali

Le ortofoto digitali a colori (Territaly 2008), seppure con un dettaglio evidentemente molto spinto per le esigenze del progetto, sono uno strumento utile per verifiche puntuali e per la validazione delle chiavi di interpretazione. Ad esempio alcuni siti di tartufaie spontanee sono posizionati in aree che non risultano avere caratteristiche idonee, ma la verifica puntuale sulle ortofoto ha consentito di appurare che si tratta prevalentemente di approssimazioni di ubicazione oppure di dettaglio delle banche dati di riferimento.

Informazioni sulla distribuzione delle tartufaie spontanee (fonte dati Unimol)

UNMOL ha censito circa 50 siti di tartufaie spontanee, che sono un utile strumento per verificare le ipotesi di relazione fra caratteristiche territoriali e potenzialità per lo sviluppo delle tartufaie.

LE GRIGLIE DI VALUTAZIONE

Dopo l’analisi dei dati disponibili sono state elaborate,per ciascuna specie, le tabelle di idoneità relativamente ai seguenti layer informativi:

- _ido_geol (attribuzione di idoneità alle 106 Unità Cartografiche, presenti nella cartografia geologica)
- _ido_quote il territorio regionale è stato suddiviso in classi di quota
- _CLCO6_IDO_

Gli strati così classificati sono stati intersecati tra di loro ed applicata una griglia di valutazione complessiva che ha tenuto conto conto delle diverse caratteristiche territoriali.
Le modalità di elaborazione della griglia risultante, sono complesse, in alcuni casi, infatti, le informazioni di uno strato informativo mettono in luce caratteristiche territoriali non discriminate, per motivi di dettaglio o di legenda, da altri strati informativi. In particolare le formazioni vegetazionali proprie delle aree prossime agli impluvi in molti casi sono relazionate a ambienti pedo-geomorfici che la cartografia geologica non coglie e quindi quando queste formazioni sono presenti la valutazione sull’idoneità geologica viene integrata da questa informazione.

Negli altri casi vengono attribuiti dei pesi alle singole caratteristiche e fatta la somma della risultante *classe* **X** *peso* i valori saranno poi classati in 3 classi di attitudine. Se uno dei singoli parametri selezionati però indica potenzialità nulla il poligono viene comunque classificato come non idoneo.
Con questa metodologia sono individuate le aree potenzialmente idonee all’attualità, ossia quelle aree che hanno ad oggi caratteristiche idonee allo sviluppo di tartufaie spontanee.

Per il *Tuber magnatum* si è tenuto conto che esistono aree prevalentemente agricole con inclusioni non cartografabili alla scala di progetto. In questo caso si è verificato che nell’unità CORINE LAND COVER relativa alle *aree agricole con spazi naturali importanti* (cod. 243), sono diffusi siti idi tartufaie spontanee. .È stata quindi creata un’unità cartografica, per le aree agricole della provincia di Campobasso ove ricorrano caratteri potenzialmente predisponenti per quanto riguarda natura dei substrati e quote.

In queste aree insiste inoltre una potenzialità che si può ulteriormente sviluppare nel caso vi siano dinamismi dell’uso del suolo, con sostituzione di aree attualmente agricole con aree occupate da vegetazione naturale.

Le elaborazioni GIS che sono state effettuate sono state: intersezione degli strati informativi, attribuzione dei valori finali, dissolve dei poligoni confinanti omogenei al fine della valutazione, eliminazione dei poligoni di dimensioni non compatibili con l’accuratezza geometrica degli stati di base, verifica delle topologia degli strati poligonali finali.

La fase di procedimento automatico è stata integrata con una revisione puntale tenendo conto di:

- ✓ orto foto digitali;
- ✓ reticolo idrografico e relative elaborazioni quali strato *buffer* per il *Tuber magnatum*)
- ✓ cartografia topografica IGMI.

Nel Geodatabase sono state elaborate delle tabelle di attitudine che classificano il territorio in 4 livelli qualitativi di attitudine per ciascuna delle specie:

A. 0 potenzialità nulla
B. 1 potenzialità scarsa
C. 2 potenzialità media
D. 3 potenzialità elevata

E. 4:aree potenzialmente idonee a medio termine (nel caso vi siano dinamismi relativi al *Land Cover*.

F. Altre aree:
- F.i. Superfici artificiali
- F.ii. Corpi e corsi d'acqua
- F.iii. Spiagge dune e sabbie (ad esclusione delle aree sabbiose litoranee che sono idonee allo sviluppo del *Tuber borchii.*
- F.iv. Rocce nude, falesie, rupi ed affioramenti
- F.v. Aree umide

Gli strati di base, le tabelle ed i file vettoriali di elaborazione finale sono state archiviati in un geodatabase (**T_CB_11.mdb**).

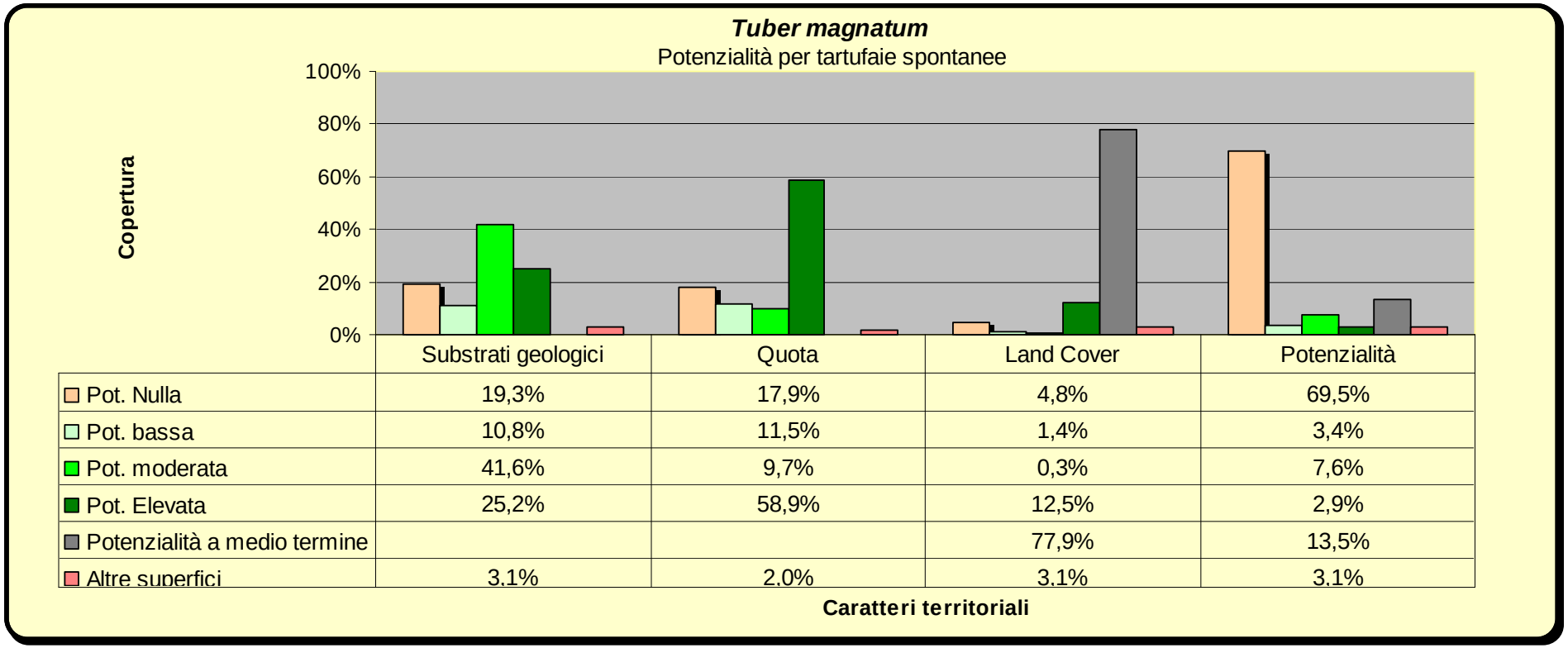
I RISULTATI PER LA PROVINCIA DI CAMPOBASSO

Nelle pagine seguenti sono riportasti sotto forma di tabelle, grafici e riduzioni cartografiche i risultati raggiunti per la provincia di Campobasso relativamente alle quattro specie in oggetto.

Le legende inserite nelle cartografie sono le seguenti

Specie	Legenda
Tuber magnatum	1)Aree a potenzialità bassa 2) Aree a potenzialità media 3) Aree a potenzialità elevata 4) Aree con potenzialità a medio termine e/o con inclusioni non cartografabili di aree potenzialmente idonee - Corpi e corsi d'acqua - Superfici artificiali (urbano, industriale, infrastrutture ecc.)
Tuber aestivum	1)Aree a potenzialità bassa 2) Aree a potenzialità media 3) Aree a potenzialità elevata - Corpi e corsi d'acqua - Superfici artificiali (urbano, industriale, infrastrutture ecc.)
Tuber Melanosporum	1)Aree a potenzialità bassa 2) Aree a potenzialità media 3) Aree a potenzialità elevata - Corpi e corsi d'acqua - Superfici artificiali (urbano, industriale, infrastrutture ecc.)
Tuber borchii	1)Aree a potenzialità bassa 2) Aree a potenzialità media 3) Aree a potenzialità elevata - Corpi e corsi d'acqua - Superfici artificiali (urbano, industriale, infrastrutture ecc.)

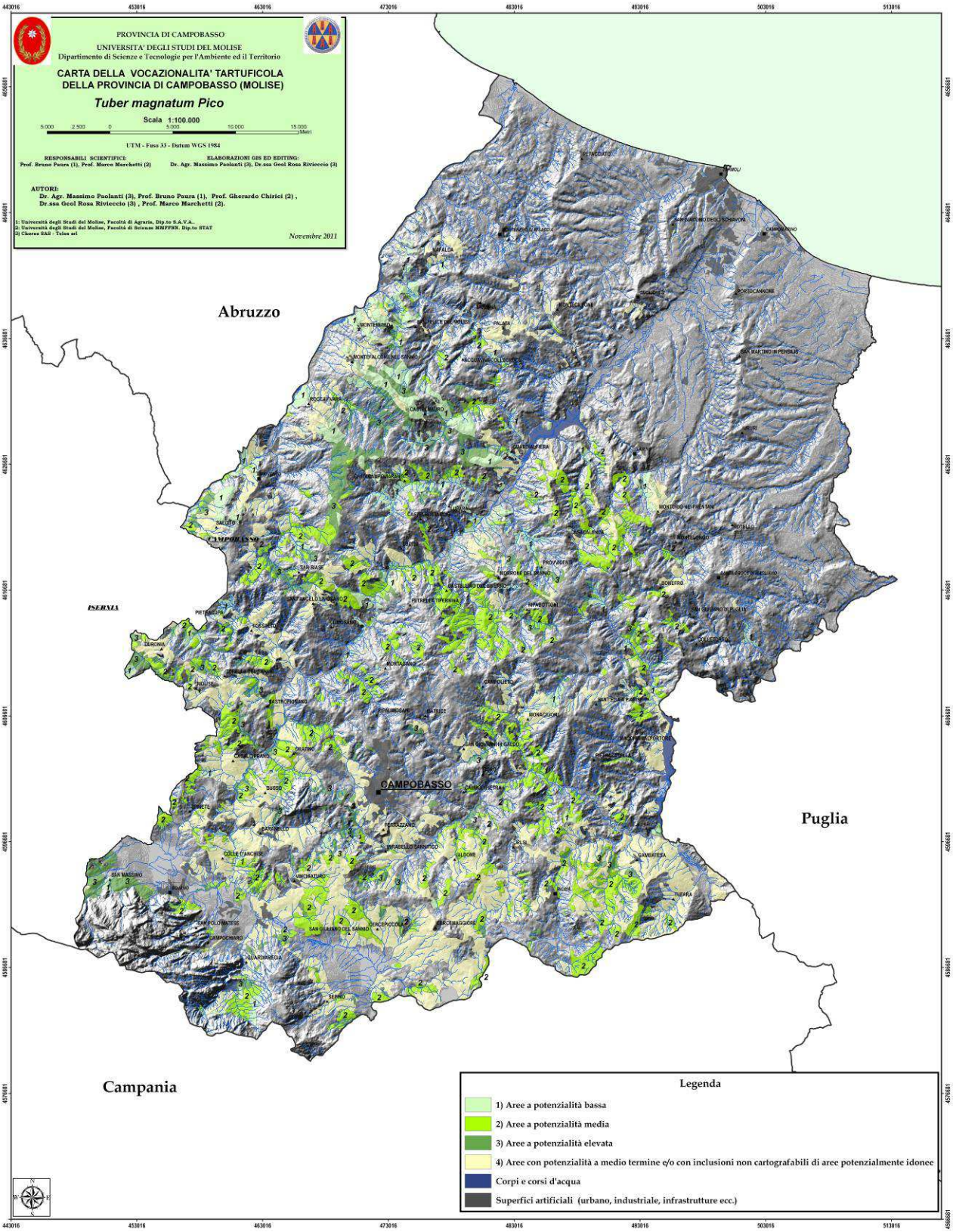
Potenzialità per il Tuber magnatum



	Substrati geologici	Quota	Land Cover	Potenzialità
Pot. Nulla	19,3%	17,9%	4,8%	69,5%
Pot. bassa	10,8%	11,5%	1,4%	3,4%
Pot. moderata	41,6%	9,7%	0,3%	7,6%
Pot. Elevata	25,2%	58,9%	12,5%	2,9%
Potenzialità a medio termine			77,9%	13,5%
Altre superfici	3,1%	2,0%	3,1%	3,1%

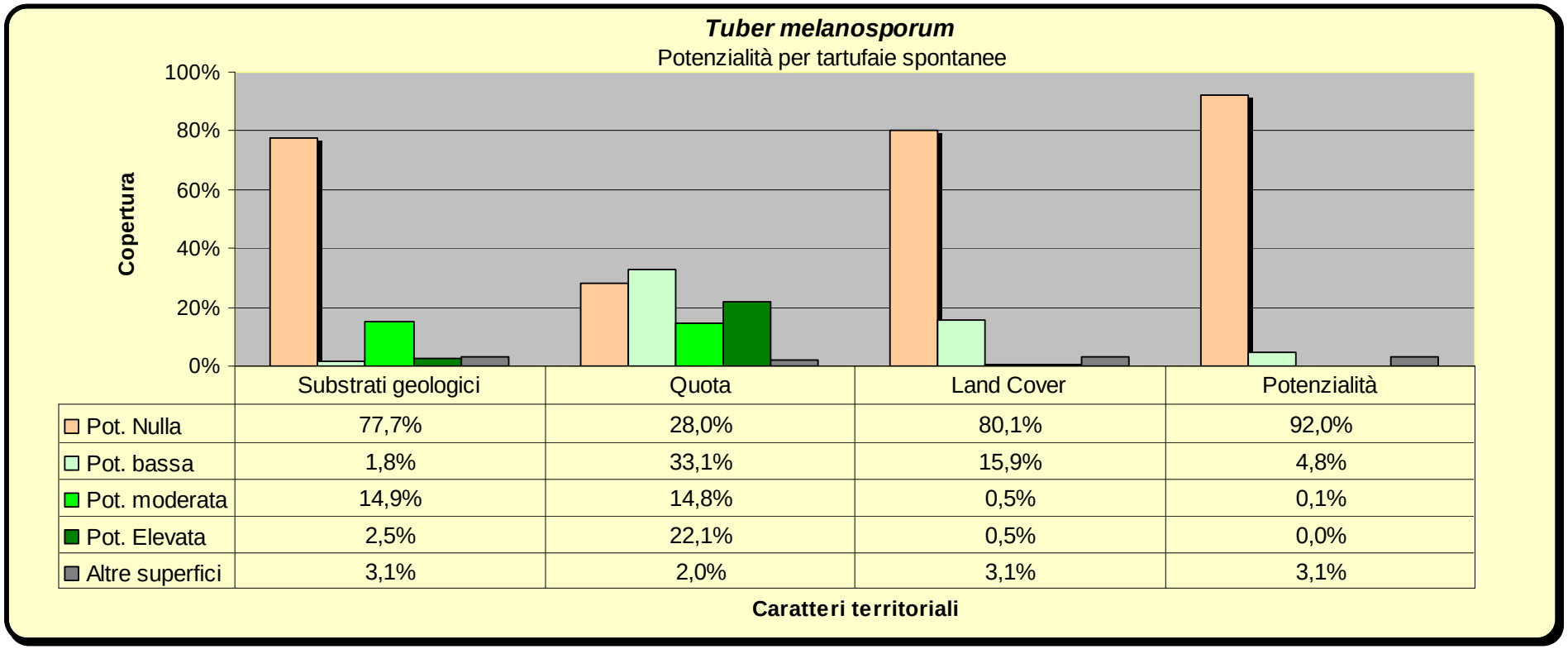
La classe Potenzialità a medio termine corrisponde ad aree agricole caratterizzate da spazi naturali importanti ove ricorrano caratteri potenzialmente predisponenti per quanto riguarda natura dei substrati e quote.

Altre superfici corrispondono a superfici non idonee allo sviluppo di tartufaie spontanee (aree urbane, corpi e corsi d’acqua, superfici poste oltre i 1.300 m slm, aree prive di suolo ecc.)



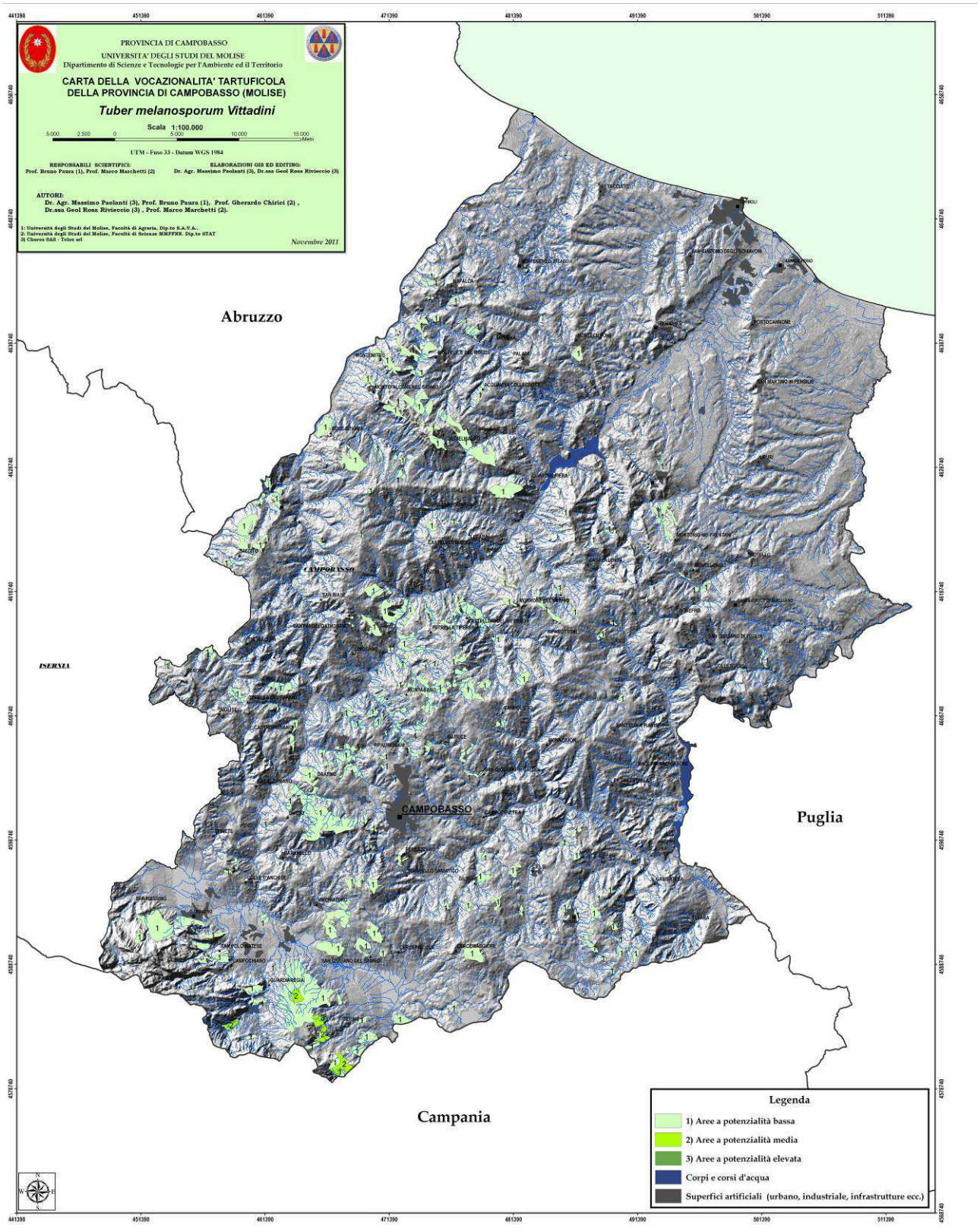
Paolanti M, Paura B, Chirici G, Rivieccio R, e Marchetti M (2011)

Potenzialità per il Tuber melanosporum



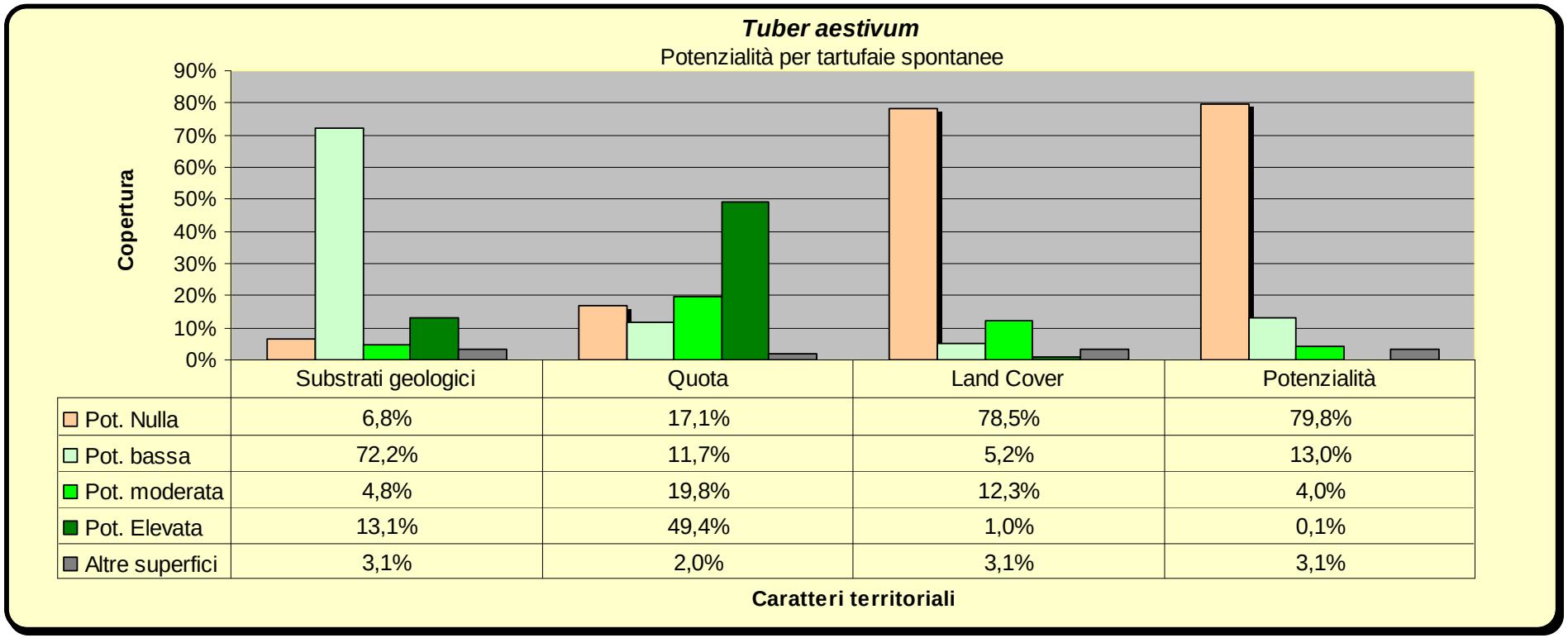
	Substrati geologici	Quota	Land Cover	Potenzialità
Pot. Nulla	77,7%	28,0%	80,1%	92,0%
Pot. bassa	1,8%	33,1%	15,9%	4,8%
Pot. moderata	14,9%	14,8%	0,5%	0,1%
Pot. Elevata	2,5%	22,1%	0,5%	0,0%
Altre superfici	3,1%	2,0%	3,1%	3,1%

Altre superfici corrispondono a superfici non idonee allo sviluppo di tartufaie spontanee (aree urbane, corpi e corsi d’acqua, superfici poste oltre i 1.300 m slm, aree prive di suolo ecc.)



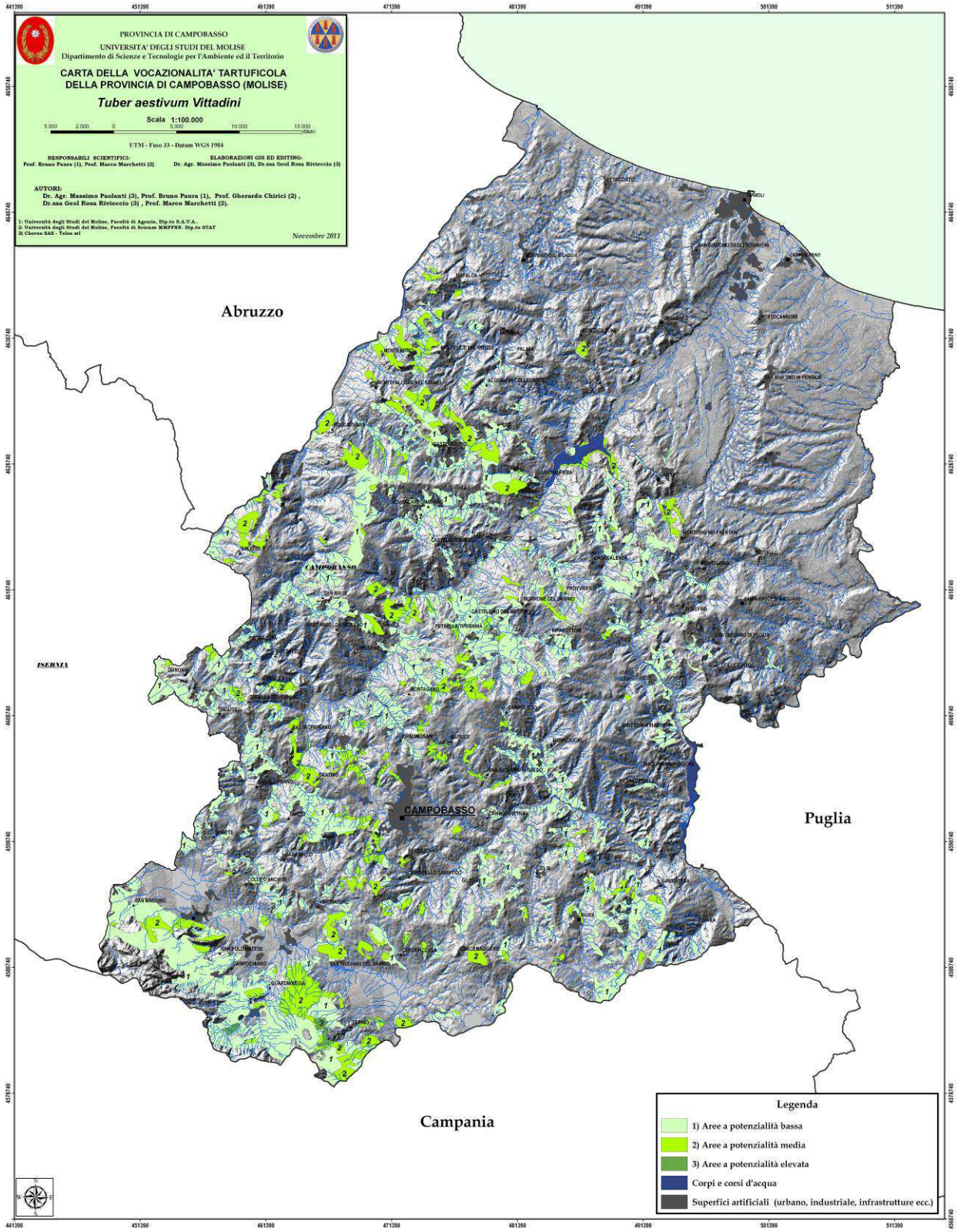
Paolanti M, Paura B, Chirici G, Rivieccio R, e Marchetti M (2011)

Potenzialità per il Tuber aestivum

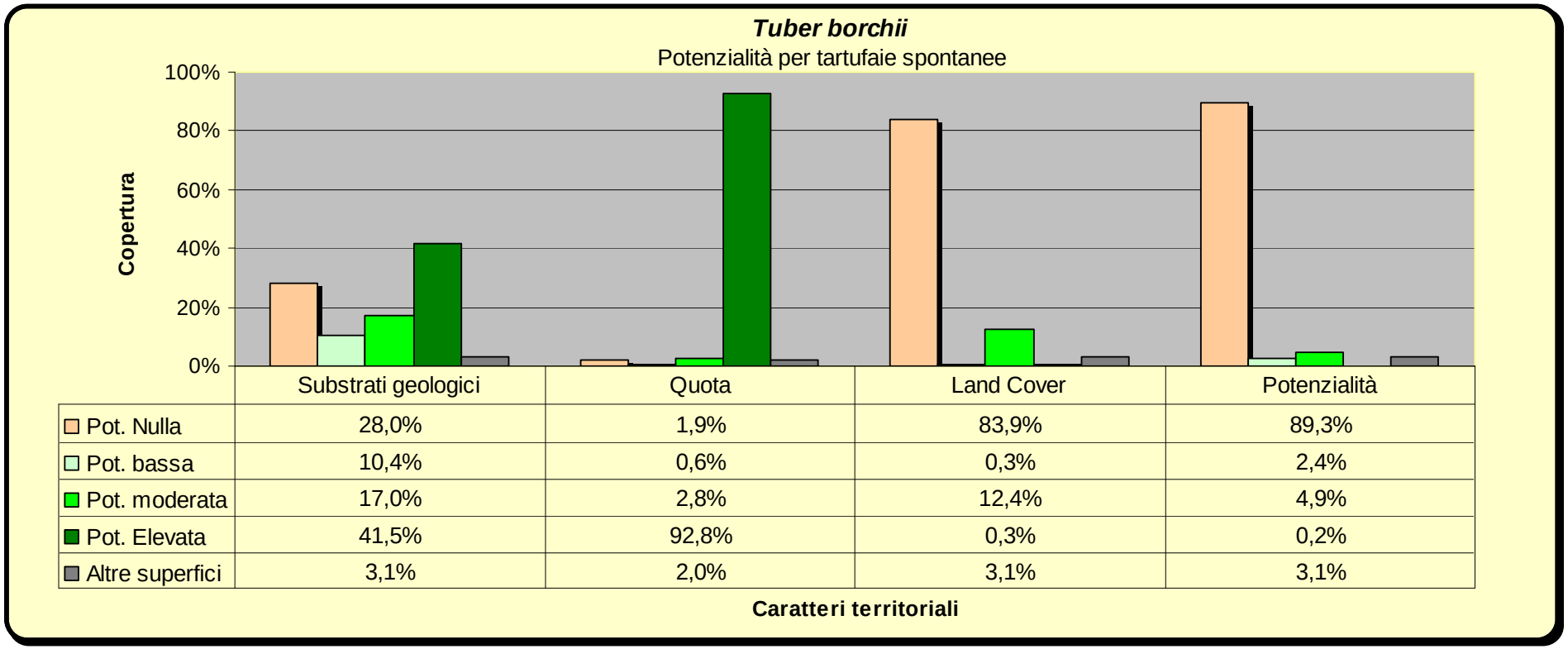


	Substrati geologici	Quota	Land Cover	Potenzialità
Pot. Nulla	6,8%	17,1%	78,5%	79,8%
Pot. bassa	72,2%	11,7%	5,2%	13,0%
Pot. moderata	4,8%	19,8%	12,3%	4,0%
Pot. Elevata	13,1%	49,4%	1,0%	0,1%
Altre superfici	3,1%	2,0%	3,1%	3,1%

Altre superfici corrispondono a superfici non idonee allo sviluppo di tartufaie spontanee (aree urbane, corpi e corsi d’acqua, superfici poste oltre i 1.300 m slm, aree prive di suolo ecc.)

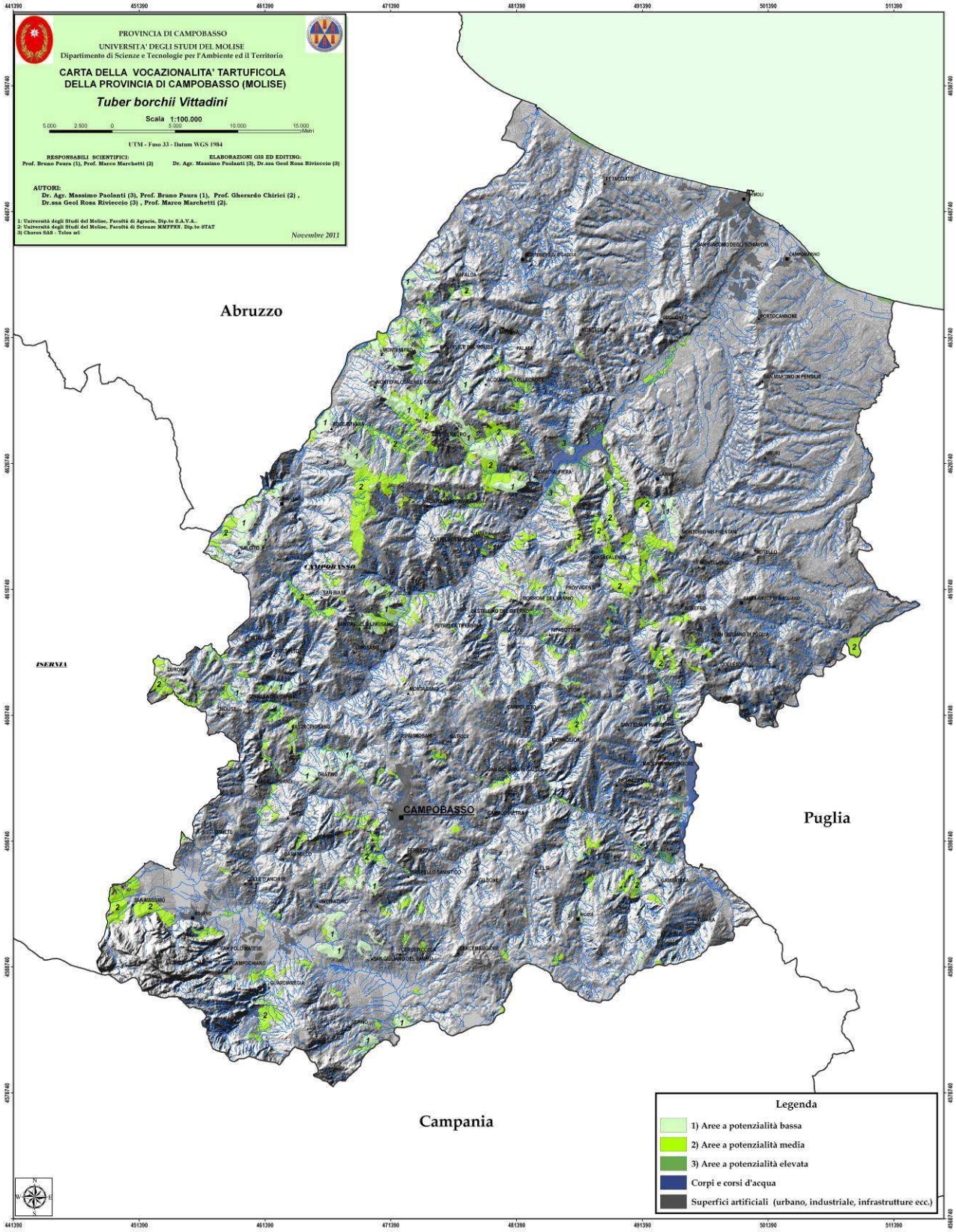

PROVINCIA DI CAMPOBASSO
UNIVERSITA' DEGLI STUDI DEL MOLISE
Dipartimento di Scienze e Tecnologie per l'Ambiente ed il Territorio
CARTA DELLA VOCAZIONALITA' TARTUFICOLA
DELLA PROVINCIA DI CAMPOBASSO (MOLISE)
Tuber aestivum Vittadini
Scala 1:100.000
UTM - Fuso 33 - Datum WGS 1984
RESPONSABILI SCIENTIFICI:
Prof. Bruno Paura (1), Prof. Marco Marchetti (2)
ELABORAZIONI GIS ED EDITING:
Dr. Agr. Massimo Paolanti (3), Dr.ssa Geol Rosa Rivieccio (3)
AUTORI:
Dr. Agr. Massimo Paolanti (3), Prof. Bruno Paura (1), Prof. Gherardo Chirici (2) ,
Dr.ssa Geol Rosa Rivieccio (3) , Prof. Marco Marchetti (2).
1: Università degli Studi del Molise, Facoltà di Agraria, Dip.to S.A.V.A.
2: Università degli Studi del Molise, Facoltà di Scienze MMFFNN. Dip.to STAT
3: Chorus SAS - Telos srl
Novembre 2011
Abruzzo
Puglia
Campania
CAMPOBASSO
Legenda
1) Aree a potenzialità bassa
2) Aree a potenzialità media
3) Aree a potenzialità elevata
Corpi e corsi d'acqua
Superfici artificiali (urbano, industriale, infrastrutture ecc.)

Potenzialità per il Tuber borchii



	Substrati geologici	Quota	Land Cover	Potenzialità
Pot. Nulla	28,0%	1,9%	83,9%	89,3%
Pot. bassa	10,4%	0,6%	0,3%	2,4%
Pot. moderata	17,0%	2,8%	12,4%	4,9%
Pot. Elevata	41,5%	92,8%	0,3%	0,2%
Altre superfici	3,1%	2,0%	3,1%	3,1%

Altre superfici corrispondono a superfici non idonee allo sviluppo di tartufaie spontanee (aree urbane, corpi e corsi d’acqua, superfici poste oltre i 1.300 m slm, aree prive di suolo ecc.)


PROVINCIA DI CAMPOBASSO
UNIVERSITA' DEGLI STUDI DEL MOLISE
Dipartimento di Scienze e Tecnologie per l'Ambiente ed il Territorio
CARTA DELLA VOCAZIONALITA' TARTUFICOLA
DELLA PROVINCIA DI CAMPOBASSO (MOLISE)
Tuber borchii Vittadini
Scala 1:100.000
UTM - Fuso 33 - Datum WGS 1984
RESPONSABILI SCIENTIFICI:
Prof. Bruno Paura (1), Prof. Marco Marchetti (2)
ELABORAZIONI GIS ED EDITING:
Dr. Agr. Massimo Paolanti (3), Dr.ssa Geol Rosa Rivieccio (3)
AUTORI:
Dr. Agr. Massimo Paolanti (3), Prof. Bruno Paura (1), Prof. Gherardo Chirici (2) ,
Dr.ssa Geol Rosa Rivieccio (3) , Prof. Marco Marchetti (2).
Novembre 2011
Abruzzo
Puglia
Campania
CAMPOBASSO
Legenda
1) Aree a potenzialità bassa
2) Aree a potenzialità media
3) Aree a potenzialità elevata
Corpi e corsi d'acqua
Superfici artificiali (urbano, industriale, infrastrutture ecc.)

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- ✓ UNIVERSITÀ DEGLI STUDI DELLA BASILICATA *Carta delle aree lucane vocate alla produzione e alla coltivazione di tartufi.* Dipartimento di Biologia, Difesa e Biotecnologie agro-forestali.

Annex B

UNIVERSITÀ DEGLI STUDI DEL MOLISE
DIPARTIMENTO S.T.A.T.
Contrada Fonte Lappone - PESCHE (IS)

Carta della potenzialità tartuficola in scala 1:100.000 della provincia di Isernia (Molise)

RELAZIONE PROV. ISERNIA

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PROF. BRUNO PAURA ([1]),
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[3] CHOROS SAS – TELOS SRL, ROMA

86090 PESCHE (IS) - Contrada Fonte Lappone - Tel. 0874 404100 - Fax 0874 404123

ALLEGATI:

CARTOGRAFIE IN SCALA 1:100.000

- ✓ Carta della vocazionalità tartuficola per *Tuber magnatum* della provincia di Isernia
- ✓ Carta della vocazionalità tartuficola per *Tuber melanosporum* della provincia di Isernia
- ✓ Carta della vocazionalità tartuficola per *Tuber aestivum* ella provincia di Isernia
- ✓ Carta della vocazionalità tartuficola per *Tuber borchii* i della provincia di Isernia

CD:

- ✓ File vettoriali delle cartografie (georiferimento UTM - Fuso 33 - Datum WGS 1984)
- ✓ Tabelle di valutazione
- ✓ Relazione

PREMESSA

L'oggetto dell'incarico assegnato a choros sas di Massimo Paolanti e C è inserito nell'ambito della convenzione che il Dipartimento di Scienze e tecnologie per l'ambiente ed il territorio dell'Università degli studi del Molise ha stabilito con la Regione Molise, che si riferisce all' *Individuazione preliminare delle Zone Geografiche di Raccolta (ZGR) dei tartufi nelle Province di Campobasso e Isernia* (Allegato A della Convenzione).

La relazione si riferisce ai risultati ed i prodotti relativi alla Provincia di Isernia.

Nel 2008 nell'ambito del progetto di ricerca "progettazione e prime azioni per la valorizzazione della vivaistica regionale"è stata svolta un'indagine finalizzata all'ecologia dei tartufi molisani", il cui risultato è stata l'elaborazione di una cartografia della *Vocazionalità* in scala 1:250.000, riferita alle principali specie di tartufo (*Tuber magnatum, Tuber melanosporum, Tuber aestivum, Tuber aestivum var. uncinatum, Tuber borchii*)

In questa seconda fase l'obiettivo è l'elaborazione, a livello provinciale, delle carte delle potenzialità per lo sviluppo di tartufaie spontanee delle predette specie tartufigene in scala 1:100.000.

Molte sono le esperienze condotte a livello di dettaglio che hanno indagato le caratteristiche ecologiche di tartufaie naturali delle diverse specie di tartufo, da queste indagini esce un quadro di relazione complesse che legano i tartufi a suoli, fisiografia, substrati, vegetazione, gestione e clima. Alcuni di questi parametri sono generalizzabili a scale meno dettagliate altri non lo sono, ed ovviamente è necessario tenere anche conto degli strati geografici disponibili.

Ovviamente sono state consultate le esperienze svolte in Italia per la mappatura di aree potenzialmente idonee a scala regionale (Abruzzo, Piemonte, Marche, Basilicata e Lombardia)

La conoscenza di siti ove sia stata accertata la presenza di tartufi allo stato spontaneo, può essere considerata un indice dell'esistenza di condizioni pedo-climatiche idonee. Tuttavia, l'assenza di tartufi in una certa area, o meglio l'assenza di segnalazioni, non è considerata un motivo sufficiente per l'esclusione della stessa dal novero di quelle potenzialmente idonee.

Le cartografie in via di elaborazione è un prodotto nel quale aree potenzialmente idonee allo sviluppo di tartufaie naturali comprendono siti idonei a tale simbionte, ma anche siti non idonei, non consentendo la scala di discriminare ulteriormente. Ricordiamo che le specie di tartufo eduli, ed in particolare il tartufo bianco pregiato (*Tuber magnatum*), sono molto selettive per quanto riguarda le caratteristiche ambientali, con forti esigenze per il loro diffondersi e soprattutto per il loro fruttificare.

La potenzialità allo sviluppo di tartufaie spontanee viene desunta dall'analisi delle caratteristiche territoriali favorevoli ricavate da ricerche condotte a livello di dettaglio. L'ecologia delle tartufaie naturali, analizzate a scala di precisione evidenza un quadro di relazioni complesse che legano i tartufi alle caratteristiche dei suoli, alla morfologia, ai substrati geologici, alla vegetazione, alla gestione agricola e forestale ed al clima.

È ipotizzabile in questa fase identificare aree con potenzialità già all'attualità ed altre con potenzialmente idonee nel caso di un cambiamento dell'uso attuale del suolo. Ovviamente in questo caso le ipotesi debbono tener conto di scenari ipotizzabili all'attualità e quindi vengono esclusi dinamiche a carico di superfici artificiali, le "*aree nude*", i corsi ed i corpi d'acqua e le aree boscate.

METODOLOGIA

Le attività che hanno coniugato le conoscenze acquisite sull'ecologia delle varie specie di tartufo da una parte e le caratteristiche territoriali dall'altra, sono ormai molte. Dall'analisi di queste relazioni, peraltro complesse, diversi sono stati i tentativi di formalizzare sotto forma di cartografia la distribuzione di ambiti potenzialmente idonei allo viluppo dei tartufi.

In tal senso si possono riportare le esperienze già maturate in Abruzzo, Basilicata, Marche, Lombardia e Piemonte.

Molto interessanti sono anche i risultati che sono messi a disposizione dagli enti di ricerca con l'elaborazione di modelli sia a scala regionale che di semidettaglio.

La maggior parte di queste esperienze alla scala regionale hanno elaborato griglie valutative che hanno applicato ad alcuni caratteri territoriali considerati particolarmente significativi per lo sviluppo dei tartufi. La tecnica generalmente utilizzata è quella dell'intersezione definendo in primo luogo le aree che esprimono condizioni considerate ostative almeno per uno dei caratteri considerati e quindi *non idonee*.

La questione principale è quindi disporre di strati informativi utili o perché informano direttamente sulle caratteristiche territoriali ecologiche considerate determinanti o perchè abbiano una correlazione più o meno stretta con gli stessi.

La disponibilità di dati sulla diffusione dei tartufi in Molise, utilizzabili ai fini della costruzione di una banca dati geografica sono scarsi, così come sono scarse sul territorio Molisano le esperienze utili ai fini di una spazializzazione cartografica.

Ai nostri fini da un punto di vista operativo sono state consultate in maniera specifica le esperienze delle regioni, con particolare attenzione a quanto elaborato nella regione Abruzzo, regione confinante a cui sono relazionabili molti ambiti territoriali del Molise.

Importante notare che in Abruzzo è presente una cartografia dei tipi forestali ed una cartografia geologica regionale ed in entrambi i casi il gruppo di lavoro è lo stesso che ha elaborato le cartografie presenti in Molise. Bisogna comunque segnalare come in Abruzzo sia disponibile una cartografia pedologica regionale ed una banca dati pedologica che archivia alcune migliaia di osservazioni pedologiche, e che nei progetti collegati alla predisposizione della cartografia sia stata effettuata anche una campagna di rilevamento pedologico in circa 200 siti.

In Abruzzo inoltre è stata predisposta una banca dati in cui sono stati censiti oltre mille siti di tartufaie spontanee: (429 siti con specie prevalente di tartufo bianco pregiato e 226 nero pregiato, 305 scorzone, 35 estivo e 97 di bianchetto). Studiando la distribuzione territoriale delle tartufaie in un territorio che presenta analogie con quello della regione Molise ha permesso di comprendere le relazioni che esistono tra alcune caratteristiche territoriali e lo sviluppo di tartufaie spontanee delle specie in oggetto, e più precisamente, dal punto di vista delle elaborazioni cartografiche, come utilizzare le banche territoriali disponibili nella regione Molise.

Le elaborazioni sono state effettuate in ambiente GIS (Arc GIS 9.3), elaborando ed archiviando i dati in un geodatabase.
Lavorare con un geodatabase aumenta l'efficacia delle nostre elaborazioni permettendo di verificare in maniera relativamente semplice ipotesi differenti.

Gli strati elaborati finali sono coerenti con i limiti regionali forniti da UNIMOL e topologicamente corretti

GLI STRATI INFORMATIVI DISPONIBILI

Nella fase preliminare.sono stati acquisiti gli strati informativi utili ai fini del presente progetto.

- Carta geologica del Molise in scala 1:100.000. (Vezzani Ghisetti, 2004)
- Uso del suolo e vegetazione:
  - ✓ Cartografia dei tipi forestali (fonte Unimol)
  - ✓ CORINE land cover 2006 (Fonte ISPRA)
  - ✓ Carta dei Tipo forestali e dell'uso del suolo (fonte UNIMOL in preparazione)
- Suoli
  - ✓ Cartografia Pedologica di semidettaglio (ERSA Molise scala 1:50.000)
- Carta del Fitoclima (GIS Natura 2005)
- DEM
- Altri dati
  - ✓ Strati topografici IGMI
  - ✓ Idrografia
  - ✓ Ortofoto digitali
  - ✓ Informazioni sulla distribuzione delle tartufaie spontanee (fonte dati Unimol)

Oltre a questi strati informativi è stata raccolta e consultata una corposa bibliografia, il cui elenco riportiamo in appendice.

Tutti gli strati informativi sono stati trattati e georiferiti in UTM fuso 33 datum WGS 1984
Solo alcuni di questi dati sono stati utilizzati per le elaborazioni che sono alla base della costituzione della valutazione di idoneità potenziale.

Carta geologica del Molise

Il documento con informazioni geologiche più attendibili è sicuramente la recente carta geologica del Molise. (A. Festa, F. Ghisetti & L. Vezzani CARTA GEOLOGICA DEL MOLISE (Scala 1:100.000). 2004-). Il documento oltre che un'attendibilità certificata che altri dati disponibili, prevalentemente di incerta fonte, non hanno, ha i seguenti pregi:

- ✓ È recente e tiene conto quindi di tutte le conoscenze anche locali disponibili
- ✓ Non necessità di un attività di correlazione fra fogli differenti
- ✓ Ha una legenda e delle note illustrative che permettono di interpretare comportamenti e caratteristiche, meglio di quanto non possano consentire altre informazione che spesso forniscono solo indicazioni generiche che al più si concretizzano in una definizione sintetica

È doveroso però sempre ricordare che, per il meccanismo proprio delle cartografia geologica che occorre sempre compiere una certa approssimazione per dedurre dalle formazioni descritte le caratteristiche dei substrati pedogenetici.

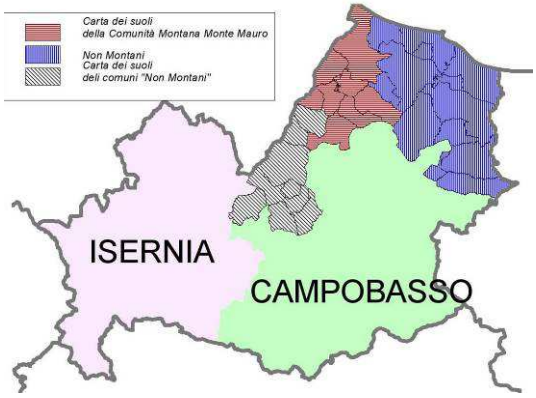
Essendo indisponibile il dato vettoriale è stato necessario elaborarlo appositamente partendo dal documento cartaceo. Si tratta di un oltre 3.100 poligoni articolati in circa 102 unità cartografiche.

Le informazioni sui suoli

Le informazioni sui suoi disponibili che sono state consultate sono le seguenti:

- Cartografia pedologica ERSAL Molise
- dati pedologici presenti presso UNIMOL
  - ✓ PedoMol_WGS32.shp
  - ✓ Pedo_wgs32_diss2.shp
  - ✓ carta_pedologica_TN_wgs33.shp

È bene precisare che i dati pedologici presenti presso UNIMOL, sono banche dati geografiche di cui non sono disponibili dati sulle fonti e metodologie, che rendono disponibile solo un set limitato di dati da cui non è possibile valutazioni su caratteristiche e qualità dei suoli, come quelle necessarie per le valutazioni sulle varie specie di tartufo considerate



Cartografia pedologica ERSAL Molise

La regione Molise non dispone di uno banca dati dei suoli uniforme per tutta la regione

Le informazioni sui suoli disponibili per la regione Molise, sono relative a cartografie pedologiche di semidettaglio che coprono circa il 28% della regione, con una notevole disformità tra le due province (42% CB e 2% IS).
Come prima fase di questo lavoro i dati presenti su supporto cartaceo sono stati acquisiti in formato digitale (Shape file ESRI)

Esiste presso ERSAM Molise anche un prodotto relativo ad un cartografia preliminare dei sottosistemi di terre, che non è però disponibile e non risulta comunque collega ad una banca dati pedologica.

Si tratta di dati molto utili da utilizzarsi per valutazioni di semidettaglio e per la costruzione di un approfondimento del modello di valutazione, sempre ricordando che solo una parte del territorio è coperta. La copertura di informazioni disponibili relative ai suoli risultano essere per la provincia di Isernia troppo scarse per poter essere utilizzate.

Uso del suolo e vegetazione

Sono disponibili due fonti dati vettoriali, una relative ai tipi forestali che interessa il territorio forestale della regione e la banca dati *CORINE land cover* aggiornata all'anno 2006 elaborata per l'Italia da ISPRA.
La Cartografia dei tipi forestali (fonte UNIMOL) e contiene 31066 poligoni, ha un dettaglio nominale in scala 1:10.000 e ad ogni poligono sono associati attributi relativi alla fisionomia, struttura e copertura.
DISTAT UNIMOL ha attualmente in elaborazione una cartografia a copertura completa che integra la carta dei tipo forestali per quanto riguarda le superfici artificiali, le aree agricole e le acque.

Per la provincia di Isernia è stato possibile utilizzare tale strato, il cui dettaglio permette di discriminare in maniera molto più efficace il territorio rispetto all'utiilizzo della banca data Corine Land Cover 1:100.000

Legenda della banca dati di uso del suolo e tipologie forestali

Uso del suolo e vegetazione	ETTARI	%
Aree edificate urbane continue (centri storici)	87	0,06%
Aree edificate urbane discontinue	1966	1,28%
Unità industriali, commerciali, rurali e agricole	729	0,48%
Discariche	6	0,00%
Siti in costruzione	62	0,04%
Rete stradale e aree associate	88	0,06%
Cave	135	0,09%
Parchi e giardini	1	0,00%
Cimiteri	48	0,03%
Impianti sportivi e per il tempo libero	70	0,05%
SUPERFICI ARTIFICIALI	**3191**	**2,08%**

Uso del suolo e vegetazione	ETTARI	%
Praterie e pascoli	14118	9,21%
Prati	6879	4,49%
Aree agricole	34938	22,79%
Seminativi	719	0,47%
Vigneti	13	0,01%
Oliveti	808	0,53%
Pioppicoltura e arboricoltura e altre colture permanenti	30	0,02%
Colture annuali associate a colture permanenti	73	0,05%
Sistemi colturali e particellari complessi	1	0,00%
Superfici principalmente occupate da agricoltura, con aree significative di vegetazione naturale	352	0,23%
AREE AGRICOLE (COMPRESI PASCOLI)	**57931**	**37,79%**
Macchia mediterranea a Fillirea	2	0,00%
Arbusteto a Rose, Prugnolo e Rovo	3379	2,20%
Arbusteto a Ginepro comune e Agazzino	183	0,12%
Arbusteto altomontano a Ginepro Nano	551	0,36%
Arbusteto a Ginestre	2325	1,52%
ARBUSTETI	**6441**	**4,20%**
Rocce nude	172	0,11%
Aree poco vegetate	750	0,49%
Sabbie (alvei fluviali)	16	0,01%
AREE NUDE O POCO VEGETATE	**938**	**0,61%**
Lecceta primitiva	37	0,02%
Lecceta termofila	181	0,12%
Lecceta mesoxerofila	1267	0,83%
Querceto a Roverella secondario	806	0,53%
Querceto a Roverella termofilo	3057	1,99%
Querceto a Roverella mesoxerofilo	13732	8,96%
Cerreta mesoxerofila	15642	10,20%
Cerreta mesofila	18931	12,35%
Orno Ostrieto primitivo	623	0,41%
Orno Ostrieto secondario	1422	0,93%
Ostrieto mesoxerofilo	2740	1,79%
Ostrieto mesofilo	1172	0,76%
Castagneto	27	0,02%
Faggeta submontana	2125	1,39%
Faggeta montana	6828	4,45%
Faggeta altomontana	1081	0,71%
Pioppo Saliceto ripariale	3645	2,38%
Robinieto ailanteto	99	0,06%
Latifoglie di invasione miste e varie	5618	3,66%
Pioppeto di Pioppo tremulo	46	0,03%
Boscaglia pioniera calanchiva	57	0,04%
Abetina pura autoctona	343	0,22%
Rimboschimento basale di conifere	307	0,20%
Rimboschimento submontano di conifere	1379	0,90%
Rimboschimento montano di conifere	551	0,36%
Querceto a Roverella termofilo Var Carpinella	354	0,23%
Querceto a Roverella mesoxerofilo Var Carpinella	271	0,18%

Uso del suolo e vegetazione	ETTARI	%
Cerreta mesofila Var Farnetto	358	0,23%
Cerreta mesofila Var Abete bianco	662	0,43%
Ostrieto mesoxerofilo Var Carpinella	1156	0,75%
Faggeta submontana Var Abete bianco	89	0,06%
AREE BOSCATE	**84606**	**55,19%**
Corsi d'acqua	96	0,06%
Corpi d'acqua	92	0,06%
CORPI E CORSI D'ACQUA	**188**	**0,12%**

Per le aree forestali sono indicate anche le informazioni relative alla struttura ed al grado di copertura.

Struttura	Codice
Fustaia P.D.	1
Boschi a struttura composita	2
Ceduo P.D.	3
Boschi infraperti	4

Copertura	Codice
> 50	0
10 - 50	1
<10	2

Analisi bioclimatica

Per quanto riguarda il clima, l'unico dato spazializzato utile per la valutazione all'idoneità è quello reso disponibile da GIS Natura (Il Fitoclima d'Italia. Ministero dell'Ambiente e della Tutela del Territorio. Consiglio Nazionale delle Ricerche, Istituto di Ecologia e Idrologia Forestale). Questo suddivide il territorio Italiano in classi fitoclimatiche italiane derivate dall'integrazione di parametri e indici climatici con le caratteristiche geobotaniche del territorio.

Uno studio condotto da Ciaschetti et alii (*Ciaschetti G., Marchetti F., Di Lena B., De Laurentiis G., Cimini G, Spinelli D. Caratterizzazione climatica delle aree a vocazione tartuficola della regione Abruzzo*), ha evidenziato che seppure in un'ampia valenza ecologica per *Tuber melanosporum* e *Tuber magnatum*. *Tuber melanosporum* mostra una chiara preferenza per l'orizzonte **Supratemperato inferiore** (Rivas- Martinez et al., 2002), mentre *Tuber magnatum* è diffuso prevalentemente nel **Mesotemperato inferiore** (Rivas- Martinez et al., l.c.).

DEM (Digitale Elevation Model)

Dal modello digitale del terreno, sono stati ricavate diverse elaborazioni. Tra queste è stato creata uno strato poligonale con intervalli di quota di 100 metri. È stata svolta una prova applicativa legando l'idoneità allo sviluppo potenziale dei tartufi a fasce di quota, scelte in base ad indicazioni bibliografiche ed in relazione alle esperienze analoghe effettuate recentemente in Abruzzo.
Analogamente possono essere fatte elaborazione sull'acclività.

Altri dati

Strati topografici IGMI

Sono stati acquisiti, georiferiti e mosaicati le tavolette IGMI 1:25.000 ed i fogli in scala 1:100.000, utili in fase di verifica dei risultati.

Idrografia

Il reticolo idrografico ha una forte correlazione con la distribuzione delle tartufaie di *Tuber magnatum*, ed quindi uno strato informativo da ritenersi utile in sede di verifica dei risultati ottenuti e per le fasi di interpretazione a video..

Ortofoto digitali

Le ortofoto digitali a colori (Territaly 2008), seppure con un dettaglio evidentemente molto spinto per le esigenze del progetto, sono uno strumento utile per verifiche puntuali e per la validazione delle chiavi di interpretazione. Ad esempio alcuni siti di tartufaie spontanee sono posizionati in aree che non risultano avere caratteristiche idonee, ma la verifica puntuale sulle ortofoto ha consentito di appurare che si tratta prevalentemente di approssimazioni di ubicazione oppure di dettaglio delle banche dati di riferimento.

Informazioni sulla distribuzione delle tartufaie spontanee (fonte dati Unimol)

UNMOL ha censito circa 50 siti di tartufaie spontanee, che sono un utile strumento per verificare le ipotesi di relazione fra caratteristiche territoriali e potenzialità per lo sviluppo delle tartufaie.

LE GRIGLIE DI VALUTAZIONE

Dopo l'analisi dei dati disponibili sono state elaborate,per ciascuna specie, le tabelle di idoneità relativamente ai seguenti layer informativi:

- _ido_geol (attribuzione di idoneità alle 106 Unità Cartografiche, presenti nella cartografia geologica)
- _ido_quote il territorio regionale è stato suddiviso in classi di quota
- _ido_T_UDS_IS11 (elaborazione originale DISTA UNIMOL)

Gli strati così classificati sono stati intersecati tra di loro ed applicata una griglia di valutazione complessiva che ha tenuto conto conto delle diverse caratteristiche territoriali.
Le modalità di elaborazione della griglia risultante, sono complesse, in alcuni casi, infatti, le informazioni di uno strato informativo mettono in luce caratteristiche territoriali non discriminate, per motivi di dettaglio o di legenda, da altri strati informativi. In particolare le formazioni vegetazionali proprie delle aree prossime agli impluvi in molti casi sono relazionate a ambienti pedo-geomorfici che la cartografia geologica non coglie e quindi quando queste formazioni sono presenti la valutazione sull'idoneità geologica viene integrata da questa informazione.

Negli altri casi vengono attribuiti dei pesi alle singole caratteristiche e fatta la somma della risultante *classe* **X** *peso* i valori saranno poi classati in 3 classi di attitudine. Se uno dei singoli parametri selezionati però indica potenzialità nulla il poligono viene comunque classificato come non idoneo.
Con questa metodologia sono individuate le aree potenzialmente idonee all'attualità, ossia quelle aree che hanno ad oggi caratteristiche idonee allo sviluppo di tartufaie spontanee.

Per il *Tuber magnatum* si è tenuto conto che esistono aree prevalentemente agricole con inclusioni non cartografabili alla scala di progetto di lembi di vegetazione quali strette fasce di vegetazione naturale legate alle incisioni minori del reticolo di drenaggio, filari ed alberate. Si tratta di superfici significative rispetto allo sviluppo di tartufaie spontanee ma non cartografabili alla scala della carta dei tipi forestali , che ricordiamo ha un dettaglio proprio di una scala 1:10.000.È stata quindi creata un'unità cartografica, per le aree agricole della provincia di Isernia ove ricorrano caratteri potenzialmente predisponenti per quanto riguarda natura dei substrati e quote.

In queste aree insiste inoltre una potenzialità che si può ulteriormente sviluppare nel caso vi siano dinamismi dell'uso del suolo, con sostituzione di aree attualmente agricole con aree occupate da vegetazione naturale.

Le elaborazioni GIS che sono state effettuate sono state: intersezione degli strati informativi, attribuzione dei valori finali, dissolve dei poligoni confinanti omogenei al fine della valutazione, eliminazione dei poligoni di dimensioni non compatibili con l'accuratezza geometrica degli stati di base, verifica delle topologia degli strati poligonali finali.

La fase di procedimento automatico è stata integrata con una revisione puntale tenendo conto di:

- ✓ orto foto digitali;
- ✓ reticolo idrografico e relative elaborazioni quali strato *buffer* per il *Tuber magnatum*)
- ✓ cartografia topografica IGMI.

Nel Geodatabase sono state elaborate delle tabelle di attitudine che classificano il territorio in 4 livelli qualitativi di attitudine per ciascuna delle specie:

A. 0 potenzialità nulla
B. 1 potenzialità scarsa
C. 2 potenzialità media
D. 3 potenzialità elevata

E. 4:aree potenzialmente idonee a medio termine (nel caso vi siano dinamismi relativi al *Land Cover*.

F. Altre aree:

F.i. Superfici artificiali

F.ii. Corpi e corsi d'acqua

F.iii. Spiagge dune e sabbie (ad esclusione delle aree sabbiose litoranee che sono idonee allo sviluppo del *Tuber borchii.*

F.iv. Rocce nude, falesie, rupi ed affioramenti

F.v. Aree umide

Gli strati di base, le tabelle ed i file vettoriali di elaborazione finale sono state archiviati in un geodatabase (**T_IS_11.mdb**).

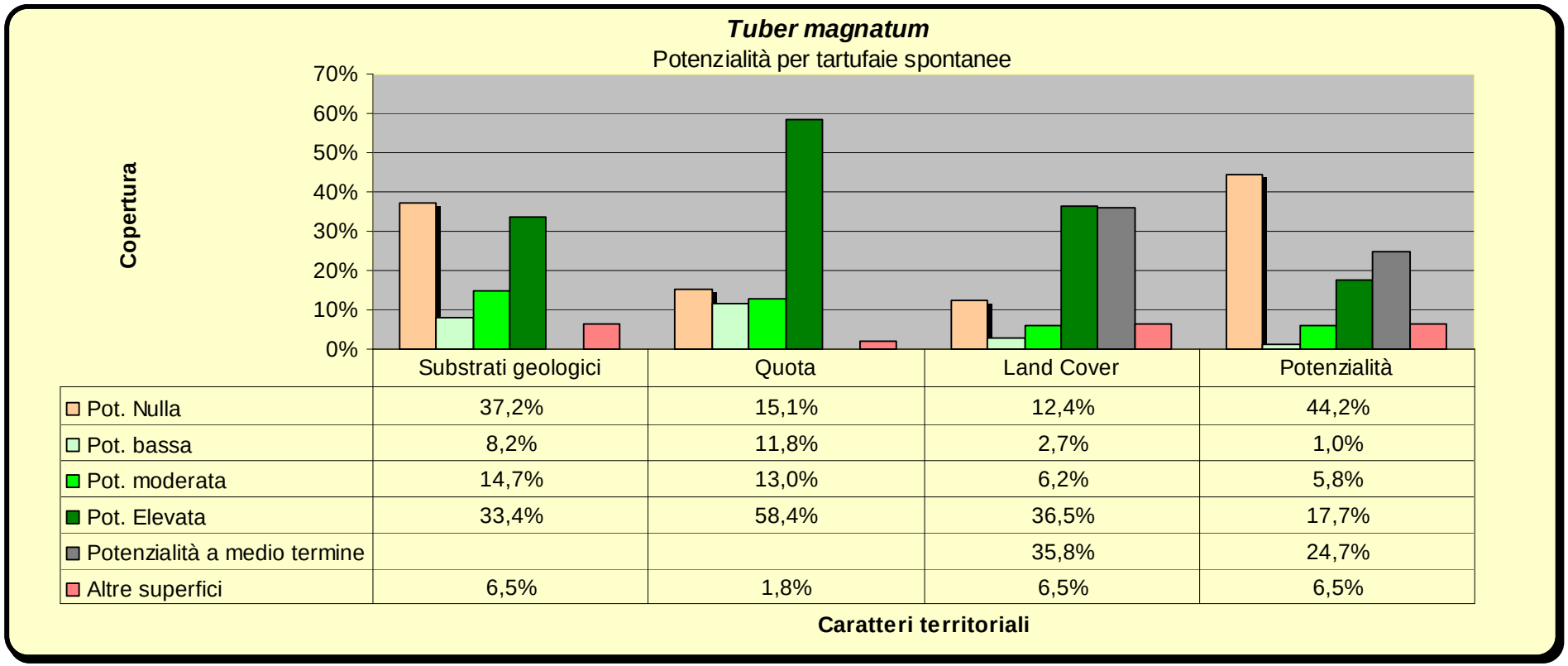
I RISULTATI PER LA PROVINCIA DI ISERNIA

Nelle pagine seguenti sono riportasti sotto forma di tabelle, grafici e riduzioni cartografiche i risultati raggiunti per la provincia di Isernia relativamente alle quattro specie in oggetto.

Le legende inserite nella cartografie sono le seguenti

Specie	Legenda
Tuber magnatum	1)Aree a potenzialità bassa 2) Aree a potenzialità media 3) Aree a potenzialità elevata 4) Aree con potenzialità a medio termine e/o con inclusioni non cartografabili di aree potenzialmente idonee - Corpi e corsi d'acqua - Superfici artificiali (urbano, industriale, infrastrutture ecc.)
Tuber aestivum	1)Aree a potenzialità bassa 2) Aree a potenzialità media 3) Aree a potenzialità elevata - Corpi e corsi d'acqua - Superfici artificiali (urbano, industriale, infrastrutture ecc.)
Tuber Melanosporum	1)Aree a potenzialità bassa 2) Aree a potenzialità media 3) Aree a potenzialità elevata - Corpi e corsi d'acqua - Superfici artificiali (urbano, industriale, infrastrutture ecc.)
Tuber borchii	1)Aree a potenzialità bassa 2) Aree a potenzialità media 3) Aree a potenzialità elevata - Corpi e corsi d'acqua - Superfici artificiali (urbano, industriale, infrastrutture ecc.)

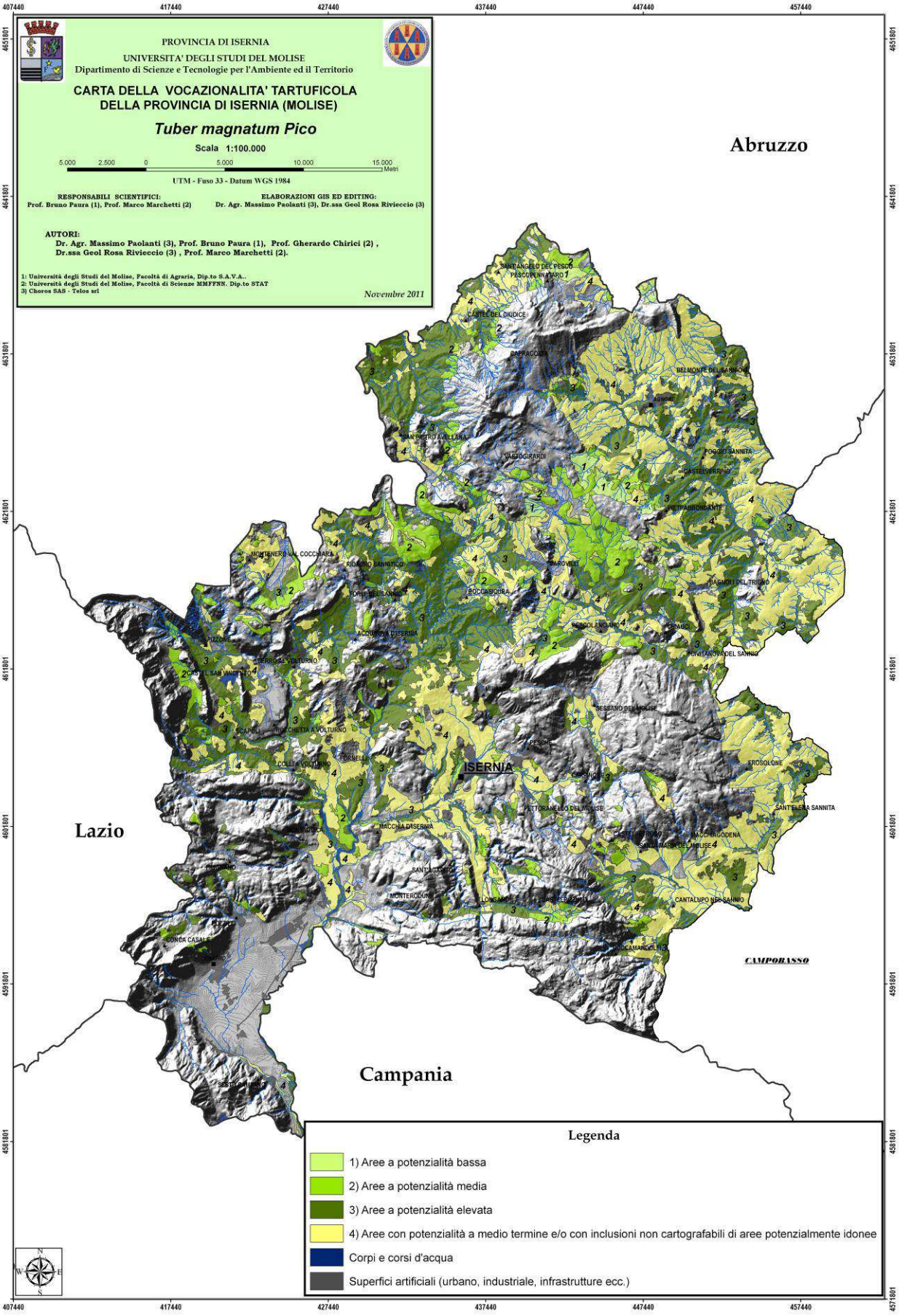
Potenzialità per il Tuber magnatum



	Substrati geologici	Quota	Land Cover	Potenzialità
Pot. Nulla	37,2%	15,1%	12,4%	44,2%
Pot. bassa	8,2%	11,8%	2,7%	1,0%
Pot. moderata	14,7%	13,0%	6,2%	5,8%
Pot. Elevata	33,4%	58,4%	36,5%	17,7%
Potenzialità a medio termine			35,8%	24,7%
Altre superfici	6,5%	1,8%	6,5%	6,5%

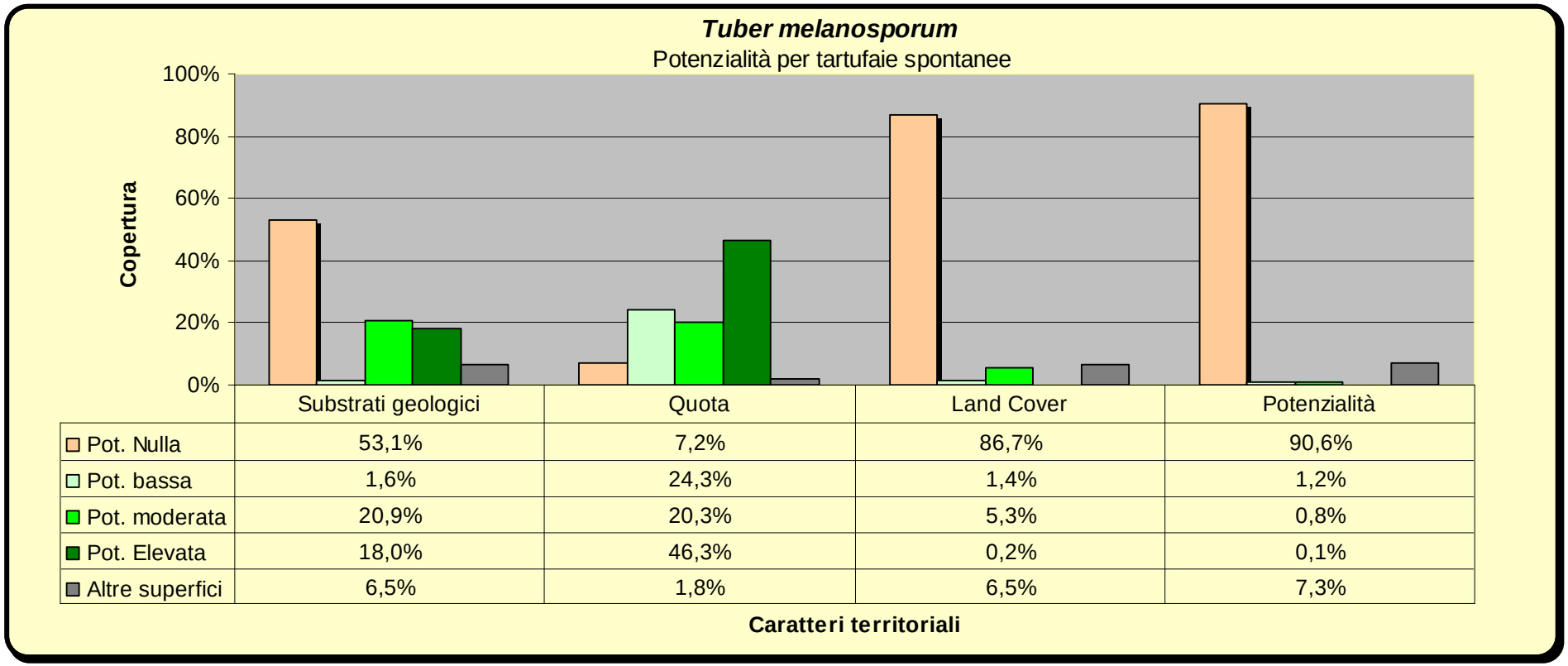
La classe Potenzialità a medio termine corrisponde ad aree prevalentemente agricole con inclusioni, non cartografabili alla scala di progetto, di lembi di vegetazione quali strette fasce di vegetazione naturale legate alle incisioni minori del reticolo di drenaggio, filari ed alberate. È un'unità cartografica per le aree agricole della provincia di Isernia ove ricorrano caratteri potenzialmente predisponenti per quanto riguarda natura dei substrati e quote.

Altre superfici corrispondono a superfici non idonee allo sviluppo di tartufaie spontanee (aree urbane, corpi e corsi d'acqua, superfici poste oltre i 1.300 m slm, aree prive di suolo ecc.)



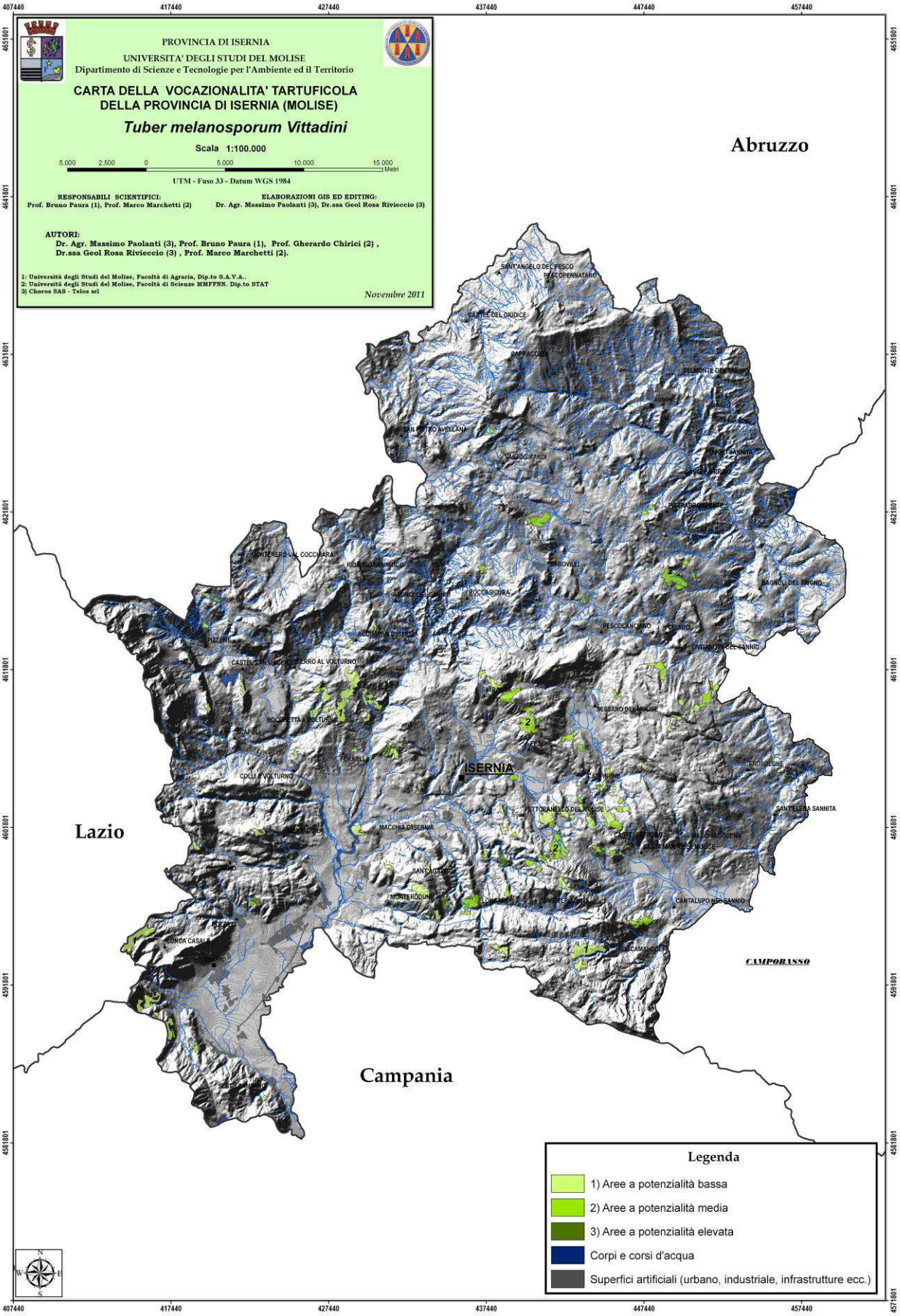
Paolanti M, Paura B, Chirici G, Rivieccio R, e Marchetti M (2011)

Potenzialità per il Tuber melanosporum



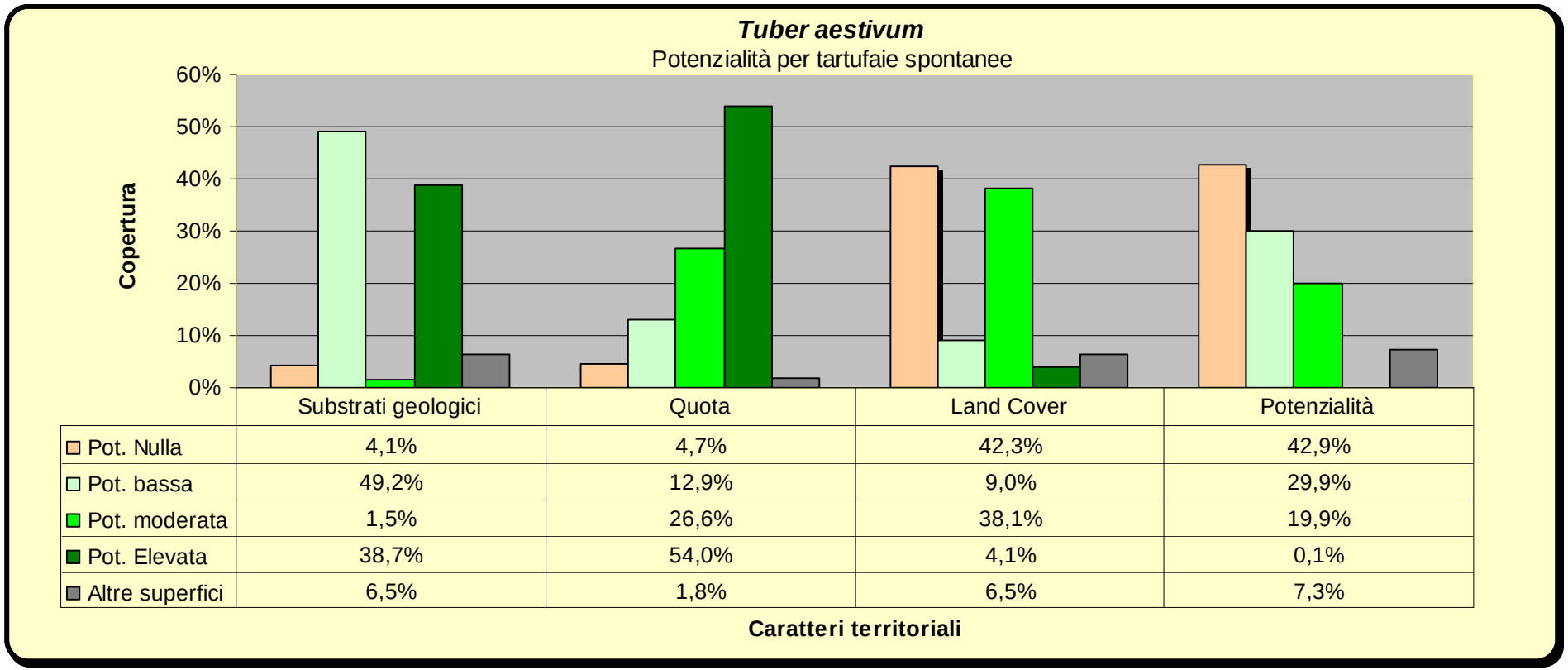
	Substrati geologici	Quota	Land Cover	Potenzialità
Pot. Nulla	53,1%	7,2%	86,7%	90,6%
Pot. bassa	1,6%	24,3%	1,4%	1,2%
Pot. moderata	20,9%	20,3%	5,3%	0,8%
Pot. Elevata	18,0%	46,3%	0,2%	0,1%
Altre superfici	6,5%	1,8%	6,5%	7,3%

Altre superfici corrispondono a superfici non idonee allo sviluppo di tartufaie spontanee (aree urbane, corpi e corsi d'acqua, superfici poste oltre i 1.300 m slm, aree prive di suolo ecc.)



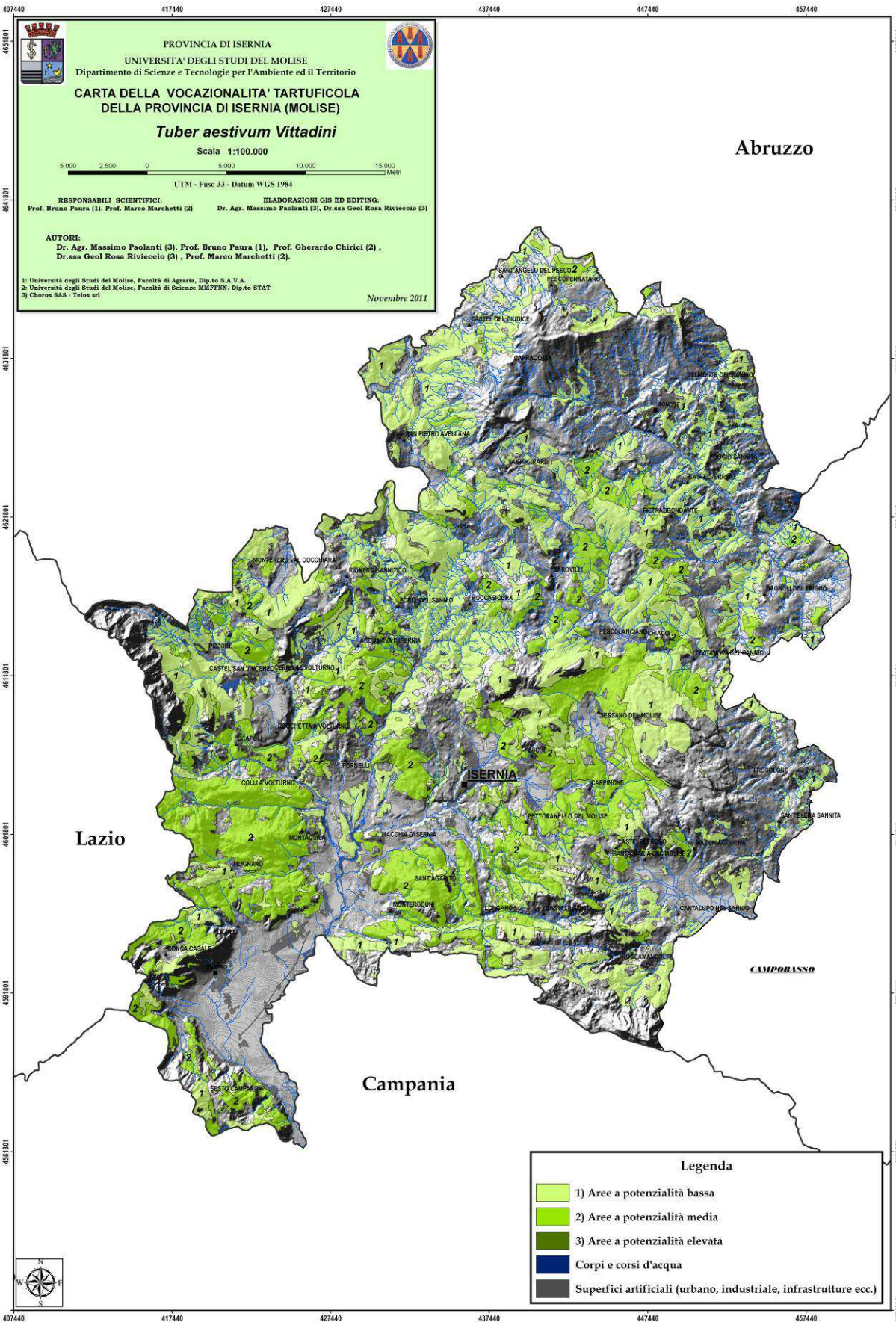
Paolanti M, Paura B, Chirici G, Rivieccio R, e Marchetti M (2011)

Potenzialità per il Tuber aestivum



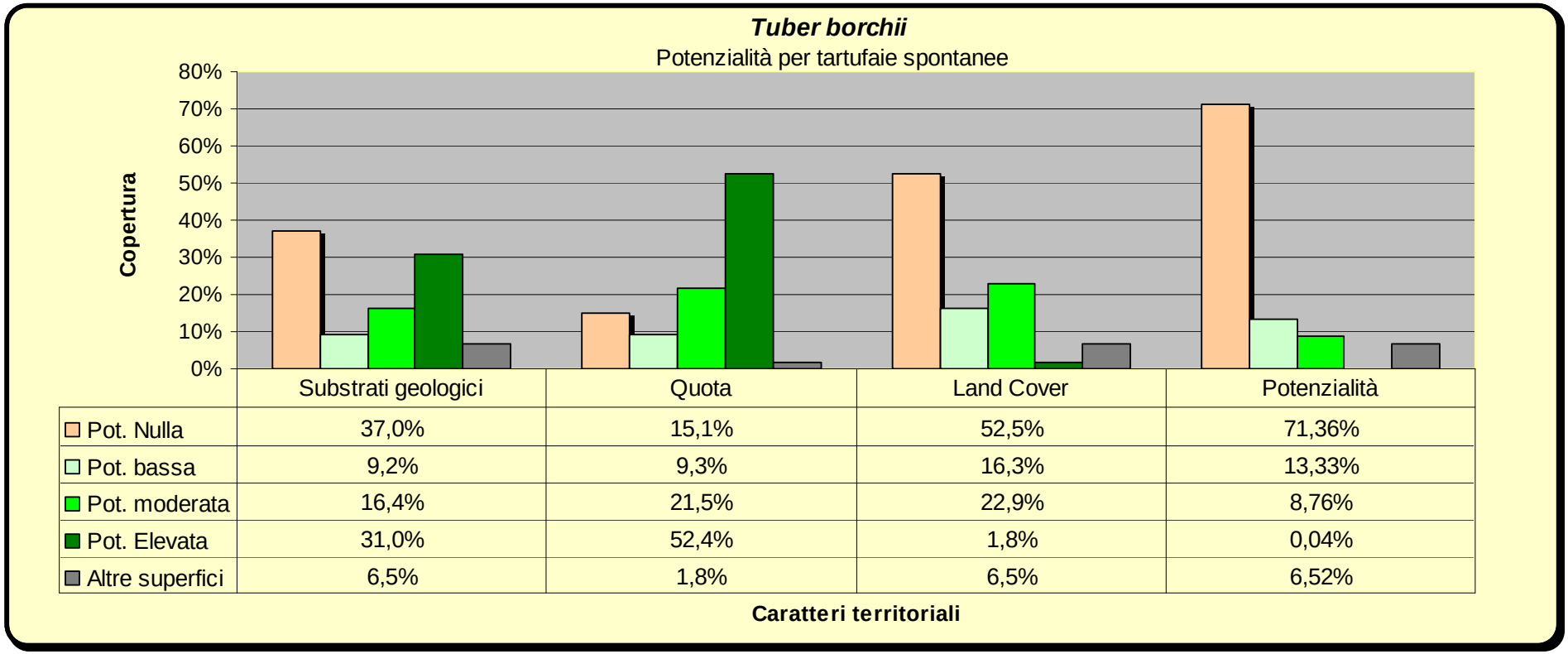
	Substrati geologici	Quota	Land Cover	Potenzialità
Pot. Nulla	4,1%	4,7%	42,3%	42,9%
Pot. bassa	49,2%	12,9%	9,0%	29,9%
Pot. moderata	1,5%	26,6%	38,1%	19,9%
Pot. Elevata	38,7%	54,0%	4,1%	0,1%
Altre superfici	6,5%	1,8%	6,5%	7,3%

Altre superfici corrispondono a superfici non idonee allo sviluppo di tartufaie spontanee (aree urbane, corpi e corsi d'acqua, superfici poste oltre i 1.300 m slm, aree prive di suolo ecc.)



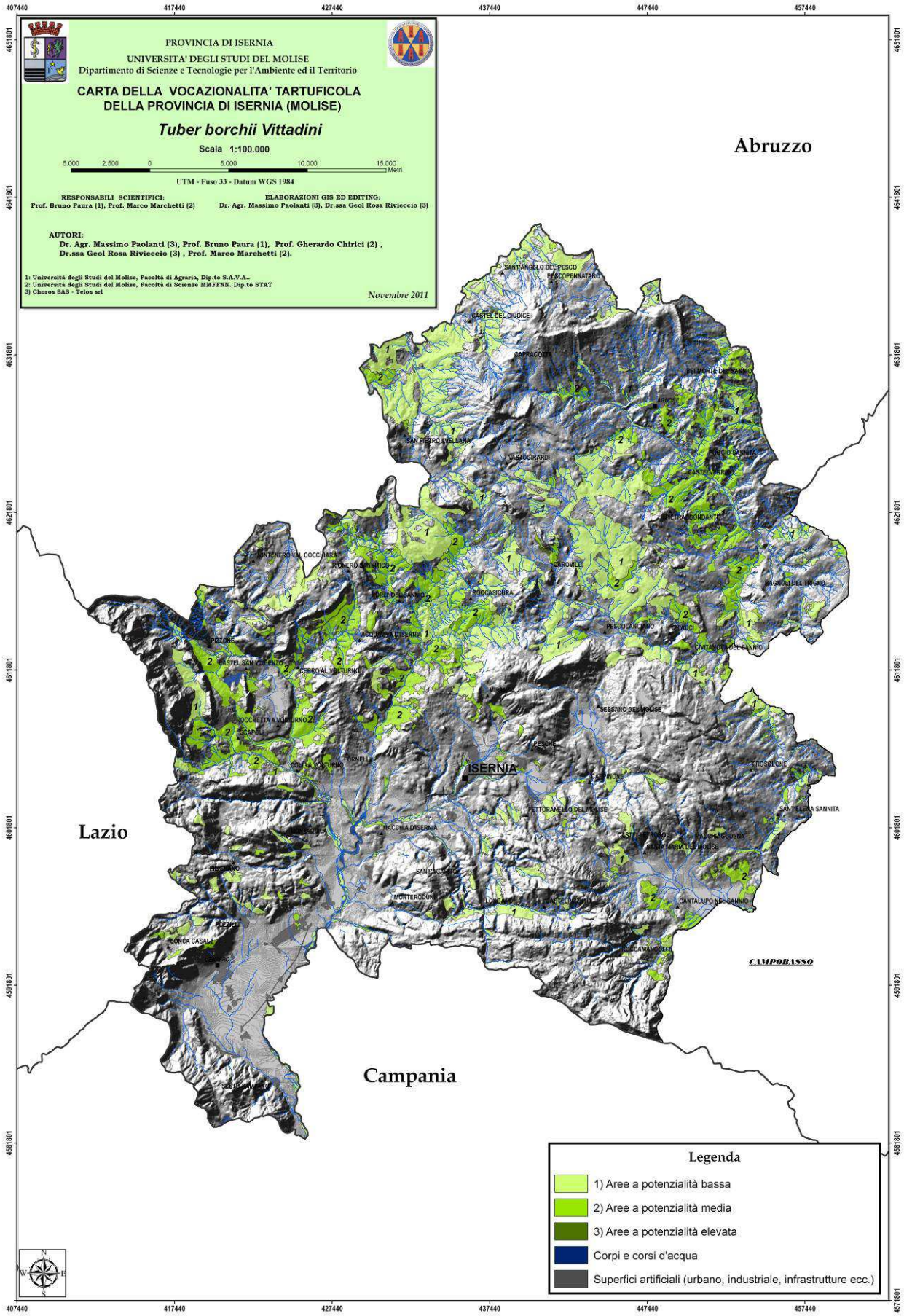
Paolanti M, Paura B, Chirici G, Rivieccio R, e Marchetti M (2011)

Potenzialità per il Tuber borchii



	Substrati geologici	Quota	Land Cover	Potenzialità
Pot. Nulla	37,0%	15,1%	52,5%	71,36%
Pot. bassa	9,2%	9,3%	16,3%	13,33%
Pot. moderata	16,4%	21,5%	22,9%	8,76%
Pot. Elevata	31,0%	52,4%	1,8%	0,04%
Altre superfici	6,5%	1,8%	6,5%	6,52%

Altre superfici corrispondono a superfici non idonee allo sviluppo di tartufaie spontanee (aree urbane, corpi e corsi d'acqua, superfici poste oltre i 1.300 m slm, aree prive di suolo ecc.)



Paolanti M, Paura B, Chirici G, Rivieccio R, e Marchetti M (2011)

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