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PHENOTYPIC CHARACTERIZATION AND ETHNOBOTANICAL STUDY OF RHIZOCARPON IN THE "MEFITE D'ANSANTO"

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ABSTRACT:

An investigation on the lichen flora was conducted in the Ansanto valley, southern Italy, in an area called Mefite, characterized by natural emission of low temperature CO_2. The collected samples were phenotypically analyzed, showing the ubiquitous distribution of the genus *Rhizocarpon Ramond* ex DC. Moreover, was described the influence of this lichen on the local population and was hypothesized a further ethnobotany application.

KEY WORD: *Rhizocarpon, Lichens, Geothermal area, "Pecorino di Carmasciano" cheese, Pollution biomonitor.*

INTRODUCTION:

Mefite d'Ansanto, southern Apennines, Italy (Latitude 40.975; Longitude 15.146) is the area with the largest natural emission of low temperature CO_2 rich gases, from non-volcanic environment, ever measured in the Earth (Chiodini *et al*, 2010). It is positioned in the center of Irpinia, at a distance of 100 Km from the Vesuvius (Naples) and Solfatara (Pozzuoli). Studies of the Italian National Research Council have found similar isotopic helium ratios ($^{3}H\backslash^{4}H$) to those of Solfatara. It is then proposed that the same source, the mantle, is feeding both systems. This is very important because the two discharges are different for dynamics, age, history and geological environment (CNR Report, 2002). The Ansanto valley is extended around the edge of the Ofanto river that ends forming a little lake with its water in contact with the fumes of sulfuric acid and carbon dioxide coming from the subsoil.

The area was colonized by the Oscans around the V – VI century BC and the natural phenomena were ascribed to the goddess Mephitis, that is the personification of the poisonous gases emitted from the ground in swamps and of the volcanic vapors (Irpinia.info). Mefite is mentioned by Virgil in the seventh chapter of the "Aeneid" that wrote: *"Est locus Italiae medio sub montibus altis, nobilis et fama multis memoratus in oris, Ampsancti valles: densis hunc frondibus atrum urguet utrimque latus nemoris medioque fragosus dat sonitus saxis et torto vertice torrens… Hic specus horrendum et saevi spiracula Ditis Monstrantur, ruptoque ingens Acheronte vorago Pestiferas aperit fauces"* (= There is a place in the center of Italy, close to high mountains, that is noble and is renowned in many districts: The "Ansanto valley". It is surrounded by a dark forest with dense foliage and in the middle there is a torrent that emits a loud sound between the stones with a swirling vortex… Here are shown a horrible cave and the glimmers of Dis, and the Acheron opens its pestiferous mouth, where a wide chasm begins). Historically, this valley was the epicentral area of the infamous 1980 Irpinia earthquake (M= 6.6). In Mefite, the emission is fed by a buried reservoir, made up of permeable limestones and covered by clayey sediments. Is estimated a total gas flux of ~2000 tons per day (Chiodini *et al*, 2010) . The gas flows along a narrow natural channel producing active fumaroles (these are vents that emit gases) which have killed over a period of time people and animals. On their surface, there are sulfur encrustations due to the sublimation of the H_2SO_4.

In general, similar conditions can be found in other geothermal sites of Italy. In fact, in literature is explained that low pH environments are generated by the sulfur emissions of the fumaroles. This is provoked by the fact that the in situ oxidation of H_2S arising from the outlets of these geothermal systems causes the production of large amounts of H_2SO_4 and, in turn, low pH on rocks and soil surrounding the offspring. Generally, the pH values remain below 2.5 for 10–25 square meters around the outflow of H_2S. Temperature, on the other hand, declines from 60°C to 25–30°C within a few meters apart from the vents (Volker AR Huss *et al*, 2002). Moreover, several researches affirm that the distribution of flora depends on pH and on the temperature. For example, is affirmed that in the geothermal sites of south Italy there are, in the close vicinity of hot springs and fumaroles where soil temperature and pH are almost constant at 40°C and 1.5, respectively, the exclusive presence of three thermoacidophilic red microalgae, *Cyanidium caldarium*, *Galdieria sulphuraria* and *Cyanidioschyzon merolae* (Merola A. *et al*, 1981; Albertano P. *et al*, 2000).On the other hand, at increasing distances pH and temperature change at temperatures below 30°C and pH values between 1.8 and 2.5. In these areas the major components of algal populations are the coccoid green algae such as *Chlorella* and the related genera (Pinto G, 1993). The interest of this project is related with the sampling of lichen species living at a distance of over 30 meters from the central lake. In this area the measured pH is between 6 and 6.5 and the soil temperature has an average of 30°C. This decision is due to the absence of macroscopically visible signs of vegetation at a shorter distance, and taking into account the hazards of the sulfur fumes from the vents.

METHODS:

The sampling was carried using the technique of the “Quadrat”. Six rectangular “quadrat plots” (or units) of the size of 5m x 15m were took in an area of 450 square meters (30m x 15m) down the side of the slope surrounding the lake. The plots covered all types of rocks occurring in that space (for example sulfur encrusted rocks, clayey sediments, tufa rocks). Was then applied the “Stratified sampling” (Stratified sampling). According with this method, were identified the interesting areas within the main body of the units and these strata were sampled separately. In literature, there is a standard formula for calculating the number of samples to be placed in each unit:

$$\text{The number of quadrats sampled in the unit} = \frac{\text{the area of the unit x the total number of quadrats to be sampled}}{\text{total area of the habitat}}$$

Table 1. Formula “number of quadrats sampled in the unit”

In this study:

Number of quadrats sampled in the unit= (75 m^2 x 6)/ 450 m^2 = 1

According to this consideration, a quadrat of 1m^2 was examined for each unit.

To get a comparison of the results, the coverage of the quadrats was measured using the method of “Braun-Blanquet” (Douglas A. *et al*, 1978). With this graduated scale, the coverage ratio of the quadrat analyzed in plot 1 was assessed as “2” (percentage of covering of the area of the "quadrat" between 5% and 25%). All the others quadrats had a “Braun-Blanquet rating” indicated as “+” (percentage of covering of the area less than 1%).

A lichen of the genus Rhizocarpon Ramond ex DC was found ubiquitously distributed in the units analyzed.

Rhizocarpon is a lichenized genus of fungi within the Rhizocarpaceae family. The recognizable characteristics are (Brodo I.M. *et al*, 2001; Annstrong R.A. and Bradwell T., 2001; Benedict J. B.,2009) the presence of a crustose thallus strongly fixed on the substratum that can be distinctly areolate or squamulose. The upper surface is whitish to greenish or deep yellow with an array of yellow or yellowish green areolae and black apothecia surrounded by a thin fungal zone of black prothallus. The areola is composed by an upper cortex that gives protection from abrasion and moisture loss, an algal layer (the photobionts usually are protoccoccoid) and a lower medullary layer, which stores food and moisture. The medulla is composed of loosely packed hyphae that form a mesh-like network around the algae. The apothecia are disc-shaped and are found near the areolae on the surface of mature lichens. Each apothecium has a layer of sac-like asci with up to eight oval shaped ascospores (two to four celled and transversely septate). These spores are surrounded by a gelatinous halo (peri spore), which helps to anchor the ejected spores to a substrate. The spores can be used in the classifying of a thallus to species level (In fact, the identification keys are based on their characteristics for example, shape, size, septation, number per ascus and color). These lichens grow predominantly on non calcareous rocks, rarely on other substrates such as hard wood. A few species are present on slightly calcareous substrates (Poelt J. *et al*, 1988).

Several publications cited Rhizocarpon as a first colonizer of areas with active vents emitting CO2. For example, The "Bullettino" of the Italian botanical society mentioned this lichen in the Solfatara of Pozzuoli (Full text of "Bullettino" - Internet Archive). Akira Shimizu found this genus in the Volcanic Highlands of Mt. Tokachi in Japan (Shimizu Akira, 2004). Five species of Rhizocarpon growing in a solfataric field are reported for Japan in the taxonomic notes of the "The Bulletin of National Science Museum" of Tokyo (Inoue M., 2001). Despite of this, relatively little is known about the ecology, physiology and growth of lichens in the genus Rhizocarpon. This is in part due to the fact that these lichens are very slow growing, cannot be removed from their substrate without being damaged and cannot readily be identified to species level in the field without chemical testing and examination of spores. Another problem is the fact that the taxonomic keys that are available for Rhizocarpons (Poelt J.,1988; Thomson, J. W.,1967) are specific to a geographic region and most have been developed for use in Europe and the Arctic. Moreover, these keys do not adequately describe the phenotypic differences that exist in other localities and cannot be applied to identify tiny thalli to species level. On the other hand, these keys are the only "gold standard" for the classification. According with them, the genus Rhizocarpon is divided into two large sub-genera, Phaeothallus and Rhizocarpon based on the presence or absence of rhizocarpic acid, also if this classification has been questioned by genetic studies (Ihlen D. and Ekman S.,2002). The subgenus Rhizocarpon can be further subdivided into four sections, Alpicola, Rhizocarpon, Superficiale and Viridiatrum. These sections can be differentiated on the base of the number of cells in a spore. In fact, Superficiale and Alpicola have uniseptate spores (two cells), differently from Rhizocarpon and Viridiatrum that contain pluriseptate spores (several cells). To get a further subdivision the keys are not sufficient but there is the need of a genotypic analysis.

The phenotypic characterization of Rhizocarpon was made according with macroscopic and microscopic observations. Macroscopically, the samples collected present a areolate crustose thallus with a greenish upper surface. The prothallus is thin, almost indistinguishable from the thallus. The apothecia are not visible. The average diameter of an areola is 0.5 mm.

The microscopic observations were made using a microscope "BRESSER Biolux AL" with Barlow eyepiece lens 16X and magnification on the objective lenses 10X. The soil containing the lichen was homogenized on a glass slide compatible with the microscope.

The microscopic characteristics attributable to Rhizocarpon Ramond ex DC are arduous to recognize through fig.7 due to the complex structure. Despite of this, a noticeable aspect is the fact that the outer rock surface appear often intruded by hyphae, which cause rupture of primary minerals, with detachment and progressive incorporation of their fragments into the thallus (as appreciable in fig.8). Dissolution features affect these minerals, that show very peculiar patterns which are suggestive of a biologically-induced control (Lee M.R. and Parsons I., 1999).

To overcome these problems, further samples of the lichen were treated with a solution of benzalkonium chloride and sodium hypochlorite 1:1 (Conte R., 2012) The treatment has flaked off the structure, making possible the observation of the layers.

The different portions were analyzed separately to put in evidence all the distinguishing features.

In Fig.10 are highlighted the non-lichenized hyphae of the hypothallus or prothallus.

Fig. 11 is a picture of the algal\lower medullary layer. A feature of Rhizocarpon is the medulla composed by loosely packed hyphae forming a mesh-like network around the algae.

Fig. 12 shows the disc-shaped apothecia; Through the bigger magnification of the image on the right (objective lenses 40X) is possible to highlight the asci inside each apothecium.

Fig. 13 is useful to put in evidence the shape and the septation of the spores. In fact, is noticeable that the ascospores are oval and uniseptate.

RESULTS AND DISCUSSION:

Overall, the phenotypic characterization permitted to follow the taxonomic keys available to distinguish the sections of Rhizocarpon. Considering the sub-genus Phaeothallus and Rhizocarpon as the same due to the studies on the genetic code, the number of cells in a spore indicates that the section of belonging of this Rhizocarpon is that of Superficiale\Alpicola. For another, there is a strong relation of this lichen and of the vegetation of the Mefite area in general, with local people. Actually, the area of the Ansanto valley, particularly a district called Carmasciano (Latitude 40.983; Longitude 15.169) is characterized by the production of a sheep cheese called “Pecorino di Carmasciano”.

As noticeable in Fig.14 , the farms (all of about 20 animals) where this cheese is produced are close to the Mefite and the sheep (mainly of the breed “Laticauda”) graze in the vicinity of the fumaroles (the red circle indicates the grazing area). A study funded by the Province of Avellino in collaboration with the Experimental Zooprophylactic Institute in Portici affirms that sulfur compounds influence the type and the attribute of the vegetation in the area (that is mainly composed by grasses such as *Phleum pratense*, *Dactylis glomerata*, *Festuca arundinacea*, *Avena bar-bata* and legumes of the type *Onobrychis viciaefolia*, *Vicia sativa* and several species of *trifolium* that grow near lichens) and this is important on the quality of milk from these pastures. In fact, the grazed grass contains a high amount of unsaturated fatty acids, which are found largely in the milk and whose oxidation leads to the formation of substances which contribute to enrich the flavor of the cheese. Moreover, this milk is particularly rich in the pro-vitamin “beta-carotene” that gives a yellowish color to the cheese, affects its aroma and has an antioxidant action. Another effect is ascribable in the influence of the sulfur to the natural and heterogeneous microflora involved in the ripening and responsible for metabolic activities that contribute to define the characteristics of the final dairy product (Coppola R. *et al*, 2006). The Ansanto valley is linked not only to the production of the "Pecorino di Carmasciano" but also to the life of shepherds and flocks. In fact, is diffused the use of the therapeutic properties of sulfur water to treat scabies in sheep. This resource was also used, in ancient time, to treat both skin diseases and rheumatism. Furthermore, people used to take the "Macra" around the Mefite lake. This was a special clay used to mark the cattle. Currently, water from Ansanto Valley is still used in therapy in a spa called "Terme di San Teodoro di Villamaina" that is fed by two springs of sulfur-carbon water coming from the Mefite. A further ethnobotany consideration can be the use of Rhizocarpon Ramond ex DC in the measuring of polluting, in agreement with several studies showing the use of lichens as pollution biomonitors. This action is due to the fact that they do not have roots and, consequently, they depend on wet and dry deposition (that contains pollutants) to take nutrients (Burton MAS, 1986). In literature there is an example in which the metal concentrations in Rhizocarpon geographicum (L.)DC have been correlated with atmospheric dry deposition in Amman city, Jordan (Ziadat A.*et al*, 2006) . The industrial pollution is often due to oxides of sulfur, especially sulfur dioxide and, therefore, can be used the ability of Rhizocarpon Ramond ex DC found in the Ansanto valley to live in a environment rich on these gasses, to estimate the concentration of these fumes.

CONCLUSSION:

Even if Rhizocarpon Ramond ex DC was classified only at sub-genus\section level, this study is important to reveal the presence of this lichen in an area never studied before. The results confirmed the capacity of Rhizocarpon to act as first colonizer in extreme regions, where the competition of other plant species is reduced (as described in literature for different zones) and have improved the available information for this genus. Another relevant aspect of this research is the consideration that ethnobotanical aspects can be evaluated also in the European Community, where the relationships that exist between people and plants are often overlooked.

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Classification:	
Kingdom	Fungi
Division	Ascomycota – Sac fungi
Class	Ascomycetes
Order	Lecanorales
Family	Rhizocarpaceae
Genus	Rhizocarpon

Table 2. Classification of Rhizocarpon Ramond ex DC

Fig.1 Mefite d'Ansanto (first part of the picture created using Google Maps)

Fig. 2 Rocks encrusted with sulfur

Fig.3 Tufa rock. Analyzed on plot 1

Legend:	
Violet	Plot 1
Brown	Plot 2
Blue	Plot 3
Green	Plot 4
Red	Plot 5
Black	Plot 6

Fig. 4 Diagram of the "Quadrat" plots

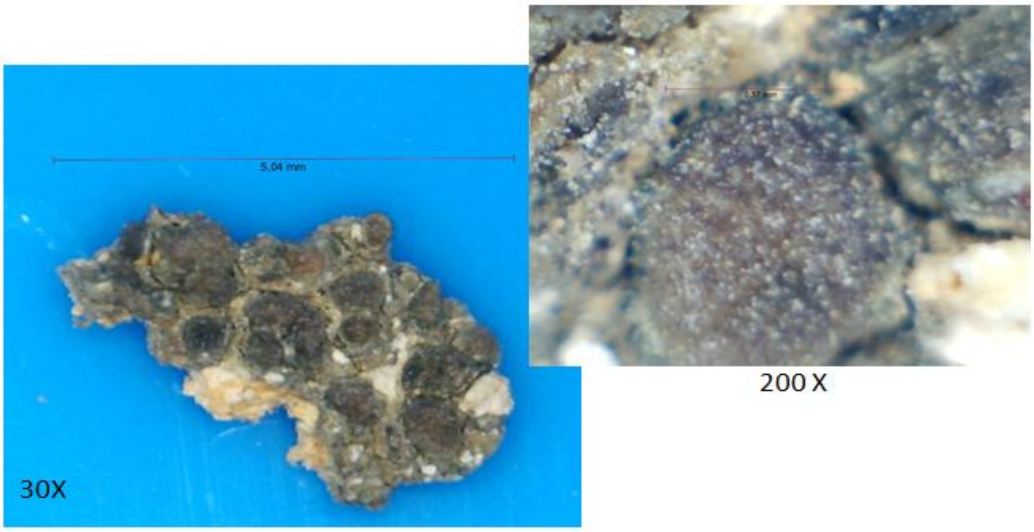

Fig. 5 Rhizocarpon collected on "Quadrat 1"

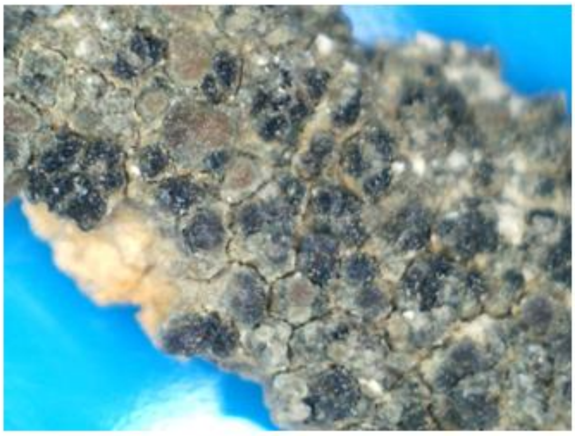

Fig.6 Macroscopic vision of Rhizocarpon

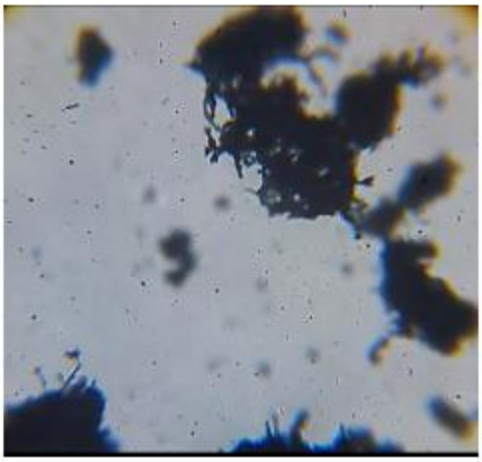

Fig.7 Microscopic picture of Rhizocarpon

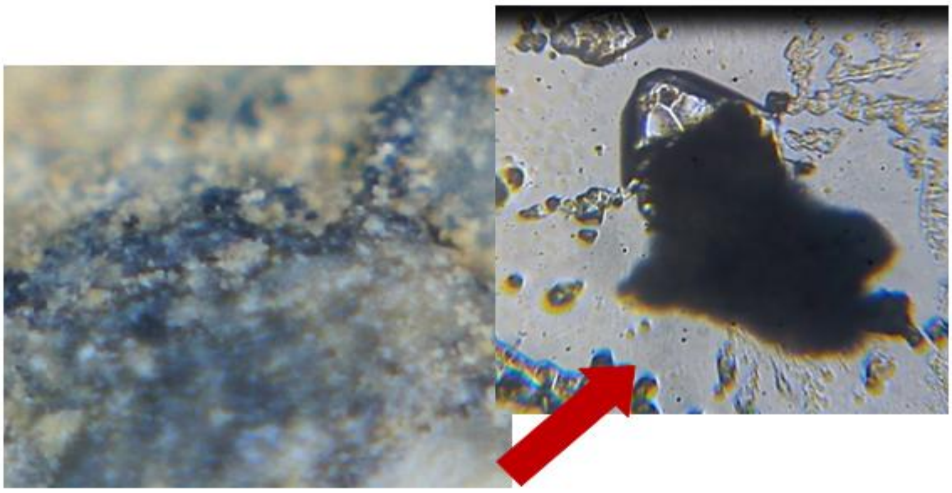

Fig. 8 Particular of the strict adherence lichen\minerals

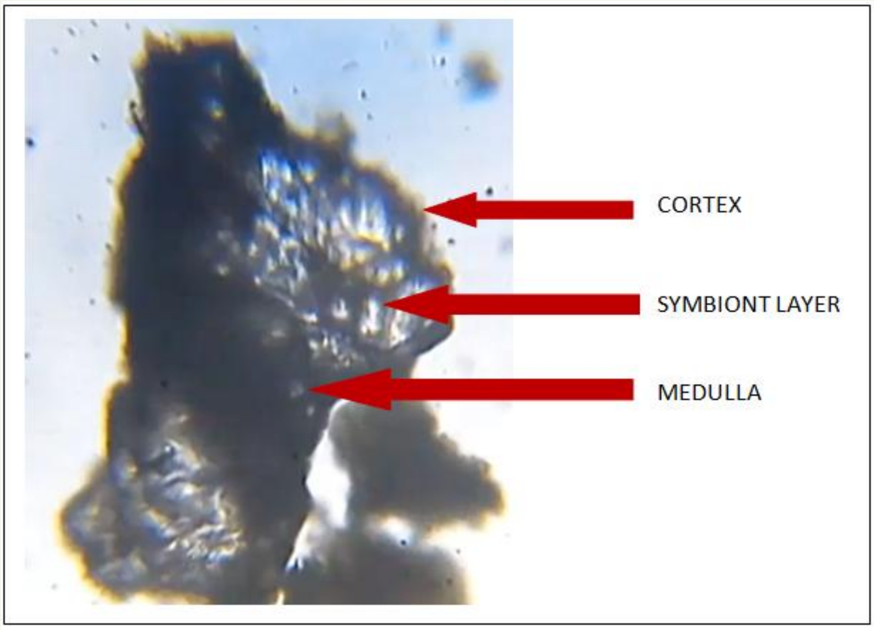

Fig. 9 Structure of the lichen after the treatment

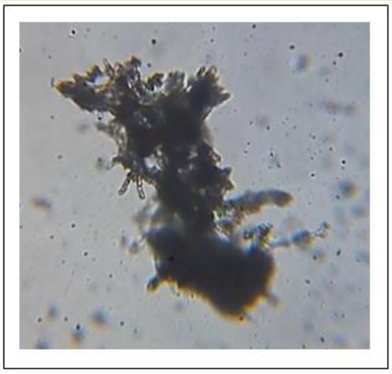

Fig. 10 Hypothallus

Fig. 11 Symbiont layer\Medulla

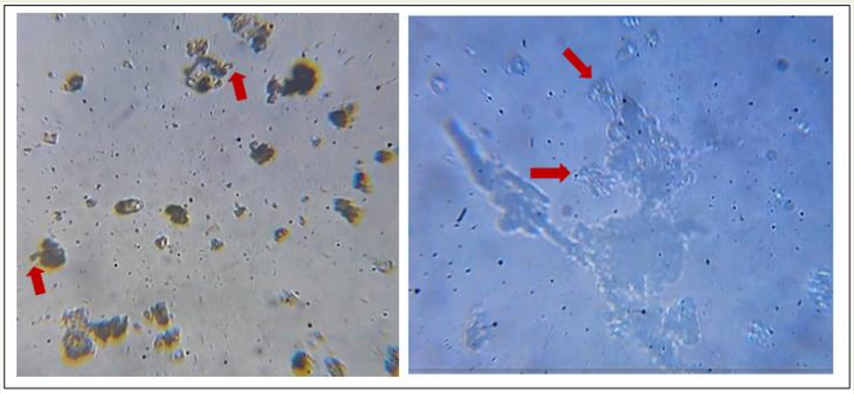

Fig.12 Fragments containing apothecia

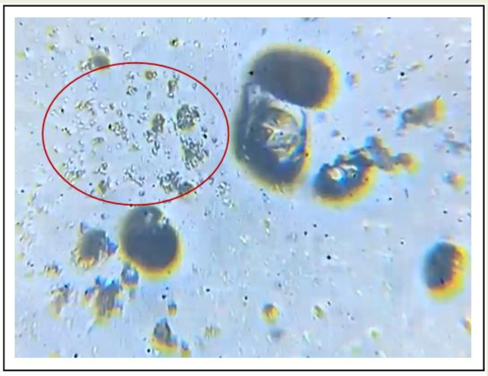

Fig. 13 Ascospores

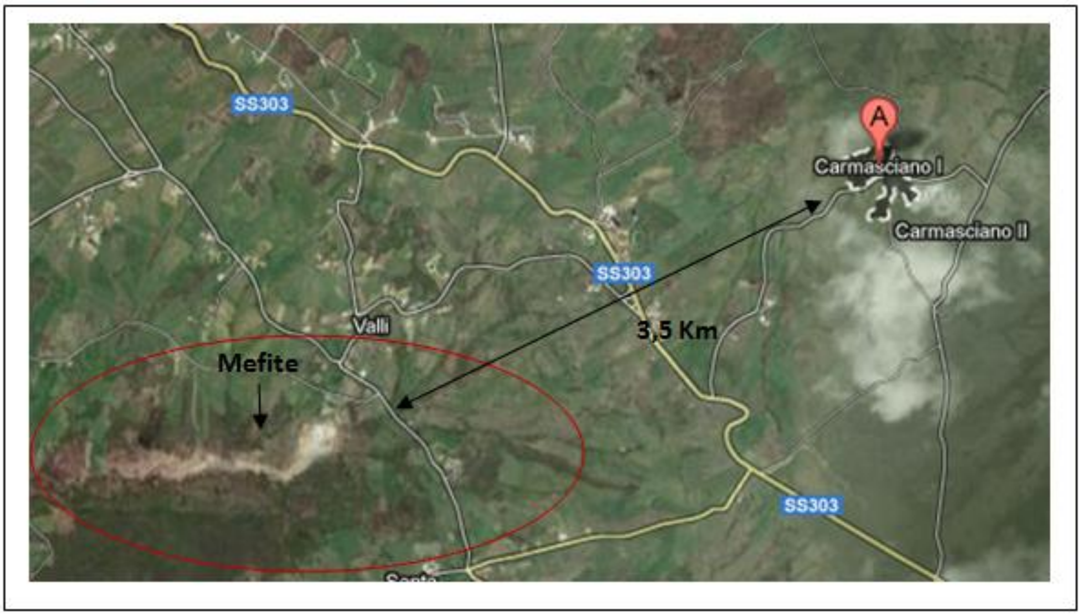

Fig. 14 Distance Carmasciano\Mefite; Picture created using Google Maps