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ORIGINAL PAPER

Instrumental monitoring of the birth and development of truffles in a *Tuber melanosporum* orchard

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Abstract Mycorrhizal symbiotic plants, soil suitability, temperature, and humidity are, by general consensus, considered decisive factors in truffle production. However, experimental approaches to define the environmental conditions that stimulate formation of truffle primordia and promote their growth to maturity have been lacking. By analysis of data of many atmospheric and soil parameters collected since 2009 within a *Tuber melanosporum* orchard, the trends of metabolic activity, detected as CO_2 production in the soil, have been identified as the most reliable parameter to indicate the 'birth' of the truffle primordia. They seem to be produced when mycelial activity is intense and undergoes water stress, after which it resumes. About 6–18 days after recovery of metabolic activity, we could collect primordia of *T. melanosporum*. Many die or develop too early and consequently rot or are eaten by insect larvae. These events occur several times during summer and autumn, those that 'sprout' in late summer or later grow steadily and reach maturity. Using a particular ground-penetrating radar (GPR) setup to discriminate truffles, we could identify individual truffles in the soil after they have enlarged to at least 6 mm in diameter and follow their growth in volume and diameter over time. These two instrumental methods (CO_2 sensor and GPR), although yet to be improved, open new important perspectives to better understand truffle biology and manage truffle orchards to support the newly acquired demonstration of the fundamental role of host plants for the nutrient transfer to the ectomycorrhiza-mycelium-fruiting body complex of *T. melanosporum*.

Keywords Truffle cultivation · Truffle phenology · Ground-penetrating radar · Carbon dioxide sensor

Introduction

The underground fruiting bodies produced by species of *Tuber* (Ascomycota, Pezizales) are known as 'truffles'. The ascomata of some species represent a delight with a high economic value. *Tuber* spp. lives in mycorrhizal symbiosis mainly with several woody plants and therefore can be grown by planting properly mycorrhized trees. The presence of mycorrhizal host plants, soil suitability, temperature, and humidity is, by general consensus, considered decisive factors in truffle production (Hall et al. 2007).

The combined roles of soil temperature and humidity have been the focus of several researchers for many years, becoming the subject of manuals as well as research (Hall et al. 2007) even if not completely understood. As for *Tuber melanosporum* Vittad., the moisture produced by summer storms or irrigation is considered crucial for fruiting. The water penetrates into the interstices of dry and well-ventilated soil, lowering the temperature and providing the moisture necessary for fungal metabolism (Bardet and Fresquet 1995).

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Based on these assumptions, Coquelin et al. (2007) proposed a model of the development of black truffle fruiting bodies, which they defined as Cycle Biologique de *T. melanosporum* (acronym for 'CBTM') in which they recognize and time the phases of (1) induction or 'birth' of fruitbodies in spring; (2) development from July to mid October, if promoted by summer rains; (3) maturation, characterized by production of melanin and aroma, from mid October to the end of December; and (4) senescence during winter, which leads to the collection of ripe truffles. In total, according to this model, production of truffles from birth to full ripening requires about 9 months. These conclusions seem to support previous observations of good *T. melanosporum* production after wet and hot summers and an empiric method of cultivation that provides adequate irrigation during periods of prolonged summer drought, according to many manuals (Pagnol 1983).

Bardet and Fresquet (1995), after several years of observations, concluded that the years with the best production of *T. melanosporum* were characterized by hot and humid spring and summer seasons. Soil temperatures had to be higher than 15 °C in April and higher than 23 °C during June through September; summer had to show marked alternation of heavy rains and drought periods not exceeding 20 days.

However, there is no other system, apart from the final evaluation of the harvest, to experimentally define the environmental conditions that (1) stimulate the formation of primordia and (2) favor fruiting body growth to maturity.

Some information exists on events that stimulate formation of fruiting bodies of several commercial species of basidiomycetes (Kües and Liu 2000), and some methods have been patented (Cantarelli et al. 2013). As regards, these farmed mushrooms, primordia's formation is induced by changing one or a combination of parameters such as temperature, humidity, deficiency of a specific nutrient (Lacourt et al. 2002), CO_2 concentration of the air, and light or physical shock (Oei 2003). It is suggested that most changes that stimulate fruiting body formation negatively affect mycelial growth, therefore less favorable conditions for the mycelial growth would favor formation of fruiting bodies. It is interesting that to be able to succeed, these shocks must occur when the mycelium has colonized the entire substrate or when it is in the maximum growth phase (Oei 2003; Cantarelli et al. 2013). A similar effect was shown in nature on chlamydospores of Glomeromycotina as a drought response after a period of favorable temperatures and moisture (Pacioni 1986).

Changes in the key metabolic processes are, most likely, the desired effects of these factors. Any change of metabolism and variation of metabolic activity, including those connected with growth and development of the fruiting bodies, can be easily measurable in terms of CO_2 production. Hence, we must take into account the measurement of CO_2 produced in the soil together with other climatic parameters generally monitored in truffle cultivation. Monitoring of soil CO2 efflux, an important part of the soil carbon cycle, has gained importance, particularly in recent years because of expected regional climate changes. It has been used to understand trends in different environmental situations, both natural and anthropogenic (Arneth et al. 1998; Delire and Foley 1999; Hirano et al. 2003; Grünzweig et al. 2009; Zhou et al. 2009).

On the other hand, the formation of subterranean fruiting bodies and their presence in soil might produce an anomaly in the soil water distribution, particularly at the interface between soil and truffle peridium. This kind of heterogeneity in water distribution could be measured with a GPR (ground-penetrating radar). The GPR is a powerful instrument widely used for many geological and engineering purposes (Yelf 2007; Jol 2009) and has also been used in agriculture and forestry to map tree root systems from Hruska et al. (1999) to Ow and Sim (2012), and also to detect the rhizomorphs of the parasite fungus *Armillaria* (Rizzo and Gross 2000).

For these reasons, we tested the usefulness of the two monitoring methods, namely, the variations of CO_2 concentrations within the truffle harvesting area over the years and the underground fruiting body response signals to ground-penetrating radar.

The present research aimed to (1) understand when the switching of the *T. melanosporum* mycelium from the vegetative to the reproductive stage occurs (flush) and (2) find a tool to follow fruiting body development in a non-destructive way.

As is well known, the growth area of *T. melanosporum* is characterized by a surface almost free of grass, known as 'pianello' in Italy and 'brûlé' in France and termed 'burned area' in this study.

Materials and methods

Orchard

The truffle orchard selected to conduct this survey is located in central Italy near the village of Roccascalegna, locality Caprilia at 230 m asl, in the southern part of the Abruzzo region (42°04′36.80N; 14°19′50.23E) (Fig. 1S, Supplementary Materials).

The truffle orchard of 2,000 m^2 was planted in 1993 with hazelnut and oak trees mycorrhizal with *T. melanosporum* and spaced at 4.5×4.5 m. The first harvest took place in winter 1999–2000.

The orchard is equipped with a spray irrigation system.

Data logging

To collect data, an automated SIAP-MICROS datalogger OLIMPO with a GPRS card was installed to transmit online

data hourly. Precipitation, solar radiation, wind, air and soil temperatures, and humidity were recorded in a single fixed position, whereas soil CO_2 was replicated inside and outside the burned area between two productive hazelnut trees. CO_2 values were measured by a Vaisala CARBOCAP© Carbon Dioxide Probe GMP343. Soil moisture and temperature probes were located at a depth of 20 cm. Data collection began in 2009, and monitoring still continues (2013).

Harvest of primordia

Based on the idea that, like other fungi, the induction of sexual reproduction is stimulated by stress factors and the research of truffle primordia took place at resumption of metabolic activity after a drought.

Soils were sampled from different areas of orchard to depths of 15–20 cm; each sample was located at two different points known as harvest points of ripe truffles. Each sampling point was monitored only once.

To check the presence of *T. melanosporum* primordia in 'stage 1' (hyphal pellets of 50–400 μm) according to Pacioni et al. (1995) definition, the wet-sieving and decanting technique as improved by Pacioni and Rosa (1986) and Pacioni (1991) was adopted. Primordia were also confirmed to be *T. melanosporum* by molecular methods (Rubini et al. 1998).

Ground-penetrating radar (GPR)

For insight into the soil, we used a GPR System model RIS-K2/0 with a shielded antenna at nominal frequency of 1,600 MHz, equipped with a metric wheel, and processing software GRED 3D developed by Ingegneria dei Sistemi (Pisa, Italy).

The investigations were performed with the GPR 'single-fold' technique with a monostatic radar consisting of an antenna module with transmission and reception functions that samples at 1024 samples/scan and full scale set to 40 ns to a depth of survey of more than 1 m.

The sample-acquired signals were previously calibrated in 2008 through iterative techniques of filtering algorithms by use of target truffles in intact surfaces to develop the best fit; and adjustments on the ground were tested with developing truffle fruiting bodies. Details of GPR settings are provided in the Supplementary Materials.

The area under radar investigation is represented by two permanent surfaces of 2×2 m and 1.8×1.8 m. Scans were made manually with a constant pitch of ~0.2 m in a direction parallel to the upper and lower contour lines from right to left, according to a Cartesian reference system chosen in consideration of a fixed-point station (see Fig. 1S, Supplementary Materials).

Scans were repeated in 2010 on 2 July, 16 July, 5 August, 9 September and 22 October at 2, 8,13, 18, 23, and 28 cm depths. Details on the permanent surfaces and setting process of GPR are provided in the Supplementary Materials.

Results

All scans were complete without significant interruption. Fortunately, 2010 proved to be an exemplary year in terms of weather events important to the development of black truffle mycelium obtained at the same time.

CO_2 trends and climate

At the outset, the metabolic activities inside and outside burnt areas clearly differed in concentration of CO_2 (ppm) in the soil. As shown in Fig. 1, the course of the two sensors shows similar trends, but sensor 1, within the burned area, recorded absolute values of CO_2 efflux almost three times higher than that produced outside. The levels of CO_2 measured by sensor 1 (inside) and sensor 2 (outside) were quite similar from January to the first half of April 2010, whereas from the second half of April to the last week of June, the metabolic activity inside rose much higher, reaching almost 16,000 ppm (May 29–June 1), almost three times the amount of CO_2 recorded outside (~6,000 ppm).

The second period of increase in CO_2 production occurred from the first days of September to December 1 with lower absolute values but with a CO_2 concentration measured by sensor 1 four times higher than that of sensor 2 (~8,000 vs ~2,000 ppm).

Between these two periods (June 1–Sept 1), the production of CO_2 tended to decline reaching the minimum on August 3 with CO_2 values of about 1,000 ppm. Heavy irrigation on July 22 and the early days (2–7) of August (Fig. 2) failed to reactivate the metabolic activity.

The trends of CO_2 with other parameters (soil moisture θ and temperature **T**) involved with metabolic activity of the soil do not significantly correlate statistically with the availability of water and the temperature values (Fig. 2).

Formation of primordia

Fruiting body primordia were not always found in samples, but when at least a single primordium was found, its development appeared related to certain pedoclimatic trends. A decrease of activity, and therefore of CO_2 concentration, due to water stress (dryness), was recorded for about a week and then a rapidly rise with the arrival of rain and a rise of soil temperatures about 20 °C, conditions that usually occur from late spring to autumn.

In 2010 (15 May, 21 May, 6 June, 23 June, and 1 October), five double samplings were made. Six times out of ten, we found primordia (stage 1, named "hyphal pellets") and

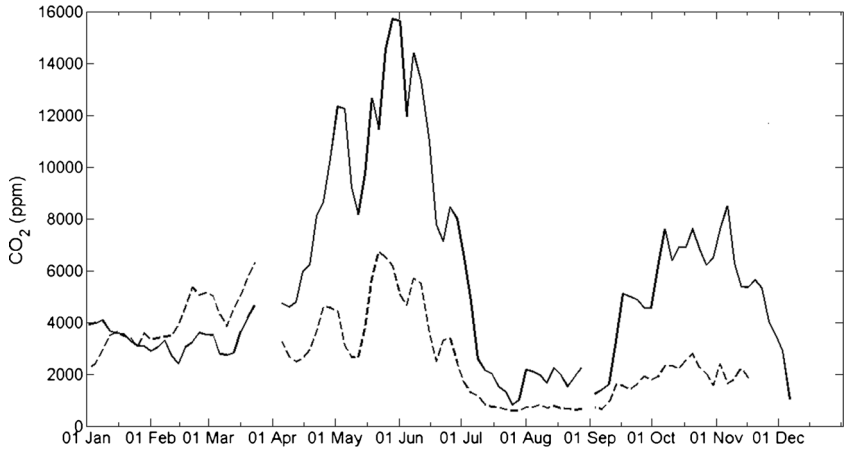

Fig. 1 Trends of CO_2 quantities recorded inside (—) and outside (—) of *Tuber melanosporum* growth and harvesting area. Hourly average, year 2010

sometimes even fruiting bodies at stages 2 ("peridial stage", ascoma with peridium and undifferentiated gleba) or 3 (ascoma with peridium and gleba with veins but no asci), maybe produced thanks to previous inductions (Pacioni et al. 1995).

Soil radargrams

Signals attributable to abnormalities of water distribution produced by truffles ≤6 mm in diameter are shown in Table 1. The relationship of these data with the evaluation of fruiting body numbers was examined by subtracting the signals present in two successive radargrams (an explanation is provided in Fig. 6S and 7S, Supplementary Materials). The signals from the 2 cm depth were excluded because they were affected by the 'main bang' effect, the first signal produced by the air/soil interface and by the risk of detecting primordia of pre-emergent fruiting bodies of nontarget fungi.

Table 1 shows that each survey showed several truffles with size detectable by GPR, but in every survey, most signals were produced by newly formed fruiting bodies. Clearly, several generations of fruiting bodies may develop or degenerate within a few weeks of each other (Fig. 6S and 7S, Supplementary Materials).

The signals also do not seem to be closely linked to the real dimensions of truffles but in fact are amplified by technical issues discussed below. Consequently, signals can be shared by two or rarely three successive radargrams. Figure 3 shows examples of radargrams obtained at a depth of 28 cm in the permanent surface 1, where two signals of truffles harvested at the end of November (27.11.2010) are circled: the truffle circled in red was mature and dug up by a dog, while the other (circled in yellow) was somewhat immature and accidentally removed during excavation. Although less well defined, the signals of these two truffles were visible from August 5 (red circle) and from September 9 (yellow circle).

Discussion

The two monitoring systems tested by experimental and objective methods appear promising to enhance understanding of the production and developmental processes of *T. melanosporum* in relation to environmental factors. Their use promises to improve methods to correctly manage the truffle orchards

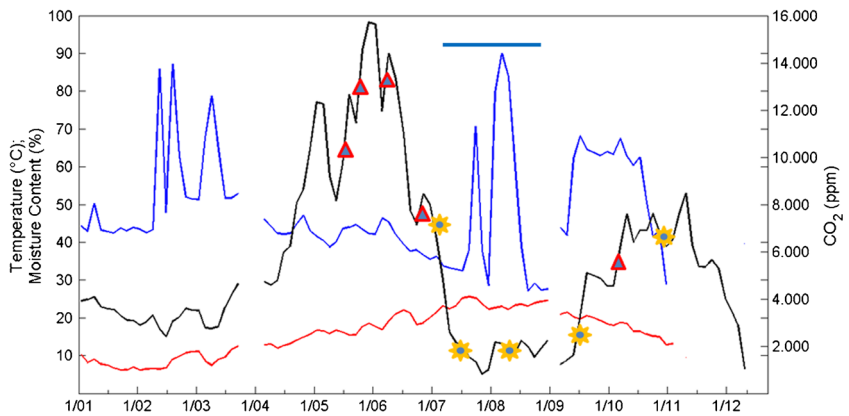

Fig. 2 Trends of soil temperature (*red line*) and soil moisture content (*blue line*) and CO_2 (*black*), year 2010, with the soil samplings (*red-blue triangles*) and GPR scans (*blue-yellow stars*). The second notches on the ordinate on the right refer to the scale of values of soil moisture content and temperature of the ordinate on the left, the *blue horizontal line* indicates the irrigations

Table 1 Truffle signals recorded in the two permanent surfaces

Day	8 cm		13 cm		18 cm		23 cm		28 cm	Total
Permanent surface 1										
02/07/2010	**27**	6	**11**	6	**8**	0	**11**	2	**6**	49
	3+		0		1+1−		1+1=		3−	
16/07/2010	**12**	5	**11**	3	**10**	3	**7**	2	**11**	38
	1− 1=		1+		1+1−		1+2−		3+	
05/08/2010	**16**	6	**13**	4	**11**	4	**10**	3	**13**	46
	1−		1−		1+1=		1+1−		1+	
09/09/2010	**17**	1	**8**	1	**12**	2	**11**	3	**7**	48
	1+2−		0		2+1−		2+		1−	
22/10/2010	**18**	7	**14**	3	**18**	3	**14**	4	**7**	54
Permanent surface 2										
02/07/2010	**20**	4	**18**	4	**16**	1	**14**	1	**6**	64
	2+1−		1− 3=		1+1−		1+2−		2+1− 2=	
16/07/2010	**12**	3	**17**	4	**17**	3	**9**	2	**11**	54
	1+2− 1=		2− 2=		1+1−		2+2− 2=		2+1− 1=	
05/08/2010	**29**	5	**22**	3	**24**	4	**24**	2	**13**	98
	1+3−		1−		1+1−		3−		1+1−	
09/09/2010	**22**	1	**15**	1	**12**	1	**12**	2	**7**	63
	1− 1=		0		2+1=		3−		1+1=	
22/10/2010	**13**	4	**13**	3	**15**	2	**15**	4	**7**	50

In the columns (bold characters) are the putative number of fruiting bodies beside the number of signals in common with the next (deeper) radargram. In the rows under each survey are the persistent signals and estimate of their size (+ increased, − decreased, = equal) compared with the previous radargram (an explanation is provided in Fig. 6S and 7S, Supplementary Materials)

In particular, this first survey reveals that

1. Induction of primordia of *T. melanosporum* is a recurring event that could be achieved with a forecasting model based mainly on CO_2 concentrations in the soil.
2. Development and growth or death of fruiting bodies in the soil can be monitored noninvasively.

Soil parameter trends and primordia production

The correlation between production of CO_2 and the soil values of moisture and temperature is still under investigation, but it is not as straightforward as one might expect in view of the previous results of Bardet and Fresquet (1995) and Coquelin et al. (2007). In our case, values of temperature (daily average above 20ºC and below 12ºC) and soil moisture (below 35–40 %) appear to be critical and can act independently on the metabolic activity of the soil and CO_2 efflux. Also, the duration of these adverse values appears to negatively influence on the production of CO_2, as shown in Fig. 2. These observations support the conclusions of Davidson et al. (1998) regarding the independence of soil water content and temperature in control of soil respiration.

In any case, the trend of CO_2 has proved to be a valuable indicator of the formation of primordia, and in fact, we have continued to collect truffle primordia driven only by the performance of this parameter (unpublished data).

The temporal variations of soil CO_2 were taken on as an expression of a putative metabolic activity of the mycelium of *T. melanosporum*. However, they may be due to mere coincidence with natural phenomena that occur with the increase of temperature and soil moisture.

Numerous investigations have focused on effects of soil temperature and water content on soil CO_2 efflux and various aspects of plant–environment interactions (Arneth et al. 1998; Delire and Foley 1999; Hirano et al. 2003; Grünzweig et al. 2009; Zhou et al. 2009). Fine root turnover is considered the major process regulating carbon (C) flux, C budget, C allocation, and C sequestration in terrestrial ecosystems and 10–30 % of net primary production in temperate forest ecosystems (Kitajima et al. 2010). In our case, the phenomenon of induction of primordia could be part of more complex interactions associated with the symbiotic life of the mycelium of *T. melanosporum* with roots of host plants. The strong difference between values of CO_2 efflux recorded inside and outside burned areas cannot be attributed only to the metabolism of the truffle, although previous studies indicated that the presence of a burned area occupied by *T. melanosporum* around a tree is related to the quantity of its mycelium in the soil (Suz et al. 2008; Parladé et al. 2013).

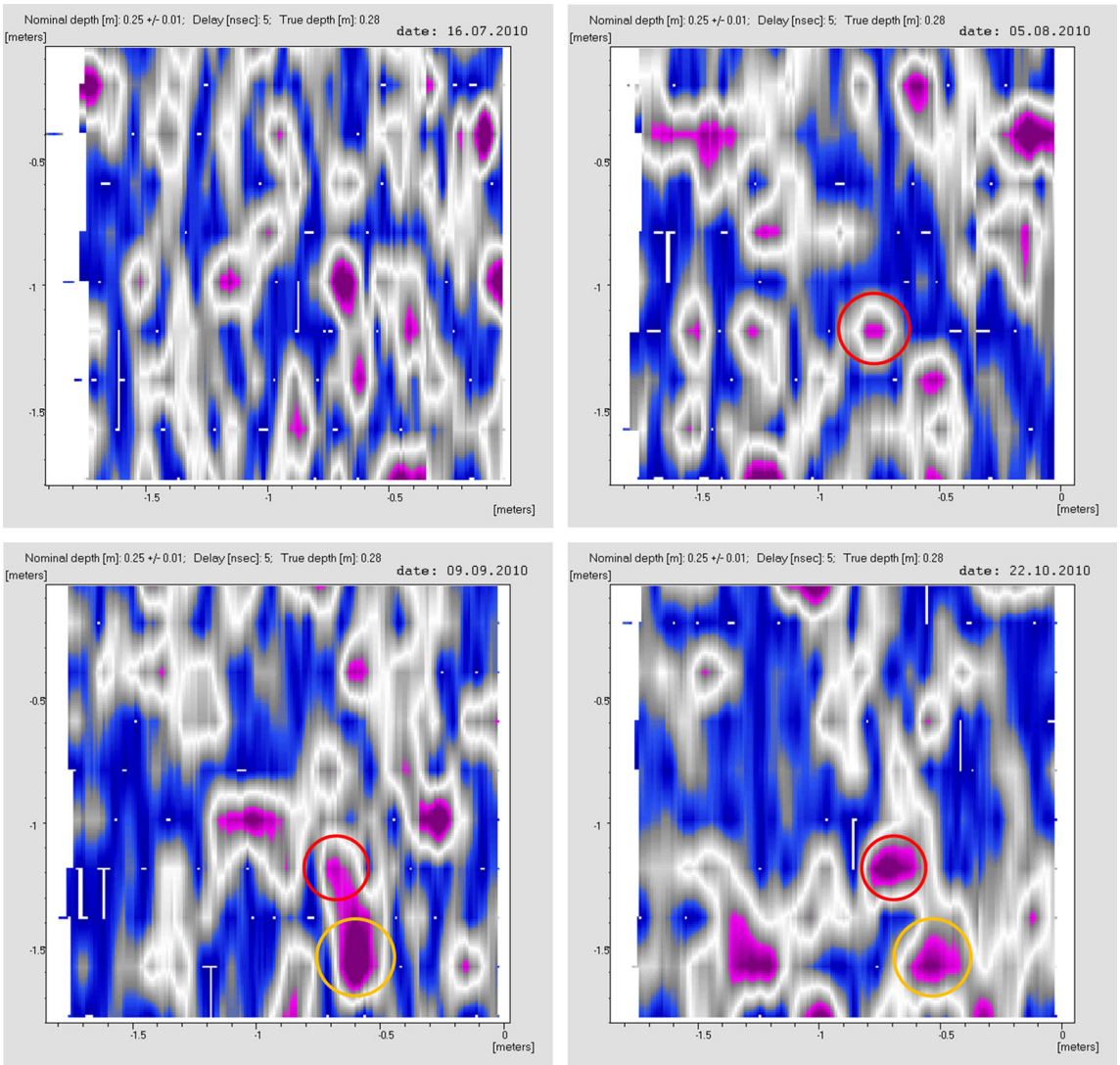

Fig. 3 Radargrams from the permanent surface 1 at 28 cm depth; the truffle signals are the *red/violet spots*. The signal of a truffle harvested matures on 27 November is circled in *red*; the *yellow circle* indicates the signal of the second truffle harvested not completely mature during the same digging

GPR

The application of the GPR technique for determination of the geometric characteristics of the reference target, as proposed in the configuration of previously reported measures, defines a good starting point to search for a valid response to the proposed aims. The time slices (Goodman et al. 1995) for the reticles of permanent surfaces and the different times of sampling should be sufficient to define a direct correlation between the response of the radar signal and the geometric characteristics of the subterranean object.

This first investigation attempt has been defined already, even in the early stages of data acquisition, some obvious problems actually affect the final result and therefore require special attention for their resolution. In particular, the interferences of the interface created by coupling the antenna with the ground (often bumpy) can generate distortions of the radar signal that results in the creation of false targets in the rough radargrams.

The second problem is represented by the inability to establish *a priori* the presence of the target and its subsequent development direction in depth with respect to the reference reticle adopted at the surface. For this study, a high-frequency-type antenna—a bow-tie with polarization pointing in the direction orthogonal to the scan profile—was used. Such device architecture offers a different sensitivity towards targets that have a direction of prevailing development as a function of the relative orientation between the antenna and the target. In other words, the response of the radar signal is exponentially attenuated as the set of target is aligned to the direction of

polarization. This defect produced radargrams that were deformed and oversized compared to the truffles in the soil.

Conclusion

In general, truffle production does not diverge much from that already known for that of other mushrooms such as Basidiomycetes, in which the conditions for mycelial growth and fruiting body development are clearly distinct by a drastic change of the environmental situation (Kües and Liu 2000; Oei 2003). If the assumption is true, the recorded trend of CO_2 concentration is mainly due to the activities of the system roots—ECM—extramatrical mycelium. Increases of emitted CO_2 provide conditions suitable for growth of mycelium, but if the CO_2 decreases, the conditions may stimulate formation of fruiting body embryos. In both cases, the roles of temperature and soil moisture are essential.

Another similarity seems to be that the induction and production of fruiting bodies in *T. melanosporum* occur in subsequent periods, similar to the flushes well known for the main cultivated mushrooms (Kües and Liu 2000).

The time needed for full maturation of fleshy mushrooms differs strongly from that for *T. melanosporum,* estimated to be several months (Pacioni et al. 1995; Olivier et al. 2012; Le Tacon et al. 2013). From our data, in fact, about 5 months are needed from induction to the maturation of truffles. From these estimates, it follows that the truffle harvests that commonly occur in central Italy from January to March are the product of inductions that occurred in late autumn. Even if it rare, some harvests have occurred in April and July, moving the hypothetical events of induction to the winter months (PG, personal information).

We believe that these monitoring systems represent a pivotal point in research regarding the reproductive biology of truffles.

This complements the newly acquired demonstration of the mode of nutrition by mycelium of *T. melanosporum*, thereby eliminating the possibility of a saprotrophic phase and reaffirming the fundamental role of host plants, in particular, for the transfer of nutrients to the ectomycorrhiza-mycelium-fruiting body complex (Le Tacon et al. 2013; Zarivi et al. 2013).

After having once again recognized the central role of the host plant, it will be necessary to deepen the study of its habit in relation to truffle symbiosis, first of all defining its photosynthetic efficiency and crop water requirements (Allen et al. 1998) to reach the goal of 'understanding truffles in the underground' (Kües and Martin 2011).

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