Chapter 8
Biological Significance of Truffle Secondary Metabolites

Richard Splivallo

8.1 Introduction

Fungal primary and secondary metabolites have an important impact on our society. Best known as mycotoxins, phytotoxins, antibiotics and natural aromas; they represent industries worth billions of dollars. Fungi are also of major importance in terms of biomass: they rank first with an estimated dry weight of 450 kg/ha, which represents 91% of the total soil biomass (microflora and microfauna) (Müller and Loeffler 1976). Yet our knowledge of the ecological significance of fungal metabolites is limited. Despite the pioneer work of Dick and Hutchinson (1966) and Hutchinson (1973) on the effect of volatile fungal metabolites on fungi and plants, this argument seems to have raised little interest in the scientific community. Since then, most studies have focused on parasitic interactions with plants (phytopathogens), while much less attention has been given to the ecological role of the metabolites of symbiotic fungi. An important group of the latter is represented by mycorrhizal fungi. Mycorrhizas are one of the oldest associations between plants and fungi. Dating back to the early colonization of the terrestrial environment (Brundrett 2002), they are classified as endomycorrhizas (arbuscular, ericoid, orchid mycorrhizas) or ectomycorrhizas depending on their ability to penetrate the host-plant root. Truffles fall in the last category of the ectomycorrhizal fungi. Best known for the complex aroma of their hypogeous fruitbodies, truffles were already known to the Greeks and the Romans, but only reached their luxury standing in the last 20 years owing to decreasing production (Fauconnet and Delher 1998; Hall and Yun 2001) and an ever-increasing demand. Despite their high commercial value, very little is known about their biology. Indeed, the unique features of mycorrhizal fungi, from their formation to signal exchange with the surrounding environment (the rhizosphere), are still poorly understood. In addition to the compounds involved in nutritional exchanges between the host plant and the fungus, various micromolecules and macromolecules are secreted into the rhizosphere. These exudates and volatile

Richard Splivallo
University of Torino, Department of Plant Biology, IPP-CNR, 25 Viale Mattioli, 10125 Torino, Italy
e-mail: ricsi17@hotmail.com

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organic compounds (VOCs) play an active role in the regulation of symbiosis and interactions with other organisms, including nonhost plants.

More than 200 VOCs and many nonvolatile compounds have been identified from various truffle species. The aim of this chapter is to discuss the ecological significance of these metabolites (VOCs and/or exudates) associated with three levels of differentiation: fruitbody, free-living mycelium and mycorrhizas. Furthermore, the possible role of these metabolites in the interaction with the host plants and nonhost plants (the so-called burnt, a zone with scarce herbaceous cover) shall be discussed.

8.2 Truffles: Life Cycle and Distribution

Ectomycorrhizal symbiosis has evolved repeatedly over the last 130 million to 180 million years (LePage et al. 1997). In boreal and temperate forests, 95% of the short roots of plants form ectomycorrhizae (Martin et al. 2001), with 5,000–6,000 species of basidiomycetes or ascomycetes—including truffles (Buscot et al. 2000; Martin et al. 2001). Ectomycorrhizae positively impact plant growth in nature (Read 1991) owing to improved nutrient uptake and protection against pathogens (Borowicz 2001; Buscot et al. 2000).

Truffles are hypogeous ascomycete fungi belonging to the genus *Tuber*, the family *Tuberaceae* and the order *Pezizales* (O'Donnell et al. 1997; Trappe 1979). Their mycorrhizal status was established worldwide in the 1960s (Harley and Smith 1983; Trappe 1962). Truffles live in symbiosis with plant roots, generally forming ectomycorrhizas. In contrast to the high degree of promiscuity exhibited by arbuscular mycorrhizal (AM) fungi towards their hosts, ectomycorrhizal fungi are rather host-specific. Indeed, truffles tend to associate with angiosperms and gymnosperms, predominantly with oaks, hazels, some species of pines, but also some species of shrubs like *Cistus*. For a complete list of the host plants of European truffle species, refer to Ceruti et al. (2003). Recently truffle mycelium has also been identified within orchid roots—even though it does not form ectomycorrhizas (Selosse et al. 2004).

The present information about truffle's life cycle is very patchy. On the basis of observations both in nature and in the laboratory, as well as possible similarities with the life cycle of other ascomycetes fungi, Lanfranco et al. (1995) proposed a model for the life cycle of truffles which can be divided into three phases: (1) a reproductive phase (fruitbody), (2) a vegetative phase (free-living mycelium—saprotrophic phase actually only observed in the laboratory) and (3) a symbiotic phase (mycorrhizas) (Fig. 8.1). Indeed difficulties arise from the impossibility to follow the full life cycle in the laboratory. Even though Fassi and Fontana (1969) reported production in pots of fruitbodies of *Tuber maculatum* in association with *Pinus strobus* (Fig. 8.2), this achievement has not been repeated since then for any truffle species! Nevertheless more insight has recently been gained into the life cycle of truffles by Paolocci et al. (2006). In an elegant experiment the authors

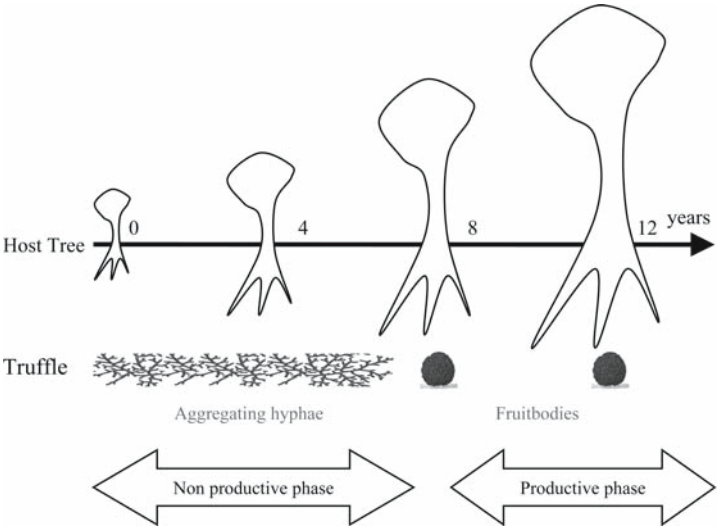

Fig. 8.1 A model of truffle's biological cycle. Associated with a young tree (nonproductive phase, 0 to approximately 7 years old), no fruitbodies are produced. During that period the fungi probably oscillates between the symbiotic and vegetative phases. Once the host has reached a certain maturity, the truffle can enter its reproductive phase (productive phase, starting approximately 8 years). The hyphae aggregate to eventually produce fruitbodies. During that period, the three phases (symbiotic, vegetative and reproductive) might succeed or coexist with each other

applied polymorphic microsatellites to compare the allelic configuration at different stages of *T. magnatum*'s life cycle (asci and surrounding mycelium in fruit bodies; ectomycorrhizal root tips). Their results suggest that *T. magnatum* outcrosses and that its life cycle is predominantly haploid. Nevertheless if outcrossing occurs, the proportion of ascocarps that do so is for the moment unknown as is how well these observations apply to other truffle species.

If fruitbodies are generally not obtainable in the laboratory, methods for obtaining mycorrhizas in 3–4 months are rather well established (Miozzi et al. 2005; Sisti et al. 1998; Zambonelli and Branzanti 1989). Success has been reported starting either with fresh fruitbodies or with mycelium grown in pure culture. The latter method (mycelium) has recently been adapted by Zeppa et al. (2004) in order to study the VOCs emitted by the mycelium/plant system before, during and after the formation of the ectomycorrhizas.

In vitro mycorrhization with *T. borchii, T. brumale* or *T. albidum* has been described for *Tilia platyphyllos, Cistus incanus, Alnus cordata, Castanea sativa, Populus alba* and *Corylus sativa* (Giomaro et al. 2002; Miozzi et al. 2005; Sisti et al. 1998; Zambonelli and Branzanti 1989, 1990).

More than 60 truffle species have been described so far (Trappe 1979), of which 20 are present in Europe (Gandeboeuf 1997). The fruitbodies and spores of two species of commercial interest are illustrated in Fig. 8.3. For a historical review and

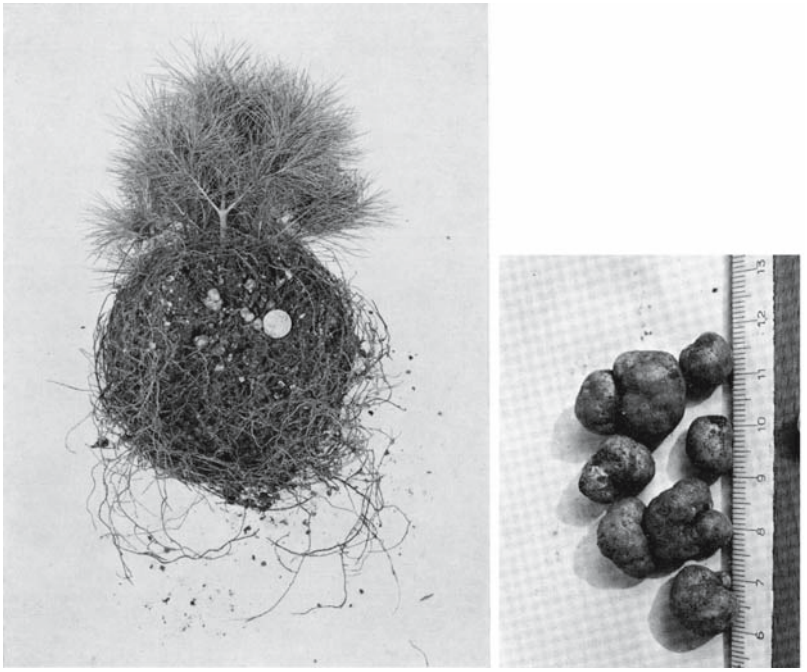

Fig. 8.2 *Tuber maculatum* and *Pinus strobus*. Association between the host and the truffle with visible fruitbodies (*left*). Fruitbodies of *T. maculatum* (*right*). (Reproduced from Fassi and Fontana 1969)

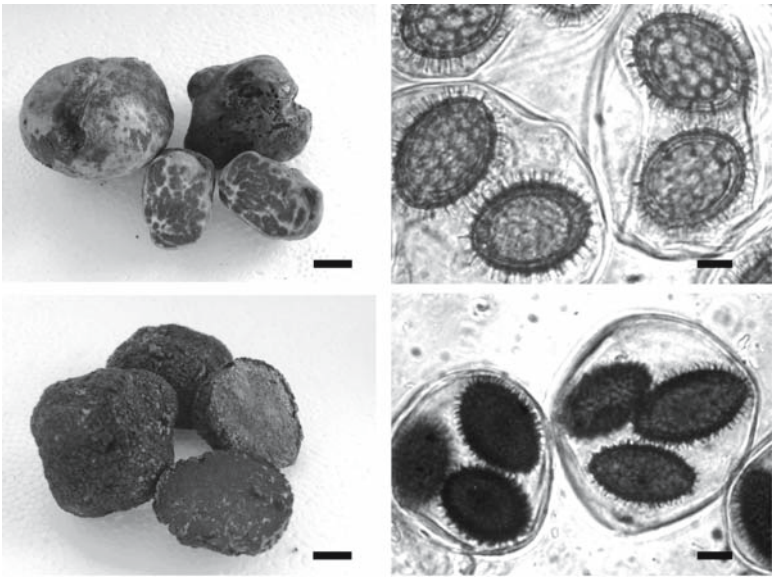

Fig. 8.3 Fruitbodies and spores of two truffle species. Fruiting bodies and spores of the white truffle *T. borchii* (top) and the black truffle *T. melanosporum* (bottom) (scale fruitbodies 0.50 cm, spores 10 µm)

identification of European truffle species, refer to Ceruti et al. (2003). The most famous and expensive ones—because of their intense and complex organoleptic properties—are *T. magnatum*, otherwise referred to as Alba's white truffle (and actually found in Italy and the Carpathian Basin), and the black truffle *T. melanosporum*, also referred to as the Périgord truffle and found mostly in Spain, France and Italy. Recent population-genetic studies suggest that *T. melanosporum* recolonized western Europe from southern Italy after the last glaciation period, and that the colonization pattern is closely related to the route followed by oak—its major host (Murat et al. 2004). However, the natural habitat of other truffle species is not limited to western Europe, but extends in the Northern Hemisphere and the Southern Hemisphere, spreading from northern Africa to Sweden towards the north (Weden et al. 2004) and the Carpathian Basin towards the east (Bratek et al. 1999). Lastly, truffles are also found in northern America (Amaranthus et al. 1999), Australia and New Zealand (where *T. melanosporum* has been recently introduced) and Asia (Yang Mei 1999).

Our knowledge of the truffle distribution is limited by the fact that fruitbodies are hypogeous and require trained dogs or pigs to locate them. Thus, the present distribution map reflects the zones where fruitbodies are collected. Mycorrhizas or nongerminated spores could tell another story, and reveal a much larger distribution than the one known today. This is exemplified by the lack of correlation between the presence of mycorrhizas and fruitbodies of *T. magnatum* observed in a truffle field in northern Italy (Murat et al. 2005).

8.3 Field Observations and Open Questions

In the Northern Hemisphere, most truffle species tend to form mature fruitbodies in the winter. In the case of *T. melanosporum*, small truffles 2 mm in diameter and reddish in color appear in June/July (Sourzat 1997). The fruitbody swells to reach its "mature" size in September/October. In the next 2 months, the peridium and gleba become darker owing to the melanization, indicating spore formation. Whether the fruitbody always remains connected with the mycorrhizas through mycelium is still unclear.

Interestingly Barry et al. (1994) suggested that the fruitbody of *T. melanosporum* and that of *T. aestivum* could absorb nutrients and take up water through de novo formed mycelial tufts at the surface of the peridium. If such a nutrition mechanism is generalized among truffles and whether it is sufficient to satisfy the full nutritional requirements of the fruitbody have nevertheless not been established yet.

It is believed that spores remain dormant—sometimes for many years—until a potential host plant gets in their vicinity. Whether spore germination in truffles involves some signaling from the host or from the fungi to the host is still not known. The mycelium from the germinated spore then comes into contact with the plant root and forms ectomycorrhizas within a few months, thus closing the life cycle (Fig. 8.1). It is not known what growth free mycelium can achieve in soil, and

how long it can survive without a host. Nonetheless, mycellia of diverse truffle species grown in the laboratory on agar and supplemented with glucose or sucrose as a carbon source display an extremely slow growth (Ceccaroli et al. 2001; Iotti et al. 2002; Saltarelli et al. 1998), suggesting similar behavior in nature.

From the planting of a young mycorrhized tree in the wild, fruitbody formation is a rather long process, and seems to be related to the age, and also the species of the host plant. For example, a young oak tree mycorrhized with *T. melanosporum* will generally not induce any fruitbody formation before it has reached 7–15 years (Fig. 8.1). Some associations might actually never do so owing to the harsh competition of truffles and other microorganisms in the soil. However, the trigger for the fruitbody production is still totally obscure. It is not clear how the age of the tree or maybe its size influences fruitbody formation. In various plantations of hazels and oaks of the same age, all mycorrhized with *T. melanosporum*, fruitbodies have been observed 2–4 years earlier under hazels than under oaks (P. Sourzat, personal communication), suggesting that some change in metabolism due to aging in the host could somehow trigger fruitbody formation.

Having briefly described the life cycle of truffles, we shall now focus on the molecules that could act as signals in the complex interaction of truffle with its environment. Before discussing their potential involvement in the interaction with host and nonhost plants, let us have a brief look at what they are.

8.4 An Overview of Truffle Metabolites

8.4.1 VOCs from Fruitbodies

VOCs emitted from truffle fruitbodies have been widely studied, mainly though for species of commercial interest such as *T. melanosporum, T. magnatum, T. borchii, T. uncinatum* and *T. aestivum*. Most of those studies focused on aroma description (Claus et al. 1981; Flament et al. 1990; Ney and Freytag 1980; Splivallo et al. 2007a; Talou et al. 1987a, b, 1989a–d), influence of storage conditions on shelf life and aroma evolution (Bellesia et al. 1996, 2001, 2002; Falasconi et al. 2005; Pelusio et al. 1995) and only recently the possible ecological role of some VOCs of *T. borchii* has been discussed (Zeppa et al. 2004).

While certain VOCs such as 1-octen-3-ol, 2-methyl-1-butanol, 3-methyl-1-butanol and dimethyl sulfide are generally common to all truffle species, other VOCs only present in trace amounts might vary in intensity and structure depending on the truffle species. Aditionally, a strong variability in the VOC blend of truffles of the same species has been demonstrated (Mauriello et al. 2004; Splivallo et al. 2007a) and can be attributed to factors such as the fruitbody's maturity (Zeppa et al. 2004), its origin (Diaz.et al. 2003) and also to associated microorganisms which might feed on the fruitbody (Buzzini et al. 2005).

To date, more than 200 VOCs have been reported from various truffle species, and that number is likely to continue growing as VOC extraction techniques such

Table 8.1 Selected fruitbody volatile organic compounds (VOCs) of some truffle species Nonexhaustive list of VOCs reported in Splivallo et al. (2007a) for the following truffle species: Tuber melanosporum (MEL), T. borchii (BORC), T. indicum (IND), T. aestivum (AEST), T. magnatum (MAGN). ND not determined, – the VOCs have not been detected so far to the best of our knowledge

Molecule	Aroma description	MEL	BORC	IND	AEST	MAGN
Fatty acid derived VOCs						
1-Octen-3-ol	Fungal	x	x	x	x	–
3-Octanone	Fungal, sweet	x	x	x	x	–
3-Methyl-1-butanol	Whiskey	x	x	x	x	–
2-Methyl-1-butanal	Green, malty	x	x	x	x	x
3-Methyl-1-butanal	Sweet, malty	x	x	x	x	x
Hexanal	Cut grass	x	x	x	x	x
Terpenoids						
trans-Ocimene	Warm, herbaceous	–	x	–	–	–
Aromatic compounds						
2-Phenylethanol	Rose	x	x	x	x	–
1-Methoxy-3-methylbenzene	ND	x	x	–	x	–
Benzaldehyde	Almond	x	–	x	x	–
Anisole (methoxybenzene)	Anise	x	–	–	–	–
Sulfur compounds						
Dimethyl sulfide	Garlic	x	–	–	x	x
Dimethyl disulfide	Rubber	x	–	–	x	x
Dimethyl trisulfide	Fecal	x	–	–	x	x
2-Methyl-4,5-dihydrothiophene	ND	–	x	–	–	–

as solid-phase microextraction and related techniques become more and more sensitive (Diaz et al. 2003; Mauriello et al. 2004; Splivallo et al. 2007a), permitting detections limits at the parts per billion level. The VOCs identified so far are simple hydrocarbons that contain functional groups such as alcohols, aldehydes, esters, ketones, aromatic groups and sulfur compounds. Some frequently reported VOCs of truffles are listed in Table 8.1, with some structures being given in Fig. 8.4.

The aim of the following section is not to give an exhaustive list of truffle metabolites, but instead to focus on the most characteristic ones (sulfur compounds, fatty acid derived VOCs) and on the classes with a major ecological importance (terpenoids for signaling, or phenolics for phytotoxicity).

8.4.1.1 Fatty Acid Derived VOCs

Most of the linear chain hydrocarbons, alcohols, aldehydes and ketones are derived from fatty acid metabolism. Among those, 1-octen-3-ol and 3-octanone have been reported for most truffle species (Table 8.1). They are responsible for the strong

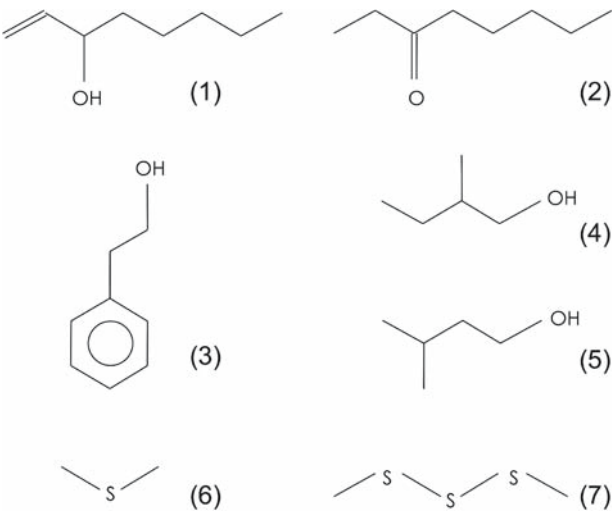

Fig. 8.4 Structure of some truffle volatile organic compounds (*VOCs*): *1* 1-octen-3-ol, *2* 3-octanone, *3* 2-phenylethanol, *4* 2-methyl-1-butanol, *5* 3-methyl-1-butanol, *6* dimethyl sulfide, *7* dimethyl trisulfide

fungal smell typical of *T. borchii*, but are also common to most other fungi (Abraham and Berger 1994; Chiron and Michelot 2005; Venkateshwarlu et al. 1999; Wnouk et al. 1983), some plants and have even been reported in fish (Ingvarsdóttir et al. 2002). 1-Octen-3-ol has recently been identified as a fungal hormone able to inhibit mycelial growth and trigger sporulation in *Penicillium paneum* (Chitarra et al. 2004). In *Pleurotus pulmonarius* mycelium grown in liquid culture, Assaf et al. (1997) confirmed that 1-octen-3-ol was directly derived from linoleic acid breakdown by a lipoxygenase. Refer to Combet et al. (2006) for a detailed review of the properties and biosynthesis of eight-carbon volatiles in fungi. 2-Methyl-1-butanol, 3-methyl-1-butanol and their respective aldehydes, all derived from fatty acid catabolism, are also well represented among truffle species (Table 8.1). They have been reported, along with dimethyl sulfide, as the major contributors to the final aroma of *T. melanosporum*. 2-Methyl-1-butanol and 3-methyl-1-butanol seem to be widespread among higher fungi (Abraham and Berger 1994: Chiron and Michelot 2005) and molds (Meruva et al. 2004), and might have phytotoxic properties (Pacioni 1991). Their production has also been reported for yeasts isolated from fruitbodies of *T. melanosporum* and *T. magnatum* (Buzzini et al. 2005), confirming the hypothesis that the VOC blends of truffle fruitbodies could be produced by more that one organism (in this case ectomycorrhizal fungi and yeasts).

Other linear-chain C6, C7, C8 and C9 aldehydes and alcohols seem also to be common among VOCs of different truffle species. For *Arabidopsis thaliana*, C6 aldehydes are known to activate defense genes and induce resistance against fungal pathogens such as *Botrytis cinerea* (Kishimoto et al. 2005). Whether the C6

compounds from truffle fruitbodies serve a similar self-defense role or might induce resistance in neighboring plants is not known.

8.4.1.2 Terpenoids

Terpenoids have only been identified recently in fruitbodies of *T. borchii* (Zeppa et al. 2004) and *T. brumale* (Mauriello et al. 2004). Unlike in many flowers or fruits (Aharoni et al. 2004), they represent a minor part of fruitbody VOCs in terms of concentration, but might be of major ecological importance. Zeppa et al. (2004) identified four monoterpenes and seven sesquiterpenes in *T. borchii*'s fruitbodies at different maturation stages, which could be involved in defense against microbes, interactions with insects and signaling with the host plant. One of these, aromadendrene, was only found in very immature fruitbodies of *T. borchii*, rendering that molecule a good marker of fruitbody maturity. Furthermore three major genes of the isoprenoid pathway, upregulated in mature fruitbodies, have recently been cloned and characterized in *T. borchii* (Guidi et al. 2006).

8.4.1.3 Aromatic Compounds

VOCs containing aromatic rings have been reported in all *Tuber* species; however, none seem to be common to all of them—maybe due to different VOC extraction techniques used in the different studies. They might somehow contribute to the so-called burnt area (area with scarce herbaceous cover) observed with some truffle species (Sect. 8.6) as simple phenolics are known for their phytotoxicity (Gallet and Pellissier 1997).

8.4.1.4 Sulfur-Containing Compounds

Sulfur-containing compounds seem to be characteristic of most truffle species (thiols, thioesters, sulfides, thioalcohols and thiophenones), but are generally present in trace amounts in fresh fruitbodies. One sulfur-containing compound, dimethyl trisulfide, has also been identified in pure mycelial cultures of *T. borchii* (Tirillini et al. 2000), while different yeast strains isolated from truffle fruitbodies also have the capacity to produce them (Buzzini et al. 2005). Most sulfur-containing compounds have very low olfactory detection limits and are thus major contributors to the final aroma of truffle fruitbodies. They derive from the catabolism of L-methionine, their major precursor (Berger et al. 1999; Spinnler et al. 2001). In *Geotrichum candidum* L-methionine is first converted to 4-methylthio-2-oxobutyric acid, which is then transformed into methanethiol, a key precursor of most sulfur VOCs (Arfi et al. 2003; Bonnarme et al. 2001a, b; Spinnler et al. 2001).

From an ecological point of view, sulfur-containing compounds might act as fumigants against microbes in decomposing roots of cabbage (Bending and Lincoln

1999) and as repellents against amphipods in marine algae (Schnitzler et al. 1998). In fruitbodies of *T. magnatum*, the concentration of sulfur-containing compounds has been reported to decrease within 2 weeks of storage at room temperature (Bellesia et al. 1996), while an increase was observed in *T. borchii* upon aging (Bellesia et al. 1996, 2001).

8.4.2 Fruitbody Non-VOCs

Nonvolatile metabolites from fruitbodies have been investigated for *T. aestivum* (Mannina et al. 2004) and *T. indicum* (Jin-Ming 2004). The authors identified sugars, polyols, amino acids, organic acids, fatty acids, sterols and lipids, among which were two sphingolipids, highly bioactive molecules known to be involved in regulation of cell growth, differentiation and apoptosis. De Angelis et al. (1996) also identified quinonoid and polyphenolic biopolymers as the major constituents of *T. melanosporum*'s melanin, and suggested a polyketidic origin.

8.4.3 Mycelial VOCs

VOCs produced by only one species (*T. borchii*) have been investigated so far. When grown either on potato dextrose agar or in liquid cultures, *T. borchii* mycelium (strain ATCC 96540) produced eight VOCs, including aromatic compounds, alcohols and a ketone, most of which have also been described from the fruitbodies of various truffle species (Splivallo et al. 2007a). Nevertheless cultural conditions strongly influence the production of volatile compounds, as exemplified by Tirillini et al. (2000), who identified 29 VOCs from submerged cultures of *T. borchii* mycelium (modified Melin-Norkans medium). Under those conditions, most of the VOCs had not been reported in truffle fruitbodies, with the exception of butan-2-one and dimethyl trisulfide described, respectively, in *T. melanosporum* (Bellesia et al. 1998a) and *T. magnatum* (Bellesia et al. 1998b). Mycelium of other truffle species has not been investigated so far, mainly owing to their poor growth.

Having considered the major group of metabolites reported in the literature, we should say a word of caution regarding their ecological significance. On one hand, they were generally identified under rather unnatural conditions (sterile system, or in the case of the fruitbody's VOCs with the fruitbodies generally washed free of soil, thus certainly under a high-stress condition). The occurrence of these metabolites should be checked in situ to understand their possible ecological role. Besides, it is likely that we see only the tip of the iceberg as far as truffle metabolite diversity is concerned. Indeed VOCs at an early stage of fruitbodies (when they are only a few millimeters in diameter) have never been investigated. Neither have VOCs been reported for advanced stages of decomposition (overmaturity). Ecologically sound

studies should thus focus on these aspects in situ, and unfortunately require the assistance of a very collaborative truffle hunter!

8.5 Metabolites Involved in Truffle and Host Plant Interaction

Signaling in the rhizosphere between plants and microorganisms is regulated by molecules which permit host–symbiont recognition and induce morphological changes in each partner. Such early signaling events have been extensively studied for *Rhizobium* and legumes, and led to the understanding that some flavonoids secreted by the plant trigger the production of the nodulation (nod) factor in the bacteria, which in turn induces morphological changes in the rooting system of the plant (Dénarié and Cullimore 1993; Heidstra and Bisseling 1996). A similar molecular dialogue has been observed in the case of the early interaction between AM fungi and their host. Indeed Akiyama et al. (2005) have recently identified a sesquiterpene lactone (5-deoxystrigol) from *Lotus japonicus* root exudates inducing branching in *Gigaspora margarita* hyphae. For a recent review on signaling between AM fungi and plants, refer to Harrison (2005).

Much less is known on the recognition events between ectomycorrhizal fungi and plants than on the AM fungi–plant interaction (reviewed in Martin et al. 2001). In the case of truffles, VOCs produced during ectomycorrhizas formation of *T. borchii* with *Tilia americana* have been recently investigated (Gioacchini et al. 2002; Menotta et al. 2004a). Twenty-nine VOCs specific to the premycorrhizal stage (where the host and the symbiont are separated by a few centimeters) have been identified as hydrocarbons, alcohols, ketones, a brominated cholesterol derivative and terpenoids, including the sesquiterpene germacrene D, as well as dehydroaromadendrene, ß-cubebene and longicyclene—which might be involved in chemotropism of hyphae towards the roots of the host (Menotta et al. 2004a). Molecular changes in mycelium during early interaction between *T. borchii* and *Tilia americana* were also investigated by Menotta et al. (2004b). Suppressive subtractive hybridization and reverse northern blots allowed the identification of differentially expressed genes in the mycelium and involved in cellular detoxification, secretion and apical growth, or general metabolism.

Nutrient availability may act as a further regulatory signal: for example, a phospholipase A, strongly upregulated by nitrogen starvation in *T. borchii* mycelium (Soragni et al. 2001), was shown to be expressed mostly during the early steps of the fungus–plant interactions (Miozzi et al. 2005). This provides confirmation of the hypothesis that mycorrhization is a response to nutrient stress. However, the road to the identification of molecular messengers involved in interaction between truffles and hosts is still long. As ectomycorrhizal fungi present much higher host specificity than AM fungi, it seems reasonable to argue that the structures of the signal molecules might be characteristic of each specific association of ectomycorrhiza and plant. The metabolites released by truffles in the rhizosphere might not

only be involved in host recognition, but might also serve other functions such as defense or competition with other organisms, as exemplified in the following section.

8.6 Interaction with Nonhost Plants

The burnt (or *brûlé* in French) is the only phenomenon where the presence of truffles mycorrhizas/mycelium is obvious. It is a zone around or near the host tree where vegetal cover is scarce (Fig. 8.5). The phenomenon, generally observed with trees mycorrhized with *T. melanosporum, T. aestivum* and *T. indicum*, is not seen with *T. magnatum*. Its occurrence with other truffle species is more controversial.

During the life cycle of the fungus, the burnt becomes apparent when the host tree is 5–10 years old, and its appearance precedes the formation of the first fungal fruitbodies by a few years. The burnt tends to form as a more or less circular zone (of a few meters in diameter) around the trunk of the host tree, and moves over years, spreading in diameter and/or moving away from the tree (sometimes as far as 15–20 m). Additionally, most herbaceous plants inside the burnt are smaller than their counterparts outside it, but some plants such as *Festuca ovina* (Mamoun and Olivier 1997) and other Graminaceae (such as *Bromus inermis, B. erectus*) seem to be less affected (Montacchini and Caramiello Lomagno 1977; Sourzat 1997).The burnt phenomenon has been known for a long time (Cicarello 1564); however, its causes still remain unclear. Explanations for the formation of the burnt have been proposed by Delmas (1983), who hypothesized that truffle mycorrhizas may compete for nutrient or water, by Plattner and Hall (1995), who suggested that *T. melanosporum* hyphae could penetrate the roots of the herbaceous plants, maybe acting as parasites, and in a series of other publications highlighting the phytotoxic effect of truffle fruitbody's metabolites (Fasolo-Bonfante et al. 1971; Lanza et al. 2004; Pacioni 1991; Papa 1980; Splivallo et al. 2007b).

In nature, the distinction between mycorrhizal and saprobic behavior is not always an easy one as the organisms involved might switch between one and the other depending on changing biotic and abiotic factors (Fitter 1991; Hibbett et al. 2000). Tibbett and Sanders (2002) reported a case of necrotrophy for an ectomycorrhizal fungus, demonstrating that *Hebeloma syrjense* P. Krast, colonizing willow roots, was able with its extraradical mycelium to find nutrient patches within the soil (dead seeds, fruits, pollen), and absorb them after digestion with exoenzymes. In the case of *T. melanosporum*, such necrotrophic and parasitic behavior (as mentioned in the preceding section) has not been clearly demonstrated. Could such a dualistic behavior—mutualistic symbiont with the host and endophytic with nonhosts—be driven by mycoeterotrophic behavior of some plants interconnected through the mycelial network of truffle? This behavior has been observed in the case of achlorophyllous orchids able to get their nutrients through the mycelial network interconnecting them with photosynthetic plants (Bidartondo et al. 2004; Girlanda et al. 2006). Could the high energy requirement necessary to produce

Fig. 8.5 The burnt. *Top*: An 8-year-old hazel mycorrhized with *T. uncinatum*—winter period (Murisengo, northern Italy). *Bottom*: A 10-year-old hazel mycorrhized with *T. melansporum*—summer period (Cravanza, northern Italy). The burnt is clearly visible in both pictures as a circular zone with scarce vegetal cover surrounding the host tree

fruitbodies induce saprobic behavior of the truffle on the herbaceous plants? In both cases why is the phenomenon (burnt) not observed with all truffle species? This question remains unanswered for the moment. Consequently further evidence must be obtained to support this dualistic behavioral theory. Its final contribution to the burnt should be quantified with other possible factors such as the competition for nutrients among the nonhost plants and truffle or the production of phytotoxic metabolites by the truffles, so far only observed in laboratory experiments.

8.6.1 Phytotoxic Metabolites in Soil from Truffle Fields?

The presence of toxic substances in "burnt" soil is supported by the retarded germination observed by Papa (1978–1979) when treating *Lepidium sativum* with aqueous extracts of burnt and non burnt soils from a *T. melanosporum* truffle field. The author however noted that if differences were obvious with 2-day-old seedlings, they had almost completely disappeared on the third day, implying either a degradation of metabolites or low starting concentrations. More recently, Lanza et al. (2004) reported reduction in primary root length of *Vicia faba* planted in soil collected from a burnt zone produced by *T. aestivum*. The authors also tested long-term toxicity (genotoxicity) using a *Vicia faba* root micronucleus test, and reported a significant increase in the number of micronuclated cells for burnt soil compared with the control. We similarly observed a reduction in root length (approximately 14%) for two consecutive years with cucumber planted in burnt soil compared with non burnt soil from a truffle field of hazels mycorrhized with *T. melanosporum* (Fig. 8.6). In contrast, no differences between burnt and non burnt soil were observed for cucumber germination (R. Splivallo, unpublished data).

One should keep in mind that neither cucumber, nor *Vicia faba* nor *Lepidium sativum* is generally found in truffle fields. Consequently, if the reduction in root length can be considered a good indicator for the presence of some inhibiting/stimulating metabolites inside/outside the burnt, herbaceous species associated with

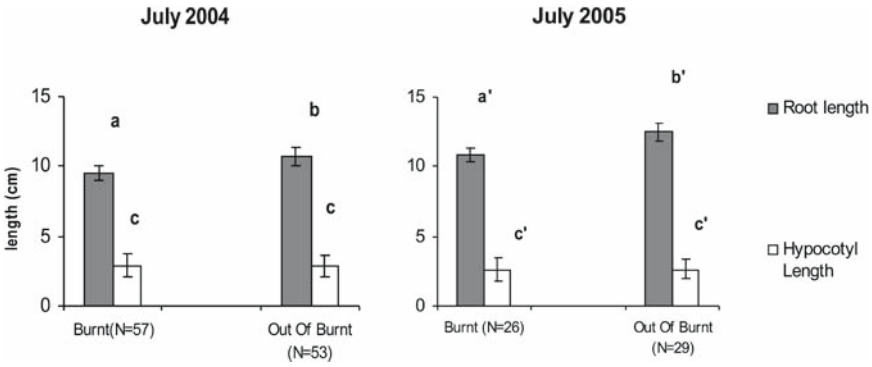

Fig. 8.6 Average root and hypocotyl length of cucumber germinated in burnt and non burnt soil. Soils samples were collected from a truffle field in Cravanzana, northern Italy. In 2004, three soil samples were taken from two different burnt zones and three soil samples were taken from just outside (approximately 2 m) the burnt zones. In 2005, eight soil samples were taken from eight different burnt zones and eight samples were taken from outside the burnt zones. For each year, the soil samples were sieved (5 mm), then pooled in two categories: burnt and out of burnt. Cucumber seeds (10 per pot) were germinated in 250 g soil wetted with 80 ml H_2O for 8 days, after which root and hypocotyl length were recorded. A significant reduction in root length was observed each year for seedlings grown in burnt compared with out of burnt soil. Hypocotyl length was not affected. The results are presented with the standard deviation (*bar*). Different letters indicate significant differences $P < 0.05$ (Kruskal–Wallis) test. *N* total number of seedlings

truffle fields should be used for an ecologically sound argument, especially as plant responses to secondary metabolites are species- and dose-dependent. Lastly, the soil collected from the field might contain very low concentrations of active secondary metabolites owing to bacterial degradation and/or owing to the physical separation from or disruption of the producing organisms (possibly mycorrhizas, mycelium) at the time of the collection. Therefore, laboratory assays might underestimate the real or long-time effect.

Neither the metabolites responsible for the effects described above nor their source has been identified yet. Truffles might potentially produce them, at one or different stages of their life cycle; there are two grounds for this. First, truffles are clearly associated with the burnt, and thus appear as obvious candidates. Second, production of phytotoxic substances by truffles has been documented in laboratory experiments.

8.6.2 Phytotoxic Metabolites from the Fruitbody

Some authors focused on the phytotoxicity of *T. melanosporum* and *T. aestivum* fruitbodies to try to explain the burnt (Fasolo-Bonfante et al. 1971; Lanza et al. 2004; Pacioni 1991; Papa 1980). Aqueous extracts of *T. melanosporum* have been tested by Montacchini and Caramiello Lomango (1977) on a series of seeds collected from truffle fields and including Graminacea, Caryophyllaceae, Lamiaceae, Scrophulariaceae, Plantaginaceae and Asteraceae. Germination bioassays with different extract concentrations always led to a reduced number of germinated seeds and reduction of root length for germinated seeds compared with the control. Similar results were obtained also with *T. melanosporum* extracts by Fasolo-Bonfante et al. (1971) and Papa (1978–1979), and for *T. aestivum* by Lanza et al. (2004), who furthermore highlighted the genotoxicity of the fruitbody using the the *Vicia faba* micronucleus test. The metabolites responsible for the phytotoxicity in the above-mentioned experiments are not fully known. On one hand, Papa (1980) reported isolating a strongly phytotoxic brown substance form *T. melanosporum* fruitbodies, however without characterizing its molecular structure. On the other hand, Pacioni (1991) tested the effect of ten VOCs characteristic of *T. melanosporum*, and reported a significant root shortening of wheat induced by 2-methylbutanol, 3-methylbutanol and 3-methylbutanal already at a concentration of 7.5 ppm of each single VOC. Similarly, lentil roots were significantly reduced at 5.0 ppm for 3-methylbutanol, 7.5 ppm for 3-methylbutanal and 10.0 ppm for 2-methylbutanol. The other seven VOCs tested, namely, dimethyl sulfide, 2-butanone, 2-butanol, 2-methylpropanol, 2-methylpropanal, 2-methylbutanal and methylanisole did not show any significant effects on wheat or lentils at concentrations of 10 and 25 ppm. We similarly tested the effect of three truffle species on cucumber. We chose *T. uncinatum* and *T. indicum* for the burnt associated with those species, while *T. borchii* was used as a negative control because it was thought it did not produce any burnt. In a first set of experiments, the fruitbodies were cut into small pieces and incorporated into the sand where cucumber was germinated, thus allowing slow

diffusion of VOCs and exudates into the sand. Root length reduction was the strongest with *T. borchii*, followed by *T. uncinatum*, while no significant difference was observed for *T. indicum* (Fig. 8.7). The trend was reversed for hypocotyl length (stimulation instead of reduction; Fig. 8.7). In order to control the effect of the fruitbody's VOCs on cucumber (and not the exudates), a second set of experiments was carried out placing the fruitbodies in a small open plastic container at the sand surface, thus only allowing free diffusion of VOCs. Exactly the same trend as in the first set of experiments was observed (with the fruitbody in the sand) (Fig. 8.7), suggesting that the VOC blends released by *T. borchii* and *T. uncinatum* are responsible for the observed root shortening and hypocotyl elongation.

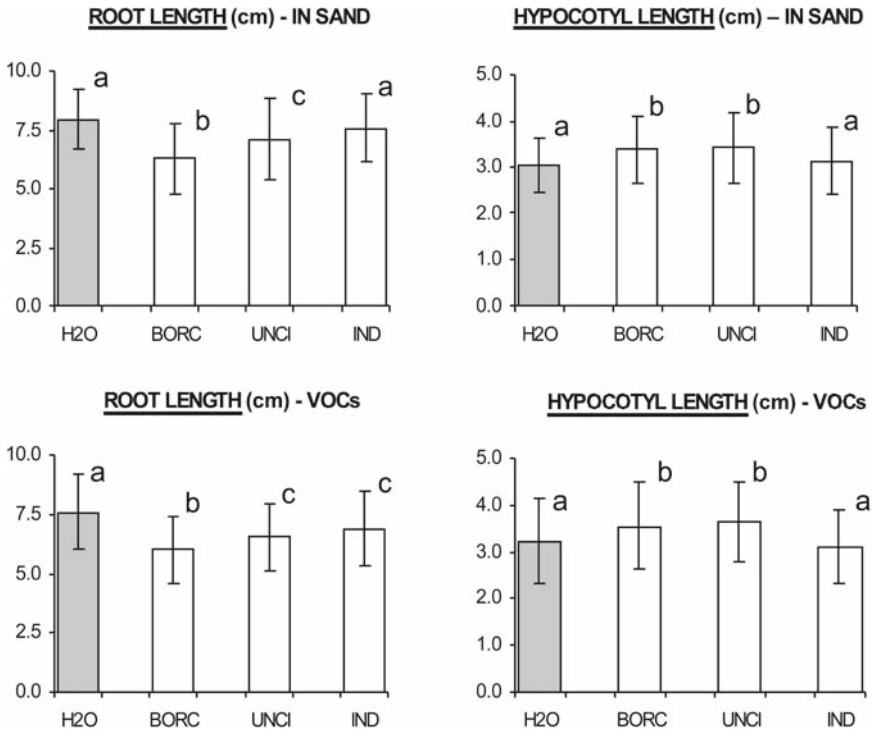

Fig. 8.7 Effect of truffle metabolites on cucumber growth. Cucumber was germinated for 8 days (ten seeds per pot with 350 g sand and 80 ml H_2O, and 1.0 g was taken from five fruitbodies of *T. borchii* (*BORC*), seven fruitbodies of *T. uncinatum* (*UNCI*) and five fruitbodies of *T.indicum* (*IND*) or without a fruitbody as the control (*H2O*). The fruitbodies (frozen at −80 °C for long-term conservation) were either chopped into small pieces and mixed into the sand or placed in a small plastic container on the surface of the sand to allow solely diffusion of VOCs. Pots were sealed to prevent loss of VOCs. All bioassays were repeated eight times, so the total number of seedlings per treatment was always more than 75. In both sets of experiments (in sand and in pots) root length was significantly reduced for all truffle species (with the exception of *IND* in sand), while hypocotyl length significantly increased in the cases of *BORC* and *UNCI*. The results are presented with the standard deviations (*bar*). Different letters represent significantly different results $P < 0.05$ (Kruskal–Wallis test)

As far as the burnt is concerned, the results described above suggest that the fruitbody is not the major cause of the burnt, as on one hand, the strongest reduction in root length was observed with *T. borchii*, which probably does not produce a burnt and, on the other hand, no reduction in root length was observed with *T. indicum*, which produces one. This is further supported by the fact that in laboratory bioassays fruitbody volatiles from various truffle species inhibited the development of both host (*Cistus incanus*) and nonhost (*Arabidopsis thaliana*) plants, suggesting that truffle fruitbody volatiles might not be involved in premycorrhizal signaling, but simply serve as defense molecules against plants (Splivallo et al. 2007b).

Further evidence from the truffle life cycle supports the fact that the fruitbody is not the initiating agent of the burnt. Indeed the burnt appears a few months to a few years before the first fruitbodies. Furthermore fruitbodies have sometimes been found well outside the burnt. Nevertheless, fruitbody metabolites might somehow enhance the phytotoxicity of the burnt, and it cannot be excluded that in truffle grounds metabolites produced by the fruitbodies are also synthesized by the mycelium and/or the mycorrhizas. Indeed, this has recently been demonstrated for 1-octen-3-ol, a volatile that strongly inhibited the development of *Arabidopsis thaliana* in laboratory bioassays (Splivallo et al. 2007b), and that has been reported from truffle fruitbodies, mycelial pure cultures (Splivallo et al. 2007a) and the symbiotic association *T. borchii*/*Tilia americana* (Menotta et al. 2004b).

8.6.3 Phytotoxic Metabolites from the Mycelium

Germination inhibition of *Sinapis alba* treated with culture broth of *T. melanosporum* mycelium was reported by Fasolo-Bonfante et al. (1971); however in the 1970s, species identification was only based on morphological observations. The experiment awaits a fresh confirmation using mycelium whose identity was confirmed by currently available molecular techniques (Douet et al. 2004).

Truffle is nonetheless not the only organism capable of producing phytotoxic metabolites. Other microorganisms, including fungi and bacteria, are indeed associated with truffle fields as we shall see now.

8.6.4 Possible Contribution to the Burnt by Other Organisms

Many plants and fungi are known to produce phytotoxic substances. In a large screening experiment for isolating new bioactive metabolites, Schulz et al. (2002) reported that 18% of endophytes fungi isolated from various soils had an antialgal/herbicidal effect, or in other words were able to produce some phytotoxic secondary metabolites in vitro. Phytotoxic substances also seem to be a common competitive weapon used by invasive plants (Barney et al. 2005).

Regarding truffles, other microorganisms are characteristic of the burnt zone, and might contribute to its toxicity. Luppi Mosca and Fontana (1977), using plate

isolation techniques to quantify and identify the saprotrophyc mycoflora in and outside the burnt (*T. melanosporum*), concluded that not only was the burnt area much "richer" in saprotrophyc flora, but that some species such as *Penicilium diversum, Penicilium restrictum* and *Acremonium breve* were strongly stimulated inside the burnt. Similar plate isolation techniques have been applied to yeast populations in truffle fields of *T. aestivum* by Zacchi et al. (2003), who identified one strain of *Cryptococcus albidus* specific to the truffle field. Bacterial population living inside the fruitbody could also produce phytotoxic metabolites. Barbieri et al. (2005) identified many bacteria living inside the fruitbody of *T. borchii*. Even though the burnt is not associated with that truffle species; it is possible that other bacterial strains associated with burnt-"producing" truffles emit some phytotoxic substances.

Lastly, AM fungi have also been known to inhibit herbaceous plant growth, especially in the interaction with a nonhost plant, probably mediated by some chemicals (Francis and Read 1995). Unfortunately, the presence of AM fungi in truffle fields (and differences in community composition inside and outside the burnt) has not been studied yet. As a consequence, whether the truffle is responsible alone or with some other organisms for the inhibition of herbaceous plants inside the burnt remains to be determined (Fig. 8.8).

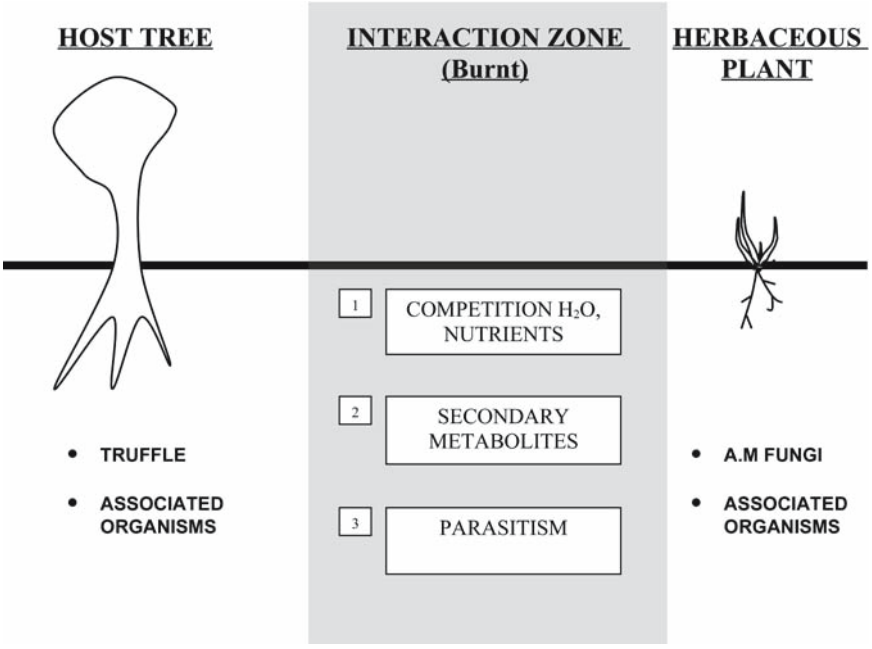

Fig. 8.8 The interactions inside the burnt. Factors possibly involved in the formation of the burnt are as follows: *1* competition for nutrients and water; *2* interactions involving phytotoxic secondary metabolites; *3* parasitism from the truffle on the nonhost herbaceous plants. *A.M* arbuscular mycorrhizal

8.6.5 Ecological Significance of Truffle Metabolites

The results obtained so far in laboratory experiments confirm that truffle fruitbodies can indeed produce some phytotoxic substances (Fig. 8.7) that might also be present in soil (Fig. 8.6). Nevertheless, those results do not reproduce the strong phytotoxic effect observed in the field (Fig. 8.5). The reasons for this can be various. First, most test plants used for the bioassays are not generally found in truffle fields, and can have different responses to phytotoxic metabolites from those of the plants typically found in truffle fields (in this case they could be less sensitive). Second, only rather short-term effects have been tested so far in laboratory experiments (days to weeks), while phytotoxicity could be induced in nature on a much longer time scale. Last but not least, the metabolites (volatiles or exudates) might be present in nature at concentrations different from those in the laboratory or might act in synergy with other unknown metabolites not present in laboratory experiments.

At this stage the ecological significance of truffle metabolites and specifically their contribution to the burnt are not known, as all the data obtained so far are once again not from field experiments but are rather from laboratory experiments. Indeed, production of secondary metabolites from fungi is known to be drastically influenced by biotic and abiotic factors (Bode et al. 2002) and consequently the metabolite production pattern by mycelium or fruitbody might drastically vary between the laboratory conditions and the field. For these reasons, investigation of the ecological roles of secondary metabolites should be possibly done *in vivo*, or in the case of the metabolites identified in laboratory experiments, their occurrence and biological role (in synergy with other metabolites present in the field) should be investigated in nature.

8.7 Conclusions

Our knowledge of the interaction of truffles with host plants is still at an early stage. Early signals between host and truffle have only been investigated recently. The genes involved in such interactions are also under investigation. The molecular and/or environmental signals triggering fruitbody formation are still completely obscure owing to the long and complex life cycle of truffles.

If truffles are a good model in which to study the interactions between ectomycorrhizal ascomycetes fungi and their host, they also offer an interesting perspective to understand the interactions with nonhost plants. Indeed bioassays with nonhost plants evidenced that truffle metabolites interact with root elongation. Experiments with soil collected from truffle fields also suggest the presence of secondary metabolites interacting with plants roots. Nevertheless, the occurrence of these metabolites and their origin in nature is not yet known. Finally, as the burnt is not reproducible in the laboratory, further investigation should be done in vivo in order

to quantify the contribution of secondary metabolites to the scarcity of the vegetal cover observed inside it, and to shed a little more light on these delicious, yet mysterious fungi!

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