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REVIEW

Impact of the competition between mating types on the cultivation of *Tuber melanosporum*: *Romeo and Juliet* and the matter of space and time

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Abstract Major breakthroughs in our understanding of the life cycles of the symbiotic ascomycetes belonging to the genus *Tuber* have occurred over the last several years. A number of *Tuber* species produce edible fruiting bodies, known as truffles, that are marketed worldwide. A better understanding of the basic biological characteristics of *Tuber* spp. is likely to have tremendous practical relevance for their cultivation. *Tuber melanosporum* produces the most valuable black truffles and its genome has been recently sequenced. This species is now serving as a model for studying the biology of truffles. Here, we review recent progress in the understanding of sexual reproduction modalities in *T. melanosporum*. The practical relevance of these findings is outlined. In particular, the discoveries that *T. melanosporum* is heterothallic and that strains of different mating types compete to persist on the roots of host plants suggest that the spatial and temporal distributional patterns of strains of different mating types are key determinants of truffle fructification. The spatial segregation of the two mating types in areas where *T. melanosporum* occurs likely limits truffle production. Thus, host plant inoculation techniques and agronomic practices that might be pursued to manage *T. melanosporum* orchards with a balanced presence of the two mating partners are described.

Keywords Ectomycorrhizas · Mating type competition · Truffle cultivation · *Tuber melanosporum* · *MAT* genes

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Introduction

The reproductive strategies of symbiotic ascomycetes belonging to the genus *Tuber* continue to puzzle mycologists, truffle growers, and hunters. Due to their texture and unique scent, the fruiting bodies produced by some of these species, known as truffles, have been highly appreciated and prized since antiquity (C. Plinii Secundi 79). Many *Tuber* species of economic interest, such as *Tuber melanosporum*, *Tuber aestivum*, and *Tuber borchii*, can now be successfully cultivated within and outside their natural distributional ranges (Chevalier and Frochot 1997; Bonet et al. 2009). Nevertheless, at both artificial and natural sites, truffle production remains de facto insufficient to meet the rising market demand for these highly praised and priced delicacies (Hall et al. 2003; Büntgen et al. 2011, 2012). How these ascomycetes propagate is a fundamental question with repercussions of high economical relevance for their cultivation and harvesting. It is therefore mandatory to glean more insight into the genetic and environmental factors underpinning the vegetative and sexual propagation patterns of truffle mycelia. In fact, for quite some time, the subterranean life cycle and limited saprotrophic ability of the mycelia of these species have hampered scientists' ability to gain further insight into the biology of truffles. Moreover, research on mycorrhizal ascomycetes lags behind that on basidiomycetes (Egger 2006), and truffle researchers cannot benefit from the existence of phylogenetically related model species. Indeed, among the edible mycorrhizal fungi with high economic value, *Tuber* species are the only ascomycetes, and all of the other species are basidiomycetes (e.g., *Cantharellus* spp., *Boletus* spp., and *Tricholoma matsutake*) (Yun and Hall 2004). Over the last 15–20 years, the advent of analyses based on molecular markers, coupled with technical progress in the isolation and fingerprinting of the various structures these fungi develop throughout their complex life cycle, has

generated evidence on the biology of truffles. Most importantly, sequencing of the genome of *T. melanosporum* resulted in a significant leap forward in comprehending the life cycles and reproductive modes of *Tuber* species.

Here, we outline the steps that have led our group to address the issue of the genetic control of sexual reproduction in *T. melanosporum*. We conclude by discussing the pragmatic implications of these findings for truffle cultivation.

The central question: do truffles require a mating partner?

Whether truffles require a mating partner to fruit has been a longstanding matter of dispute among mycologists. The reproduction mode of fungi is governed by the *MAT* locus (Fraser and Heitman 2003). According to their mating strategies, filamentous ascomycetes can be sorted into two main categories: homothallic and heterothallic. All known heterothallic ascomycetes exhibit a single *MAT* locus, with two alternative genes, *MAT1-1-1* and *MAT1-2-1*, being found in different strains (Metzenberg and Glass 1990; Debuchy et al. 2010). These two genes encode transcription factors with an alpha (MAT1-1-1) and a HMG (MAT1-2-1) domain. In these fungi, same-clone mating is prevented because only haploid cells that carry different alleles at the mating type locus can fuse (Murtagh et al. 2000; Kronstad 2007; Paoletti et al. 2007; Billiard et al. 2012). Thus, haploid heterothallic fungi require a partner with an opposite mating type to complete their life cycle. Conversely, in homothallic fungi, syngamy can occur between genetically identical haploid cells. True homothallic taxa harbor either only one *MAT* gene (Wik et al. 2008) or, more commonly, both *MAT* genes at the same haploid nucleus; these two genes can be either linked or not linked (Butler 2007). Thus, homothallic fungi do not exhibit any genetic impediment to either of these two reproductive modes but can opt to outcross or self-fertilize according to environmental conditions.

The impossibility of mating truffle mycelia under controlled conditions has precluded the investigation of their mating strategies through direct approaches. Furthermore, the poor conservation of *MAT* genes across fungi has frustrated attempts to isolate these genes through homologous cloning (Rubini et al. 2007). Hence, an indirect approach based on co-dominant molecular markers has been employed. These markers allow the unequivocal distinction of homozygous and heterozygous genotypes (Piepho and Koch 2000). When co-dominant simple sequence repeat (SSR) markers were used to assess the presence of different alleles at several microsatellite loci in DNA isolated by grinding the gleba and the spores of *T. melanosporum* truffles together, the absence of any pattern of heterozygosity was interpreted as a clear marker of homothallism or even exclusive selfing (Bertault et al. 1998, 2001). Likewise, no apparent heterozygous genotypes were detected in *Tuber magnatum* fruiting bodies screened using SSR markers (Rubini et al. 2004). However, when the *T. magnatum* SSR data were subjected to population genetics analyses, the occurrence of extensive exchange of SSR alleles between individuals from the same or even geographically close populations clearly emerged (Rubini et al. 2005). Because gene flow among individuals is a marker of outcrossing, the *T. magnatum* population genetics data set the stage to reconsider the life cycles and reproductive strategies of truffle species. Experiments were performed to test the hypothesis that the lack of heterozygous patterns observed in *T. magnatum* stemmed from a methodological bias due to the difficulty of disrupting the spores of this species and analyzing the DNA contributed by these structures. Under this scenario, the DNA recovered from truffle ascocarps is contributed mainly, if not exclusively, by the gleba, which is made up of haploid hyphae of a uniparental origin. Specific procedures to disrupt these spores to isolate their DNA were therefore needed. Hence, a new strategy designed to achieve differential recovery and analyses of DNA from pools of spores versus DNA from the surrounding gleba within single *T. magnatum* ascocarps was developed. The availability of this tool turned prior suspicion into factual evidence: a methodological bias was inherent in the approaches applied previously; i.e., in some truffles, the DNA from pools of spores displayed additional alleles at several SSR loci with respect to those exhibited by the gleba. Conversely, regardless of the allelic configuration displayed by the spores, the gleba always presented a single allele per SSR locus (Paolocci et al. 2006). Likewise, some, but not all, *T. melanosporum* truffles exhibited these dual SSR patterns when the asci were genotyped separately from the corresponding gleba (Riccioni et al. 2008). These dual SSR patterns found in individual *T. melanosporum* and *T. magnatum* truffle samples led to the following conclusions: (1) the gleba of truffles is formed by homokaryotic hyphae, (2) the additional alleles displayed by the spores must be contributed by a sexual partner, and (3) the gleba of truffles is formed by haploid hyphae contributed by only one of the two sexual partners. While these studies demonstrated the uniparental origin of the hyphae that form the gleba of truffles, they did not provide definitive clues regarding the mating systems present in these species. Indeed, most of the *T. melanosporum* and *T. magnatum* ascocarps screened did not exhibit clear markers of non-selfing. At this stage, the hypothesis that truffles are obligatory outcrossing species remained far from being settled. The mating system question was finally solved when the sequencing of the *T. melanosporum* haploid genome was completed, and the first *MAT* genes for a truffle species were identified (Martin et al. 2010). The gleba of all truffles investigated showed either the *MAT1-2-1* or *MAT1-1-1* gene, according to PCR screening based on primer pairs designed for both *MAT* genes. In the corresponding asci, spores carrying

either the *MAT1-1-1* or *MAT1-2-1* gene were found. Therefore, it was concluded that the black truffle is a heterothallic fungus (Rubini et al. 2011a). The cloning of the two *MAT* genes in *T. melanosporum* was a seminal step in identifying their orthologs in other truffle species of economic interest through homologous cloning and demonstrating that are all heterothallic as well (Martin et al. 2012; Belfiori et al. 2013). In summary, truffles are obligatory outcrossing organisms. As such, mating between strains of different mating types is their unique mode of sexual reproduction (Fig. 1).

Heterothallism in truffles: practical repercussions

The first attempts at truffle cultivation date back to the sixteenth century (Ciccarelli 1564). However, the first host plant inoculation with a truffle species under controlled conditions was realized only in 1967 (Fassi and Fontana 1967). The inoculation of host plants with *Tuber* spp. via spore suspension has been an established nursery practice for decades, except for *T. magnatum* (Rubini et al. 2001). This procedure has propelled the implementation of plantations for the cultivation of the most profitable truffle species. In Europe, truffle plantations have been extensively established since the 1970s, despite the fact that basic knowledge on truffle biology at that time was still in its infancy (Chevalier and Dupré 1988).

Many biological and environmental factors interact to yield a productive or non-productive truffle orchard. The paucity and inconsistency of truffle production at many cultivated sites have been ascribed to poor quality of the plant material involved, both in terms of the mycorrhization level and/or plant robustness/fitness; to suboptimal soil and environmental conditions, i.e., water deficiency; or to a combination of both factors. However, due in part to the assumption of selfing (Bertault et al. 1998), the importance of truffle sexuality has been completely overlooked, and each individual host plant has been regarded as a self-sufficient production site.

Currently, the management recommendations made by truffle researchers to truffle growers have revolved around criteria for selecting suitable cultivation sites and agronomic practices to achieve or maintain certain standards of ecological parameters in truffle stands, i.e., maintaining an optimal soil pH, structure, and humidity for fungal species growth. Concerning the quality of plant material, in addition to features such as height, root neck diameter, and the absence of damage, pathogens, and deformation, growers have mainly

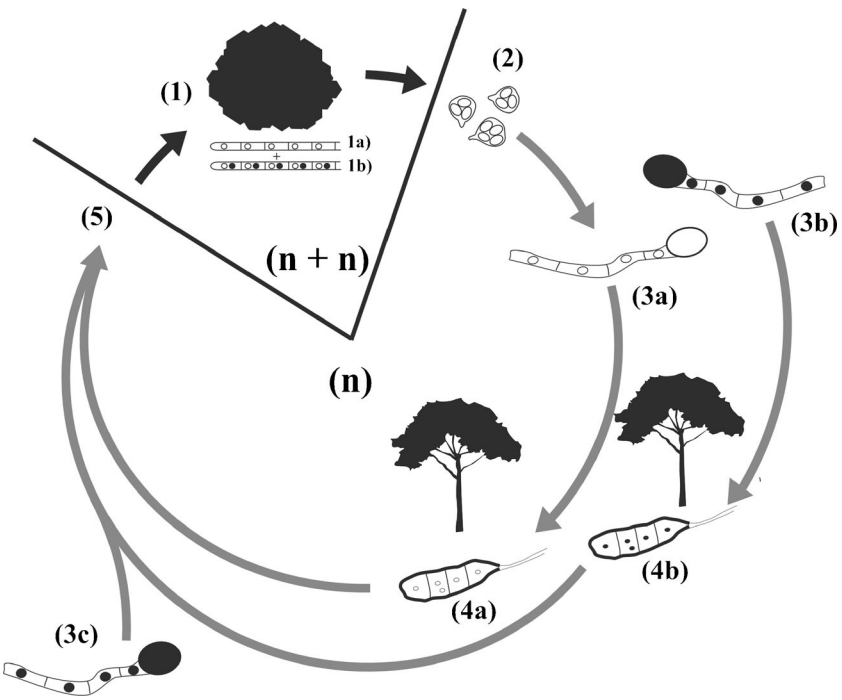

Fig. 1 The *T. melanosporum* life cycle. The haploid (*n*) phase (*gray arrows*) of the life cycle is predominant, while the dikaryotic (*n*+*n*) phase is limited (*black arrows*). The nascent fruiting body (*1*) is composed of uniparental maternal hyphae (*1a*) and dikaryotic hyphae (*1b*) derived through fertilization. Dikaryotic hyphae differentiate the ascus mother cells where karyogamy and meiosis take place, resulting in the formation of mature asci containing haploid ascospores (*2*). At maturity, the fruit body is composed of gleba of maternal origin and asci enveloping a variable number of plurinucleate ascospores. The ascospores germinate in the soil, producing primary mycelia (*3a*, *3b*) that colonize host plant roots, forming ECMs (*4a* and *4b*). Host plants can sustain genetically different *T. melanosporum* strains on their roots, provided that all of the strains share the same mating type. The haploid hyphae emanating from ectomycorrhizas (*4a*) are fertilized by hyphae of the opposite mating type (*5*) and behave as the maternal partner in the cross. The male partner is of uncertain origin: it may be a mycelium developing from ectomycorrhizas of a neighboring tree (*4b*) or a free-living mycelium derived from a germinating ascospore (*3c*)

been concerned with achieving sufficient levels of mycorrhization by the desired truffle species and the absence of other "contaminating" mycorrhizal fungal species on the outplanted host. Hence, growers from many countries have been asking for a means of checking the quality, via morphological or morphological/molecular-assisted analyses, of the plant material sold by nurseries to determine whether the percentage of truffle and truffle-competing mycorrhizal fungi in a given lot of plants passes arbitrarily set threshold levels. However, because of heterothallism, the additional aspect of the distribution of the two mating partners, both on single plants and at cultivation sites, has emerged as a new, critical aspect to be considered. For truffle fruiting to occur, the two sexual partners have to sense each other and synchronize their sexual reproductive programs to mate. Consequently, fruiting is clearly a critical step in the life cycle of truffles, even when the two mating partners share the same host.

The distributional patterns of *T. melanosporum* strains on their hosts are likely the result of competition between different mating types

Preliminary studies on truffle ectomycorrhizas (ECMs) carried out using SSR markers have shown that regardless of the species involved, *Tuber* spp. ECMs are formed by haploid hyphae (Paolocci et al. 2006). Notably, the ECMs harvested from a given root branch are genetically identical, suggesting that host root colonization is achieved through the spreading of a single haploid mycelium and that mycelia from different strains do not interfere with each other in this process. Cell surface factors likely act at the pre-fusion level, preventing the formation of anastomosis between strains to maintain the genetic integrity of each strain during vegetative growth (Iotti et al. 2012).

Given these findings and because of heterothallism, recent studies on *T. melanosporum* have tested whether the distributional pattern of ECMs with different mating types is a variable affecting and limiting truffle production. First, the distribution of the two mating types was investigated on ECMs from a natural *T. melanosporum* plantation. Surprisingly, this screen revealed the presence of ECMs showing identical mating types and SSR profiles underneath individual host plants. Plants harboring either *MAT1-2-1* or *MAT1-1-1* ECMs were productive, and the collected fruiting bodies exhibited gleba (the maternal tissue of the ascocarp) with identical SSR and *MAT* profiles to the ECMs collected nearby. Because the spores from the same ascocarps could conversely display additional SSR alleles with respect to those found in the gleba and these additional alleles have not been detected in any of the ECMs sampled in orchards, it can be concluded that (a) ectomycorrhizal strains behave as the maternal partner in the mating process, (b) *T. melanosporum MAT1-1* and *MAT1-2* strains are equally competent in forming male and female reproductive structures, and (c) the male partner can be "erratic" (Rubini et al. 2011b). This preliminary screen essentially documented the presence of a single strain per investigated plant in a natural truffle-growing area. However, the possibility remained that these results suffered from a sampling bias due to the impossibility of recovering and genotyping all of the ECMs underneath the plants. To circumvent this problem, a *MAT*-based analysis was employed to trace the mating types of ECMs obtained from seedlings inoculated under controlled conditions, according to the procedures routinely followed in nurseries. These analyses demonstrated that segregation between strains of opposite mating types takes place secondarily. In each nursery-inoculated host plant, ECMs of both mating types are initially present, but 12–18 months after seedling inoculation, a single mating type can dominate and/or replace the other mating type (Rubini et al. 2011b). Segregation of the two mating types was later confirmed in studies conducted in *T. melanoporum* plantations in Australia and Europe. In Australia, in relatively young black truffle stands, Linde and Selmes (2012) noted that approximately half of the plants investigated sustained ECMs of a single mating type, with a skewed representation of the two mating types being found at the sites. In two older plantations (approximately 25 years old) in France and in Italy, Murat et al. (2013) performed a capillary analysis of single ECMs collected around productive host plants and revealed the presence of ECMs with different SSR profiles on different root branches of most of the investigated plants. Significantly, however, all of the ECMs underneath an individual plant showed the same mating type. These data lend strong support to the contention that in open field conditions, host plants tend to sustain either a single mycorrhizal strain or multiple strains, provided that they all share the same mating type (Fig. 1). As a consequence of this spatial segregation, the distribution of the two mating types in truffle fields is patchy and often unbalanced.

At first glance, this mating type-dependent competition appears to be a paradox: truffle strains of different mating types compete for persistence on the same host plant, but they still need each other to complete their life cycle. In comparison with asexual reproduction, sexual reproduction represents a costly propagation process for all species, in terms of both the energy spent to find a partner and the genetic load incurred, i.e., the exchange of parasitic genetic elements and transmission of diseases (Otto and Lenormand 2002). It has been argued that the spatial segregation of *T. melanosporum* strains according to their mating types could act to decrease the probability that sexually compatible partners will meet as a strategy to lower the odds of mating (Selosse et al. 2013). Under this hypothesis, the *MAT* locus of *T. melanosporum* most likely acts together with other polymorphic loci in self/non-self recognition phenomena. Genetic non-self recognition systems, such as vegetative incompatibility, operate in

many filamentous fungi to regulate hyphal fusion between genetically dissimilar individuals (Choi et al. 2012). Vegetative incompatibility is thought to protect resources within hyphae from exploitation by non-kin individuals (Debets and Griffiths 1998). Indeed, in *Neurospora crassa*, *Sordaria brevicollis*, and *Ascobolus stercorarius*, the *MAT* locus is one of the loci controlling vegetative incompatibility (Glass et al. 2000). In *T. melanosporum*, commitment of the *MAT* locus in the vegetative incompatibility phenomenon is a hypothesis that awaits experimental verification. Although the genetic bases of the competition between strains with different mating types are not yet understood, recent findings show that this competition results in limitation in the spatial distribution of sexually compatible *T. melanosporum* strains. This limitation likely represents more than just a possible bottleneck of truffle fructification.

Juliet, where is your Romeo?

With "Tartuffe," Molière (1664) elevated the underground, muddy clod that is the truffle to the rank of literature. Indeed, recent genetic findings in black truffle harken to another eternal piece of theater, "Romeo and Juliet" (Shakespeare 1597). This tragedy metaphorically recapitulates the vegetative and reproductive tactics of this fungus.

During the mating process, the fungal strains on root tips act as maternal partners because ECMs are responsible for allocating plant nutrients to feed the developing fruiting bodies (Zeller et al. 2008). Because of mating type-dependent spatial segregation, the male *T. melanosporum* partner, "Romeo," must migrate to find its "Juliet," i.e., a root-colonizing strain of the opposite mating type. Alternatively or concomitantly, "Juliet" could spread into the soil to come into close proximity to "Romeo." But how far can a root-colonizing strain grow to find its partner(s)? And how relevant is "timing" in this process?

The distributional range of black truffle genets on roots is quite limited. Murat et al. (2013) showed that the maximum genet sizes in two orchards were less than 5 m, with many genets being limited to a single root sample. However, little information is available regarding the spatial and temporal distributional patterns of the hyphae that depart from these mycorrhizas to explore the surrounding soils in search of water, nutrients, and, of course, a male partner. However, in most cases, black truffles are harvested within the burnt area, or brulé, around the plant trunk, so it is conceivable that strains that colonize host plants do not or cannot explore the soil beyond a certain distance from the roots. Consistent with this view and with the migration of the male partner, black truffles collected around the same plant share the same maternal partner, but not necessarily the same male partner (Riccioni et al. 2008; Rubini et al. 2011b; Murat et al. 2013). Thus, the quintessential question of truffle sexuality concerns the origin, distribution, and persistence in the soil of the "Romeo" partner(s). Sampling soil cores from orchards are of paramount importance to answer this question. Our inability to detect multilocus genetic profiles of fungal samples from the soil without isolating and growing them in vitro has limited such analyses to evaluating a single locus at a time (i.e., *MAT* or ITS) (Zampieri et al. 2010, 2012; Parladé et al. 2013). With respect to the mating types, the truffle mycelia in soil samples around productive plants display the same mating type as the nearby ECMs or, more rarely, both mating types (Rubini et al. 2011b; Zampieri et al. 2012; Murat et al. 2013). A more in-depth analysis is therefore needed to explore the spatial and temporal dynamics and the identity of the mycelia or of other fungal structures sampled within soil cores. Those strains that show a mating type opposite to the type present on the plants are of particular interest because they represent potential sexual partners. The prevailing hypothesis is that for truffles harvested in winter, fertilization occurs early in spring, and the nascent truffle remains quiescent or develops very slowly until fall (Sourzat 1997). According to this view, the presence of the male partner(s) next to the ECMs of a given host appears to be critical in spring. Extensive analyses of strain biodiversity both within and around the brulé of productive and unproductive hosts across seasons will help us to model the quantitative and qualitative fluctuations of "Romeo" strains in the soil. Doing so will also support our hypothesis that gamete limitation occurs more frequently, in terms of both the local distribution and/or seasonality, in unproductive compared to productive sites.

However, a basic question remains: what is the source of truffle DNA in soil samples? Resolving this issue warrants a deeper understanding of the fertilization steps in the truffle life cycle. DNA is likely not contributed by intact spores present in the soil due to the difficulties of breaking these structures. It is therefore conceivable that this DNA is provided by mycelia that spread from the ECMs on surrounding plants and/or by mycelia originating from the soil spore bank. It is noteworthy that in addition to generating differentiated or undifferentiated hyphae, several ascomycetes can produce passively mobile cells known as microconidia or spermatia, which act as male fertilizers (Selosse et al. 2013). However, the occurrence of these structures has been reported in various *Tuber* species, but not in *T. melanosporum* (Urban et al. 2004; Healy et al. 2013). Unfortunately, the available information on the sexual structures that develop in *Tuber* species remains insufficient because reports of ascogonia are not common (Callot 1999). Thus, the fungal structures involved in the fertilization step remain a major mystery of the truffle life cycle.

Perspectives for black truffle production

How can we encourage the mating of sexual partners if genetic forces operate to split them apart on their hosts? Intriguingly, long before the life cycle and mating strategies

of truffles were revealed, truffle hunters and growers were experimentally inoculating soil around host plants with mature ascocarps in an effort to enhance truffle fructification. However, the inoculation of soil with spores can be a time-consuming, expensive, and risky practice in relation to truffle production. Mycelia originating from these spores likely exhibit a limited temporal window for interacting with mycelia from ECMs of the opposite mating type at a site. As the genome of *T. melanosporum* presents a restricted repertoire of CAzymes for degrading plant cell wall polysaccharides, strains of this species are not expected to survive in the soil for a long period (Martin et al. 2010). In keeping with this in silico prediction, the monitoring of strain dynamics in plants grown "in-pot" has shown that once a given *T. melanosporum* strain is displaced from the roots, its persistence in the soil is seriously jeopardized (Rubini et al. 2011b). Consequently, the ability of a given mycelium originating from the germination of a spore to develop sexual structures could be very limited temporally.

Thus, the fructification step in *T. melanosporum* requires strains of different mating types that have to be not only in the same place but also sexually compatible *at the same time*.

Under these scenarios, it can be inferred that the chances of mating are higher between strains of opposite mating types that colonize two host plants close to each other than between a strain colonizing a root tip and a mating partner that originates through the germination of a spore/cell in the nearby soil. Indeed, root-colonizing strains of both mating types could allocate resources provided by their hosts to develop sexual structures more than once to favor their reciprocal mating. Currently, despite the limitation mentioned above, direct spore dissemination and/or agronomic practices such as soil tillage or pasturing of animals that might favor the dispersion of spores or other fertilizing cells at the site remain the most feasible and simple strategies for alleviating gamete limitation in natural and cultivated *T. melanosporum* grounds. However, the patchy and unbalanced distribution of the two mating types in truffle stands could be counterbalanced more efficiently and permanently by outplanting host plants harboring ECMs with the limiting mating type in each soil patch.

The balance of the two mating types is also of relevance when new truffle stands are established. In theory, plants harboring *MAT1-2-1* or *MAT1-1-1* strains should be numerically and spatially equally distributed at a site to facilitate reciprocal mating of the fungal partners. To achieve balance of the mating types, host plants should be checked to determine the mating types of their ECMs prior to outplanting. Unfortunately, it is not possible to predict which of the two mating types outcompetes the other on plants inoculated with spores, and screening individual plants to identify the mating types of their symbiotic strain(s) is not feasible due to the cost. Thus, alternative strategies should be pursued to produce plants with known and certified mating types on their roots (Martin et al. 2012). The use of plants inoculated with either *MAT1-2* or *MAT1-1* in vitro cultivated mycelial strains represents the best approach. Preliminary experiments carried out at Consiglio Nazionale delle Ricerche Istituto di Bioscienze e Biorisorse (CNR IBBR) in Perugia have shown that this host inoculation practice is technically feasible for *T. melanosporum* mycelia of both mating types (Rubini et al., unpublished results). We plan to use plants subjected to this procedure to establish experimental fields in which the hosts harboring *MAT1-1-1* or *MAT1-2-1* strains will be differently spaced so that we can trace the spatial and temporal propagation patterns of mycelia of both mating types at the sites and retrieve fundamental information on environmental factors that contribute to shaping these patterns. The long-term goal is to spatially distribute the two mating types to limit the number and extent of unproductive areas in truffle stands.

Nurseries use spores as inocula for the production of mycorrhizal plants for truffle cultivation. They generally employ small or low-quality truffles as spore donors, while the best fruiting bodies are sold in the market. If traits of economic relevance, such as the aroma and size of truffles, are under genetic control, then the propagation of strains of minor value is encouraged (Selosse et al. 2013). In light of heterothallism and the life cycle described above, there are a few additional considerations concerning the use of spores as inocula: (a) the strains originating through the germination of meiospores can exhibit genetic traits that are different from each other and from the two parental strains due to the recombination and chromosome segregation that can take place between the two parental genomes at meiosis, and (b) because the origin of the male partner that fertilizes the root-colonizing strain is unknown, the traits conferred by this mating partner are also unknown. In summary, the use of spores for the inoculation of the host plants might not guarantee the propagation of strains with superior traits, even when the spores come from truffles of the highest quality. As an alternative to spores, mycelia grown in vitro can be used as inocula. These mycelia can be isolated from mycorrhizas adapted to specific microhabitats and/or from fruiting bodies with a superior quality/size to encourage the propagation of strains that retain useful traits. In particular, if truffle characteristics of economic relevance, such as size and aroma, are primarily determined by the gleba, which is the most abundant tissue in a fruiting body, then isolation of mycelia from the gleba of large-sized, high-quality truffles would permit the propagation of strains of superior value. Moreover, many plants colonized by the same fungal strain can be produced using in vitro grown mycelia. However, caution is needed when using plants inoculated by this approach in large-scale cultivation programs because there is a risk of propagating only a few fungal genotypes due to the difficulty of isolating and growing truffle strains in vitro. Therefore, the long-term feasibility of using plants inoculated with in vitro grown mycelia for large-scale truffle cultivation

programs will greatly depend on developing protocols and media that guarantee the isolation of mycelia from many ECMs or fruiting bodies as possible, along with the rapid growth of *T. melanosporum* mycelia in vitro. Protocols for exploiting the saprotrophic ability of truffle mycelia while minimizing the insurgence of genetic mutations during in vitro cultivation are also needed. Indeed, various transposable elements (TEs) present in plants, yeast, and *Drosophila* can be activated under stressful conditions (Feschotte et al. 2002; Daboussi and Capy 2003), including abiotic stresses (e.g., temperature, irradiation, oxidation) or biotic stresses (e.g., tissue culture, isolation of protoplasts, or pathogen infection) (Potter et al. 1979; Arnault and Dufournel 1994; Hirochika et al. 1996; Sacerdot et al. 2005; Sehgal et al. 2007; Rakocevic et al. 2009). By jumping from one locus to another, TEs can induce a wide array of mutations (Casacuberta and González 2013). How truffle genomes respond to in vitro cultivation has yet to be addressed. However, as the genome of *T. melanosporum* is the available fungal genome showing the highest density of TEs (Martin et al. 2010), maintaining the genome integrity of this species is mandatory. This is important not only for maintaining the capacity of a given fungal strain to form viable mycorrhizas and enter into the sexual phase but also for preserving the genetic traits determining the production of flavor and/or the size of the ascocarps. We are currently maintaining a collection of in vitro grown black truffle mycelia at CNR IBBR, with the goal of preserving the biodiversity of this species and propagating strains with the aim of retaining favorable genetic traits, e.g., the flavor and size of the fruiting bodies and the adaptability of the fungus to different environments. Maintaining viable strains of different provenances for all *Tuber* spp. of economic interest will be important to potentially enhance quality standards for future truffle production and preserve truffle biodiversity. When introduced into areas where truffles grow spontaneously, strains with unknown origins and traits could represent a serious threat to local strains. Whenever possible, regardless of the protocols that are followed, local strains should be employed as inocula. Indeed, large-scale population genetics analyses carried out in *T. melanosporum* have recently shown that there is a high rate of genetic diversity among fruiting bodies of different provenances (Murat et al. 2004, 2011; Riccioni et al. 2008). The results of these studies contrast with the contention that *T. melanosporum* is a species with minimal genetic diversity over its geographical range (Bertault et al. 1998). Furthermore, they allow us to predict that genetic and environmental influences together explain the differences in several traits, including the famous organoleptic properties of the black truffle, observed across its geographical range. The sequencing of the *T. melanosporum* genome has recently provided mycologists with a plethora of markers (Murat et al. 2011). These markers will help us to test the possibility of typing truffle populations according to their provenance as a means of tracking the origins of inocula and encouraging the introduction of native strains at cultivated sites whenever possible.

How do we gain insight into the biotic and abiotic factors that determine the entry of *T. melanosporum* into the sexual reproduction program?

As outlined in the previous paragraph, the co-occurrence of opposite mating types in close proximity in the environment is a prerequisite for syngamy to occur. However, both mating types must be able to sense each other and synchronize their life cycle. The specific biotic and abiotic factors that trigger the sexual pathway for both mating partners remain to be identified. In ascomycetes, complementary pheromones and receptors allow the mating program to be switched on when conspecific haploid cells are ready to enter into the sexual phase (Perrin 2012). The majority of genes controlling sexual reproduction in yeasts and other filamentous ascomycetes are indeed conserved in the *T. melanosporum* genome (Martin et al. 2010; Rubini et al. 2011b). The in silico identification of these genes suggests that a pheromone pathway operates in *T. melanosporum* (Rubini et al. 2012). The availability of powerful tools such as whole-genome custom oligoarrays and RNA-seq-based methods has opened new investigative avenues for shedding light on the genetic and environmental factors that are important for the entry of truffle species into the sexual phase. For example, through an oligoarray analysis, Zampieri et al. (2011) recently showed that the cold stress response in *T. melanosporum* mycelia involves extensive transcriptomic changes and suggested that a cold period is necessary to initiate the development of truffle ascomata. These new tools, together with the availability of *T. melanosporum* isolates of different mating types, will help us to monitor how and to what extent different environmental factors, such as cold, light, and nutrient starvation, affect the transcription of *MAT* genes as well as that of downstream genes in the mating and pheromone signaling cascades.

The soil microbial ecology in the rhizosphere and within the brulé might also play a role in governing the transition from the vegetative to the sexual phase in truffles. The soil microbial community might favor or interfere with soil exploration and nutrient uptake by both mating partners. Interestingly, bacteria and mycophagists are both thought to play a critical role in softening the cell walls of truffle spores in the soil and thus promoting their germination (Barbieri et al. 2007). Additionally, bacteria, yeasts, and other fungi found in fruiting bodies may play a critical role in the development of truffle primordia, as they emit volatile compounds that could help the nascent fruiting bodies to interact with the surrounding environment and attract animal vectors for spore

dispersal upon maturation (Buzzini et al. 2005; Pacioni et al. 2007; Antony-Babu et al. 2013).

Integrating findings from disciplines ranging from genomics to molecular ecology and field ecology will allow us to uncover the environmental factors that allow the mating type program to be switched on in these symbiotic fungi.

Concluding remarks

The last several years have witnessed a major breakthrough in our understanding of the life cycle of truffles. The identification of heterothallism in *Tuber* spp. and the mating type-dependent spatial segregation of *T. melanosporum* strains on their hosts represents a tremendous, fundamental advancement in our understanding of how to ultimately improve truffle cultivation. However, additional research will be needed to trace the population dynamics of truffle strains in the field. Our recent findings suggest that gamete limitation can affect the production of black truffle stands, and we should therefore develop inoculation techniques and agronomic practices to counteract this phenomenon and, thus, potentially improve truffle productivity. Finally, recent progress on the vegetative and sexual propagation patterns of *T. melanosporum* is paving the way to conduct similar studies in other truffle species. In particular, it would be conceptually and pragmatically significant to determine whether the competition phenomenon observed between *T. melanosporum* strains of different mating types also occurs and plays a role in orchestrating the distributional patterns of not only other truffle species but other heterothallic symbiotic ascomycetes as well.

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