Advances in molecular genetics have increased knowledge of Tuber species' life cycle and population genetic structure, indicating ways to improve yield

Advances in molecular genetics have increased knowledge of *Tuber* species' life cycle and population genetic structure, indicating ways to improve yield

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Abstract

Truffles are possibly the only high-value cultivated organisms for which some aspects of the habit and life cycle have only recently been elucidated or remain unknown. Molecular techniques have helped explain the biological basis for some traditional empirical management techniques, such as inoculating soil with ascospores to improve yield, and have enhanced the detection of competitive or pathogenic soil microorganisms. Improved precision of assessment of the quality of inoculated seedlings is now possible. New knowledge of the genetic structure of populations has indicated that as trees age, the genotypes of mycorrhizae on inoculated trees change, and that there are large differences in the number of female and male genotypes participating in ascocarp formation. The plasticity of *Tuber* species has also been revealed, with maternal genotypes growing as an ectomycorrhiza in host tree roots and as surface mycelium or an endophyte in roots of adjacent non-mycorrhizal species. Refinement of management techniques has resulted from applying the new information, and the tools are now available to resolve the many outstanding gaps in our knowledge of *Tuber* biology.

Keywords: Truffle cultivation, Ectomycorrhizal fungi, Mycorrhizal helper bacteria, Truffle traps, Root inoculation

Introduction

Truffles are underground fruiting bodies of Ascomycota that form ectomycorrhiza (ECM) with the roots of their host plants (Murat et al. 2005). There are 20,000 ectomycorrhizal species (Zambonelli and Bonito 2012), and commercial edible truffles belong to the genera *Tuber* (the true truffles) and *Terfezia* (the desert truffles). Truffles have a long history of human consumption. They were eaten by Romans, Etruscans, Greeks, Egyptians and Babylonians, while in the Middle Ages, there is a record of them being served at the wedding feast of Charles VI of France in 1385 (Mustafa et al. 2020).

The first record of cultivation of a mycorrhizal fungus was early in the 19th century (Hall et al. 2009). In 1800, Joseph Talon discovered that oak seedlings growing under trees that produced Périgord black truffles (*T. melanosporum*) could be transplanted, and trees would yield truffles after 5-10 years (Chevalier and Frochot 1997; Dupont et al. 2017; Zambonelli et al. 2015). Then, in 1847, Auguste Rousseau of Carpentras, Vaucluse successfully introduced Périgord black truffle to a *Quercus ilex* plantation, but exactly how he inoculated the trees is not known (Dupont et al. 2017; Zambonelli and Bonito 2012). The development of modern culture techniques was initiated by Palenzona (1969), who successfully inoculated *Corylus avellana* with *T. brumale*, *T. aestivum* and *T. melanosporum*. Clean seedlings were inoculated with spores and raised in a shade house or greenhouse for a few months before planting in the field, and this method is still used today (Zambonelli et al. 2017). Trees inoculated in this way may produce truffles usually 5 –7 years after inoculation, but the time may range from 3 –10 years (Bonet et al. 2006; Zampieri et al. 2012), with yield slowly rising to a maximum after 15–20 years (Eslick 2017). Truffle plantations, in addition to yielding valuable truffles, also offer the additional benefits that the host species may produce nuts, timber, carbon credits, promote biodiversity conservation (Therville et al. 2013; Zambonelli and Bonito 2012), and provide habitat for invertebrate and vertebrate fauna, shade and a range of other ecosystem services and functions (Kotze et al. 2022).

Knowledge of successful inoculation techniques launched modern truffle cultivation in the 1970s, firstly in countries where *Tuber* species are native (France and Italy) (Reyna and Garcia-Barreda 2014; Wang 2012), but cultivation soon spread to other European and extra-European countries with appropriate climates such as Spain, South Africa (Dullstroom and Western Cape), Chile (Panguipulli, Duao, Chufquen, Quepe and Traiguén) and Argentina (Lobería) (Hall et al. 2017). In New Zealand (NZ) and Australia, the first truffle orchards were established in the mid-1980s; the first harvests were *T. melanosporum* from Gisborne NZ in 1993 and Tasmania in 1999. The primary hosts utilised were initially *C. avellana* and *Quercus robur*, but these days, a combination of *Quercus suber* and *Q. ilex* is usual. The most commonly cultivated truffle species is *T. melanosporum*, but the industry in several countries has recently been expanded to include species such as *T. aestivum* and *T. borchii* (Hall et al. 2017), while *T. himalayense* and *T. indicum* cultivation is limited to China (Huang et al. 2021; Li et al. 2018; Lu et al. 2021). The first reports of successful ascocarp formation from cultivation of the most expensive truffle, *T. magnatum,* have recently come from France following 4.5 years of growth of inoculated seedlings of *Q. pubescens* (Bach et al. 2021).

Despite the significant value and importance of truffles as a commodity, certain crucial aspects of their life cycle remain unknown or have only recently been determined. It is inherently difficult to study an underground

organism that does not undergo sexual reproduction in vitro, and some information may not have been shared, but kept as trade secrets due to the value of the product. Molecular analysis has given information on the gametic components of ascocarps, the structure of mycelial populations in the soil and as ectomycorrhizas, and the presence and abundance of competing soil organisms. It is also valuable for the quality assurance of inoculated seedlings as the success and extent of mycorrhizal formation can be checked using not only morphological identification of the mycorrhiza (Andrés-Alpuente et al. 2014) but also molecular techniques to accurately identify the species and avoid the errors that may arise using morphology.

Environmental DNA (eDNA) sampling now provides a rapid and sensitive method of studying species present in any habitat (McColl-Gausden et al. 2023). Molecular strategies such as denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), amplified rDNA restriction analysis (ARDRA) and quantitative real-time PCR (qPCR) are now available and can identify different *Tuber* species in soil and quantify their presence at very low abundance (Iotti et al. 2012a; Napoli et al. 2010; Parladé et al. 2013). The distribution and abundance of different genotypes in the field can be analysed using simple sequence repeat (SSR), and mating types can be distinguished (Leonardi et al. 2020). The composition of the soil microbiome can be studied using metabarcoding (Mello et al. 2011; Suwannarach et al. 2021; Van Elsas and Boersma 2011), and key metabolic processes revealed using metabolomics and integrated transcriptomics (Li et al. 2023).

This review focuses on how advances in molecular genetics have increased our knowledge of the truffle life cycle, genotype distribution and abundance in truffle populations, the presence of competing soil organisms, and how this information can help improve yield.

Life Cycle of the Truffles

Habitat and asexual reproduction

It has long been known that *Tuber* species can grow symbiotically and form ectomycorrhiza on certain perennial host species such as *Quercus* spp. and *Pinus* spp. (Fischer et al. 2017). Molecular techniques have recently revealed that for several *Tuber* spp., hyphae from the mycorrhiza of the main host can also occur as loose masses on the root surface or as an endophyte in non-ectomycorrhizal species (Schneider-Maunoury et al. 2020). For example, *T. aestivum* and *T. melanosporum* mycelium were detected in the roots of species distributed in 29 families of Angiosperm (Gryndler et al. 2014; Schneider-Maunoury et al. 2018; Schneider-Maunoury et al. 2020). Asexual reproduction is largely through mycelial growth, and field observations have shown conidia (mitospores) to occur in *T. oligospermum* and *T. borchii* (Urban et al. 2004). Mitospores have been observed under in-vitro conditions of *T. japonicum* (Nakano et al. 2022), but mitospores have not been recorded for *T. melanosporum* (De la Varga et al. 2017). The distribution of *T. melanosporum* genotypes in roots and soil may thus rely on only ascospore dispersal (from sexual reproduction), and the species displays narrow spore dispersal and low heterozygosity in populations (De la Varga et al. 2017).

Sexual reproduction

Most ascomycetes are heterothallic and hermaphrodite, reproducing sexually by producing antheridia and ascogonia from haploid mycelium (Bennett and Turgeon 2016; Le Tacon et al. 2016; Pöggeler et al. 2006). However, there are rare examples of Ascomycete species showing trioecy (hermaphrodite, male and female genotypes) (Benjamin 1986; Bennett and Turgeon 2016; Leslie 1995; Pöggeler et al. 2006). Until recently, it was considered that truffle species such as *T. melanosporum* were homothallic or self-fertile (Bertault et al. 1998; Linde and Selmes 2012), but it is now known they are heterothallic, and self-fertilisation does not occur. Molecular analysis of natural and orchard populations indicates that some *Tuber* species have partially evolved from the ancestral hermaphrodite condition to trioecy (Selosse et al. 2017). For example, *T. melanosporum* is mainly dioecious (female and male gametes produced from separate genotypes) with occasional hermaphrodites (De la Varga et al. 2017). The genetic makeup of the gleba and the spores of an ascocarp enables the identification of the genotypes of the female and male partners involved. Hyphae from the mycorrhiza in the host tree contribute to the female gamete and forms the gleba. The spores are a mixture of genotypes, that of the male gamete, the female gamete, and recombinants. Once the female genotype has been determined, analysing spore tissue using simple sequence repeat (SSR) based fingerprinting enables subtraction of the female genotype to deduce the male genotype (Rubini et al. 2011a). In a *T. melanosporum* orchard in France, De la Varga et al. (2017) recorded only 1.5% hermaphrodite genotypes out of a total of 219, while in an Italian field study of *T. melanosporum,* 13.3% of genotypes were hermaphrodite (Rubini et al. 2011b). In *T. aestivum*, hermaphrodites may also be rare. Staubli et al. (2022) reported 3.2% and 3.9% hermaphrodite genotypes in two districts of southern Germany. In *T borchii,* they may be more common, as in an experimental mycelial inoculation trial using five genotypes; one was shown to be hermaphrodite (Leonardi et al. 2020).

Table 1 Data illustrating the difference in numbers of male, female and hermaphrodite genotypes and their mating type detected in late spring 2009 and winter 2009–2010 in natural *T. melanosporum* grounds under *Ostrya carpinifolia* and *Quercus pubescens* (Rubini et al. 2011a)

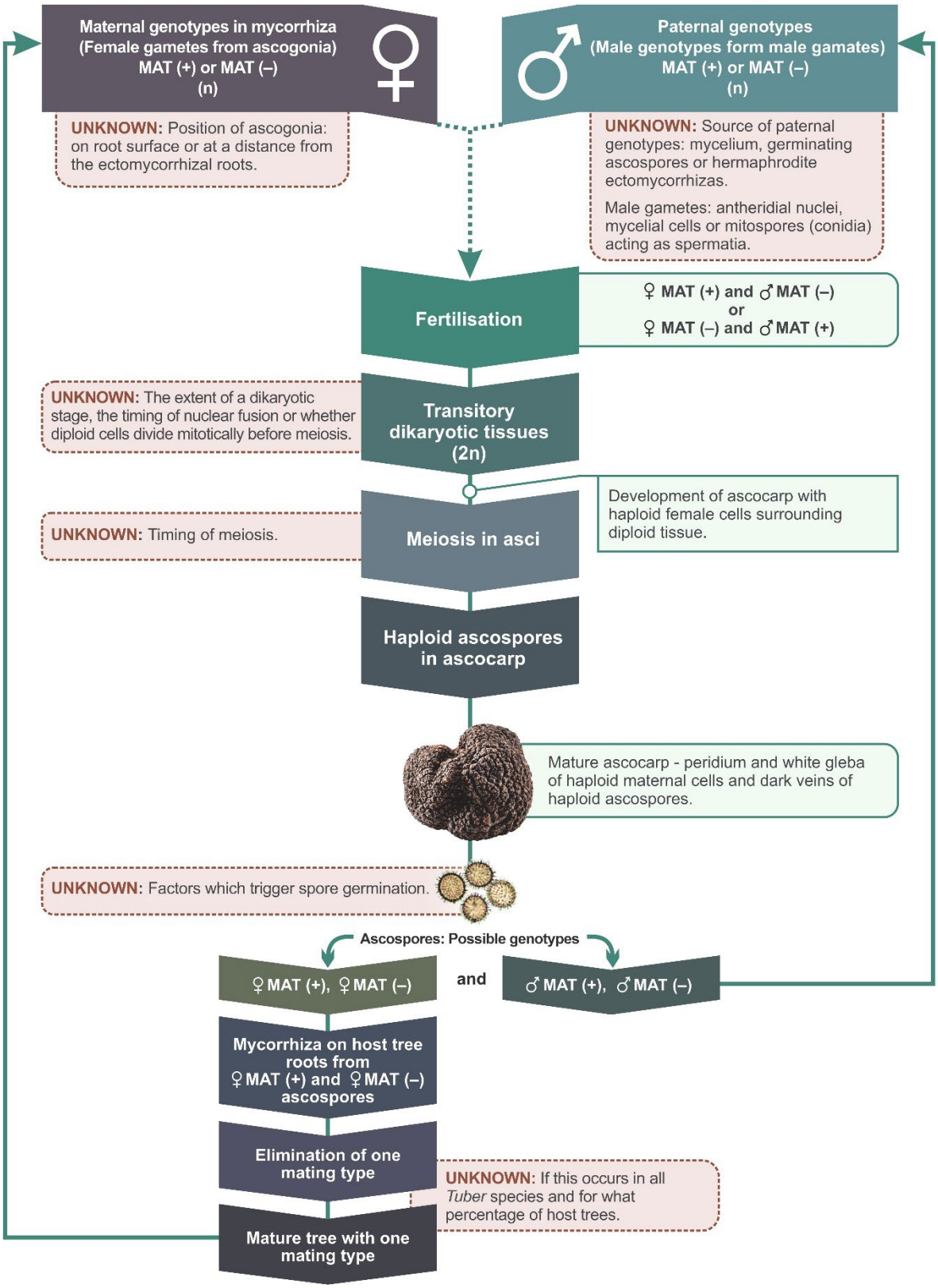
Male		Female		Hermaphrodite	
Genotype number	**Mating type**	**Genotype number**	**Mating type**	**Genotype number**	**Mating type**
IV	MAT -	I	MAT +	II	MAT +
VIII	MAT -	III	MAT -	VI	MAT -
X	MAT -	V	MAT -		
XI	MAT -	IX	MAT -		
XII	MAT +				
XIII	MAT +				
XIV	MAT +				
XV	MAT +				
XVI	MAT +				

There is no firm evidence that hermaphrodite genotypes produce male as well as female gametes from mycorrhizas on the host tree, but the possibility cannot be discounted. In an orchard of *T. borchii* inoculated with mycelium, after nine years, an ascocarp was found with the female genotype from one host tree and the

paternal genotype of the ectomycorrhiza on the neighboring tree (Leonardi et al. 2020). It is possible that mycelium from the ectomycorrhiza became detached from the root and formed an independent, small male colony, but the proximity of the ascocarp with the male genotype the same as the ectomycorrhiza on the neighboring tree would suggest that ectomycorrhizal mycelium produced the male gamete. As most Ascomycota are hermaphrodite it remains possible that *Tuber* species are not genetically trioecious, but hermaphrodite with the environment determining whether female or male gametes are produced. The appearance of dioecy may be due to insufficient sampling to detect each genotype as both a female and a male partner. The environment and high level of nutrition of the ectomycorrhizal mycelium may determine the production of female gametes, whereas a sparse (possibly saprophytic) mycelium in the soil produces male gametes. In both vascular plants and animals, there are many examples of environmentally determined sex (Charnov and Dawson 1989).

There are two mating types (idiotypes), and mating occurs only between gametes with different mating types (Rubini et al. 2014). The mating types can be distinguished using direct PCR methods as they can be amplified using mating type (MAT) specific primers (Rubini et al. 2011a). Different authors use various abbreviations to designate the mating types: MAT 1-1 and MAT 1-2 (Qin and Feng 2022), MAT1-1-1 and MAT1-2-1 (Linde and Selmes 2012) and MAT(+) and MAT(−) (Rubini et al. 2011a), MAT 1-1 and MAT 1-2-1 (Selosse et al. 2017). This paper will use MAT(+) and MAT(-). Female gametes can carry MAT(+) or MAT(-), as can male gametes, and MAT(+) and MAT(-) occur equally frequently in female and male gametes (Rubini et al. 2011a). Female and male gametes from a hermaphrodite will have the same mating type precluding self-fertilisation (Rubini et al. 2011a). It appears that certain genotypes may be more prevalent as maternal gametes and others as paternal ones. For example, from 15 genotypes analysed by Rubini et al. (2011a), 60% formed only paternal gametes, 26.6 % only maternal and 13.3% formed maternal and paternal gametes (hermaphrodites).

Fig. 1 Life cycle of true truffles. (Only dioecy is illustrated here, but hermaphrodites occur in low frequency in several species) (Artwork by Jodi Burgess (http://jajographics.com.au))



Ectomycorrhiza and production of female gametes

Female gametes are produced from the ectomycorrhizal mycelium in host species, which has been proven by molecular genotyping using microsatellite markers (De la Varga et al. 2017). After seedling inoculation with a mix of spores carrying different genotypes with the two different mating types, mycorrhizas of both mating types are formed in the roots of each plant. However, in some cases, as trees mature, one mating type becomes predominant, if not exclusive, to each host tree (Qin and Feng 2022). It is not known how many genotypes of the same mating type may be present in a single host plant, but Taschen et al. (2022) observed that there may be a large number of maternal genotypes in the same brûlé, suggesting the presence of multiple mycorrhizal genotypes in a single tree. This is difficult to confirm in an orchard or forest, as after several years of growth, the roots of adjacent trees may be mixed during root collection unless roots are carefully traced back to the trunk (Meinen et al. 2009; Smith et al. 2014). It is known that in other host species, there is a turnover of mycorrhizal species as trees mature (Kubisch et al. 2016; Mason 1975). Therefore, the comparative competitive ability of maternal *Tuber* genotypes may differ in the hosts' roots at the seedling, sapling and mature stages.

The timeline and extent of elimination of one mating type varies in different environments and for different truffle and host species. Rubini et al. (2011a) found both mating types of *T. melanosporum* were present six months after inoculation of *Q. pubescens* seedlings, but after 18 months, half the seedlings had only one mating type, while in native forests of *Ostrya carpinifolia* and *Q. pubescens,* some mature trees had *T. melanosporum* mycorrhiza from both mating types. Zampieri et al. (2012) found that only one mating type of *T. melanosporum* was present in the soils under productive and formerly productive *Q. pubescens* trees. The dominance of a single mating type in mature trees has also been found in Australian truffle orchards (Linde and Selmes 2012). Two-year-old *T. melanosporum* inoculated seedlings of *Q. robur* and *Q. ilex* purchased from different nurseries in Australia initially had both mating types present. However, 50% of *Q. robur, Q. ilex* and *C. avellana* trees when 5-9 years old showed only one mating type (Linde and Selmes 2012). This applied to both productive and unproductive plants. Rubini et al. (2011a) reported that opposite mating types tend to separate from each other in an orchard, and the minimal distance between opposite mating types of *T. melanosporum* in Spoleto was 50 m, with mostly one mycorrhizal genotype in each location. These field data were supported by in vitro experiments. For instance, vegetative compatibility (merging of colonies) of *T. borchii* and *T. melanosporum* mycelial cultures was found only within the same isolate (Iotti et al. 2012b; Sbrana et al. 2007). If the natural distribution of mating types at tree maturity is for large areas to be colonised by the same mating type, there could be an advantage in designing orchards so that large areas are planted with seedlings of the same mating type (but a range of genotypes). This would require the use of mycelial inoculum, but it would be interesting to determine if this distribution resulted in maximum production being reached earlier.

Analyses of the genotypes of mycorrhiza of the roots of the host plant have revealed much lower genetic diversity than that recorded amongst the male gametes involved in the production of ascocarps. The same female genotype can produce ascocarps over several years (De la Varga et al. 2017), whereas this is unusual for a male genotype. The lower genetic diversity in the ectomycorrhiza compared to the male gametes is not unexpected; if the host trees have been inoculated as seedlings, it is likely they have been provided with spores

of only a few genotypes, whereas in the soil where the species is native, there would be a wide reservoir of genotypes able to produce male gametes.

It thus appears that the female gametes are formed from the ectomycorrhiza in the host plant, and one tree may host an unknown number of genotypes but possibly only one mating type. There is no evidence that eliminating one mating type from a host tree impacts yield. Where mycelial inoculation is possible, the opportunity to select elite female genotypes that yield the largest ascocarps should be utilized.

Disentangling the contribution to yield and truffle quality of various environmental factors and *Tuber* genotypes will be easier in regions where the species is not native. In these regions, it is possible to plant replicates of trees inoculated with the same *Tuber* genotype to assess the relative contribution of environmental factors (host vigour, soil nutrient levels) and *Tuber* genotype on truffle size and aromatic production without trees being colonised by strains other than those used in the inoculum. Some detrimental effects of inoculation have been reported, such as stunted lateral root growth and sparse shoot development in *Quercus* inoculated by *T. melanosporum* (Garcia-Barreda et al. 2016). Molecular techniques should be applied to determine whether the growth depression was caused by a particular genotype(s) of *Tuber* or ecotypes of the host species and, if so, to avoid using these combinations.

Production of male gametes

A critical gap in knowledge is the source of the male gametes and whether a deficit of male gametes is responsible for some low yields. It has been suggested that male gametes could derive from germinating ascospores, mycelium in the soil, mitospores or associations with understory species (De la Varga et al. 2017). As mentioned above, molecular analyses have found a wide diversity of male genotypes in ascocarps. As most male genotypes are detected in only one ascocarp in one year (De la Varga et al. 2017), they are probably present in the soil for only a short period and do not persist after producing male gametes. This indicates that most male gametes are unlikely to come from a persistent endophyte or saprophytic colony, but likely arise from short-lived mycelium from germinating ascospores in the soil. Vilanova et al. (2017) made two key observations; (1) inoculated trees in the nursery and two-year-old trees in the field have viable ungerminated spores near their roots, (2) following the application of spore material in the field, peak fructification does not occur until two years later. The time of germination and length of viability may differ for spores that produce ectomycorrhiza and those that produce male gametes. Spores that produce mycelium developing male gametes may have a longer period of dormancy than those that develop into maternal ectomycorrhizas. Taschen et al. (2022) suggest that a comparison of the effect of adding spores of different ages to truffle traps and assessing the genotypes in the ensuing ascocarps may provide some insight into the importance of spore dormancy in the production of male gametes.

One aspect that is difficult to reconcile with the concept that the male mycelium is a short-lived soil saprophyte is that in newly established orchards in regions where *Tuber* is exotic, the inoculated seedlings are out-planted and generally yield ascocarps only after 3-4 years, without additional application of spores to the soil. If the males are short-lived, the source of the gametes in these new orchards needs to be explained. As pointed out above, there is scant evidence of hermaphrodite genotypes growing as mycorrhiza, producing male gametes (De

la Varga et al. 2017). Inoculated seedlings are grown in relatively aseptic potting media for up to a year. After hosts are out-planted, the male genotypes must survive as either spores or saprophytes for a further three or more years before female reproductive maturity is reached. In refrigerated storage, truffle spore viability decreases after two years (Zambonelli et al. 2010), but as noted above, Vilanova et al. (2017) found some viable ungerminated spores on the roots of two-year-old trees. In newly planted orchards of truffles as an exotic species, an indication of whether it is possible for spores from the initial inoculum to survive until fructification could be gained from analysis of the genotypes of the spores in the inoculum compared to those in ascocarps eventually produced by the inoculated plants.

Tuber spp. can also grow endophytically in non-ECM host species (Schneider-Maunoury et al. 2020). Thus far, only mycelium of the female genotype on a nearby host tree has been detected these endophytic relationships, but it remains possible that male genotypes may also grow in this way. They could form small associations with each genotype in only one root tip, making them difficult to detect. Such endophytic associations may be a source of male gametes, but more research is required to verify this hypothetical growth mode for the male genotype.

Ascocarp formation

One area of uncertainty is what triggers the initiation of the production of sexual gametes in truffles. Le Tacon et al. (2016) suggest climatic factors such as rainfall and low temperatures, as they noted a positive correlation between winter rainfall and truffle production in France. The dispersal of the male gamete to the female gametangium and the gamete fusion process for truffles are unknown. There are two possibilities. The most likely is that hyphae from the mycorrhizal roots extend into the soil until they encounter hyphae of the opposite mating type, and this contact stimulates gametogenesis. *Tuber* species produce non-motile male gametes (Le Tacon et al. 2016), as do all Ascomycetes, so the formation of male gametes at any distance from the female gametangium would require passive dispersal, which would be a weak point in the reproductive cycle. After fertilisation, the fruiting body forms over a period of weeks (Le Tacon et al. 2016). Within the fruiting body, the peridium and the sterile white veins are haploid and of the maternal genotype. The dark gleba is a mass of haploid spores derived from meiosis and possibly subsequent mitotic proliferation (Selosse et al. 2017). Whether or not there is a dikaryotic stage after fertilisation, whether the diploid zygote undergoes divisions before meiosis, the exact timing of meiosis and whether haploid spores undergo mitotic divisions before maturity is unknown. Although the first paper indicating outcrossing in truffles was for *T. magnatum* (Paolocci et al. 2006), most life cycle studies have been carried out with *T. melanosporum* (De la Varga et al. 2017; Le Tacon et al. 2016; Rubini et al. 2014; Selosse et al. 2017) and *T. aestivum* (Staubli et al. 2022) and details for other species need to be confirmed. Ascocarp growth depends mainly on organic nutrients obtained directly from the host roots rather than the soil organic matter or dead plant tissues (Le Tacon et al. 2016; Le Tacon et al. 2013; Mello et al. 2017).

Spore germination for ectomycorrhizal mushrooms in vitro has rarely been reported (Murata et al. 2015), and to date, no successful mycelial growth from *Tuber* spp. spores has been documented. For some species, particular symbiotic bacteria may be essential (see below). It is possible that spore dormancy can be broken by the

presence of root exudates, as clearly spores inoculated onto host seedling roots germinate, or that dormancy can be broken from passage through an animal gut. Germination of truffle spores isolated from porcupine feces was reported (Maun 2009; Ori et al. 2018; Tian et al. 2021). It has not been possible to induce gamete formation from in vitro cultures. When mycelial cultures derived from the gleba are used, even in combination of MAT+ and MAT-, both strains will be of the female genotype. Experiments using co-culture of either a female strain and a hermaphrodite strain, or of two hermaphrodites of MAT + and MAT - may be more successful in stimulating gametogenesis in vitro.

Cultivation techniques to improve yield

Improved yield can be achieved by increasing the mycorrhizal root mass on the host and/or ensuring the abundance of male gametes (Taschen et al. 2022). A traditional technique still used by modern truffle farmers is to rip the zone between rows of hosts and add spore inoculum into holes around the tree (Fischer et al. 2017). Anecdotal evidence suggests this is successful for increasing yield, but there does not appear to be sufficient experimental data with appropriate replication and controls. The benefit could arise from adding the spores or stimulation of lateral root formation by the host tree.

A more recent technique is the addition of substrates and generally spores in pits dug in the root zone of well-established host plants. These are known as truffle nests, Spanish wells, Catalan holes, spore traps, truffle wells, and truffle traps (Fischer et al. 2017; Murat et al. 2016; Taschen et al. 2022) and will be referred to here as truffle traps. The techniques evolved from traditional practices used by wild truffle hunters who used spot application of charcoal hearth soils or decomposed organic matter to improve truffle yield (Garcia-Barreda et al. 2020).

The provision of truffle traps can increase truffle formation after two years and has the additional benefit that truffles may preferentially form in the region of the truffle trap, simplifying harvest (Taschen et al. 2022). More than ten commercial substrates have been used in the truffle traps (Garcia-Barreda et al. 2020) (Table 2), and there are insufficient data to determine the optimal medium, but the effectiveness of peat as a substrate in the traps may vary according to soil pH. It is assumed that these substrates provide a habitat attractive to host roots and mycelium of both male and female hyphae, increasing the chance of successful sexual reproduction. Introducing truffle spores to truffle traps might be expected to provide abundant paternal partners, but Franco-Manchón et al. (2018) reported the addition of spores incorporated with peat-based substrates to truffle traps did not significantly alter the amount of *T. melanosporum* mycelium present. However, as the male genotype is thought to produce only a tiny amount of mycelium, a significant increase in mycelial abundance need not accompany an increase in the availability of male gametes. Taschen et al. (2022) found that the introduced spores were involved in ascocarp formation but that overall yield was not increased. The effectiveness of truffle traps can be affected by the soil texture, host age, the root structure of the host plant in the truffle trap location, and the amount of mycelia present in the soil (Garcia-Barreda et al. 2020). It can be hypothesised that in truffle orchards in countries where the species is not native, placing spores in truffle traps would provide the male partner of ascocarps more frequently than in orchards in regions where *Tuber* species occur naturally. As non-inoculated truffle traps can induce an increase in yield, suggesting that soil disturbance and root damage during

digging that initiates the development of more lateral roots with mycorrhiza is the beneficial effect (Taschen et al. 2022), similar to the effect of ripping between trees. No study has compared the effectiveness of truffle traps and ripping. Molecular analysis of the fungal diversity of the holes with different additives, with and without the addition of spores, has not been undertaken but would provide insight into the relative importance of the technique for the female and male genotypes. Truffle traps would also appear to be a valuable habitat for searching for the production of male gametes and the early stages of sporocarp development using conventional microscopic techniques.

There is frequently no undergrowth or only stunted plants under the canopy of host trees of truffle species such as *T. melanosporum*, *T. aestivum* and *T. indicum* - a phenomenon known as brûlé. The area of brûlé grows larger each year (Innangi et al. 2020; Streiblova et al. 2012), which is a sign that the inoculated mycorrhiza is well established in host roots. The exact mechanism of plant growth suppression remains unresolved, but fungal exudates are likely to be involved. Some of the volatile organic compounds produced by *T. melanosporum,* and possibly other *Tuber* species are allelopathic (Napoli et al. 2010; Pacioni 1991; Schneider-Maunoury et al. 2020). In addition, some truffle farmers deliberately remove all undergrowth chemically or mechanically. In contrast, other growers selectively maintain certain species, such as *Festuca ovina*, which is considered to have a positive impact on *T. melanosporum* yield (Taschen et al. 2020). Molecular techniques have proven that roots of non-ectomycorrhizal plants from a broad taxonomic range can form associations with hyphae derived from ectomycorrhizas of *T. melanosporum* or *T. aestivum* (Schneider-Maunoury et al. 2020). Evidence from both the field and experiments in rhizotrons shows that the presence of other vascular plant species can increase the abundance of truffle mycelium in the soil (Taschen et al. 2020). De la Varga et al. (2017) determined that while most male genotypes were found in just one ascocarp in a single year, 11.6% appeared to be from a perennial mycelium, which persisted for as long as four years. As mentioned above, it is possible that these perennial genotypes were growing as endophytes or at least in association with the roots of understorey species. More studies are required on the most beneficial understorey species and the effect of the brûlé on the presence of *Tuber* mycelium and other microbes. Mello et al. (2013) reported differences in the occurrence and abundance of bacteria groups, such as *Firmicutes*, *Actinobacteria* and *Cyanobacteria*, in and outside the brûlé and Liu et al. (2023) found that understory species affect the abundance of soil microbial groups. The availability of molecular techniques has opened up new possibilities for managing truffle orchards to increase productivity through manipulating plant and microbial species in the understory, but current evidence suggests that complete elimination of undergrowth is not desirable.

Although Mediterranean forests of *Pinus sylvestris* and *P. nigra* subsp. *salzmannii* have a high proportion of roots colonised by *T. melanosporum,* fruiting bodies are not produced, and *Pinus* spp. are not recommended as hosts for *T. melanosporum* (Garcia-Montero et al. 2007). What is the reason for this? Do the pine roots not stimulate the formation of male gametes, or does the soil not provide a favourable habitat for the survival of male partners? If the latter, the placement in *Pinus* plantations of truffle traps containing truffle spores and possibly litter from *Quercus* or *Corylus* may result in successful male gamete production and, thus, ascocarp formation.

Table 2 Composition of truffle traps and their effect

Host relationship	Truffle trap composition	Comments	Reference
T. melanosporum, Q. pubescens	*T. melanosporum* ascomata (250g), honey (350g), horticultural vermiculite (50l) and organic compost (50l)	95% of harvested truffles from inside the traps after two years	(Murat et al. 2016)
T. melanosporum *Q. ilex*, *Q. faginea*	European sphagnum peat-based substrate (Turbatruf ® from Projar: black peat - white peat - coir - perlite mix 11:5:3:1) with *T. melanosporum* spores (0.1 g dry fruitbody per litre of the substrate)	Increased the number of truffle fruit bodies per dig, earlier harvests and less damage by leiodes beetles	(Garcia-Barreda et al. 2020)
T. melanosporum, *Quercus* spp.	Crushed truffle material	Truffle harvest percentage inside the traps ranged from 0-89.4% depending on geographic locations and tree age	(Taschen et al. 2022)

Inoculation methods

Ascospore suspensions are most commonly used for the inoculation of seedlings. This has the advantages of simplicity and speed. The major problems are the possibility of contamination with pathogens, pests and other ectomycorrhizal competitors (Iotti et al. 2016). Mycelial inoculation, being aseptic, removes these problems and using molecular methods allows control of the ratio of mating types used in the inoculum to maximise the potential for both mating types MAT(+) and MAT(-) to be present on different trees and optimise yield. Mycelial inoculation has been used successfully for *T. borchii* on the hosts *Corylus avellana*, *Pinus pinea*, *Q. pubescens* and *Q. robur*. The first ascocarps were harvested from trees inoculated around eight years previously (Iotti et al. 2016). Leonardi et al. (2020), also using *T. borchii* mycelial inoculation of five single-genotype strains, found significant differences in productivity between strains. The 18 harvested ascomata analysed were derived from the inoculated genotypes with the addition of two other maternal genotypes originating in the field. There were 14 new paternal genotypes recorded and two that were the same as an inoculated strain, indicating these strains were hermaphroditic. Hermaphrodite genotypes could be one way of increasing the availability of male gametes, particularly in regions where *Tuber* is non-native. Leonardi's experimental mycelial inoculations also included treatments in which all five strains (three MAT + and two MAT -) were used on the same seedlings. From the mixture, only two MAT + strains were present on the eight-year-old trees, indicating a range of competitive abilities amongst strains. Thus, mating type, productivity and competitive ability can be selected and utilised when using mycelial inoculations. Mycelial inoculation also opens up the possibility of designing experiments to assess the relative contribution of environmental factors (climate, host vigour, soil nutrient levels) on truffle size.

Evaluation of the success of root inoculation

Molecular methods complement traditional methods of assessing the success of seedling inoculation and ensuring only high-quality plants are used for outplanting. Andrés-Alpuente et al. (2014) present an analysis of the different traditional techniques. Most are based on morphological identification, which can give erroneous results because a single ectomycorrhizal species may produce multiple morphotypes, which can vary with the age of the root tip, host species, fungal strain, and environmental conditions (Zambonelli et al. 2012). In addition, different species may produce mycorrhiza very similar anatomically and morphologically; for example, the pattern of the mantle of *T. borchii* cannot be distinguished from those formed by other white truffle species (Giomaro et al. 2000). Misidentifications have led to unintentional introductions of species of lower economic value, such as *T. brumale* and *T. indicum*, which can compete with *T. melanosporum* in the field when introduced as seedling contaminants (Bonito 2009; Murat et al. 2008). Molecular techniques also allow the detection of other mycorrhizal or pathogenic species that may be missed in a visual inspection. The cost of including molecular checks for quality assurance before outplanting is worthwhile for such a long-term investment and high-value product.

Mulching

Mulching is traditionally used to increase truffle production by controlling weeds, adding nutrients, and retaining soil moisture. Leaves, straws, branches, or plastic films have been used and differentially affect mycorrhiza abundance and truffle production (Zambonelli et al. 2005). Piñuela et al. (2021) found white mulch (plastic) increased the mycorrhizal growth of both *T. aestivum* and *T. melanosporum.* The success of white mulch is due to the reflection of light and maintenance of cooler soil temperature and higher soil moisture (Díaz-Pérez and Dean Batal 2002). Zambonelli et al. (2005) compared the effect of mulching with black mulching cloth, wheat straw, sub-watering cloth and an aluminised cloth on *T. aestivum* (syn. *T. uncinatum*) in a truffle orchard. Sub-watering cloth increased *T. aestivum* mycorrhization and depressed the competitive mycorrhizal species. Black mulch and straw depressed the *T. aestivum* mycorrhization and stimulated the competitors, so they are unsuitable for truffle orchards, at least in the first few years after planting (Zambonelli et al. 2005). qPCR techniques were used to compare the mycelium quantity among different mulching materials (Piñuela et al. 2021). Whether removing litter, keeping it on the surface or burying it by ripping is best for ectomycorrhizal growth of *Tuber* spp. and suppression of competitive ectomycorrhizae requires further study.

Pest species and competitors

Contamination of orchard soil by unwanted ectomycorrhizal fungi, including the undesirable *Tuber* species, which outcompete the commercial species, is an ongoing problem for truffle producers. For example, *T. brumale,* which outcompetes *T. melanosporum* in host roots, has been introduced into Eastern Australia (DPIRD 2021). Other ectomycorrhizal species are likely in truffle orchards, including *Tomentella* spp., *Suillus* spp., *Helvella* spp., *Scleroderma* spp., and *Inocybe* spp. (Ori et al. 2023). Control of diseases of the host species is also essential. For example, *Armillaria* root rot, present in many truffle-growing areas of the world, causes dieback of oaks (Davari and Askari 2005; Kelley et al. 2009; Kim et al. 2022). Molecular analysis of the soil of

potential truffle orchards to identify the mycorrhizal and pathogenic species present enables growers to avoid unsuitable sites or to initiate early and effective control of the pest and competitor species. It will also be possible to monitor potential pathogens or competitors on materials introduced as fertilisers, mulches or truffle trap materials. Certifying that truffle orchards providing truffles for inoculum for nurseries are free of key pests and diseases is highly desirable. Certification could require metabarcoding of soil DNA to prove *Tuber* inoculum does not carry unwanted organisms.

Fungi from mycorrhizas on trees native to an area may compete with *Tuber* species. This is likely to be less of a problem when the host plants are cultivated outside of their natural range, but in Australia Brown (1998) found that ectomycorrhizal fungi from eucalypts may form mycorrhiza with *C. avellana.* Another native mycorrhizal species, *Scleroderma* spp. competes with *T. melanosporum* on *Quercus robur* trees in New Zealand (Hall and Yun 1998). Using molecular techniques to determine the composition and dynamics of the soil fungal microbiome may offer the opportunity to manipulate the soil conditions to benefit the *Tuber* species and disadvantage the competitors. For example, Brown (1998) suggested that in Tasmania, applying phosphorous may improve the competitive ability of *T. melanosporum* compared to native fungi.

It cannot be assumed that all other soil and endophytic fungi will be disadvantageous for truffle production. Both endophytes and ectomycorrhizas utilise the carbon source of host plants (Martin et al. 1987; Wang and Dai 2011), and thus there will be competition between other endophytes and *Tuber* spp. for the host root habitat. For example, the abundance of arbuscular mycorrhizal fungi on the understory plants of *T. melanosporum* truffle brûlé decreases in the presence of *T. melanosporum* (Taschen et al. 2020). However, some endophytes may be desirable or essential for maximum host health and growth as endophytes may increase the fitness of host plants by improving tolerance to biotic and abiotic stress and decreasing water consumption (Rodriguez et al. 2009). Investigating the endophytic species of highly productive host trees may elucidate the importance of other endophytic species.

Mycorrhizal helper bacteria that enhance truffle production or quality

Complex microbial communities inhabit the mycorrhizae, the peridium, and the gleba of truffle ascocarps. Fruiting bodies may be colonised by a few hundred species of bacteria, and populations reach 10^7–10^8 cells per gram of truffle. The three commercial truffle species are associated with beneficial bacteria termed Mycorrhizal Helper Bacteria (MHB) (Piñuela et al. 2020). Some bacteria may be obligate or beneficial symbionts for the growth of *Tuber* species in vitro. Growth of mycelium of *T. melanosporum* and *T. brumale* stalls without the presence of *Rhodopseudomonas* spp. (Le Roux et al. 2016) and *Bradyrhizobium* spp. isolated from ascocarps promoted *T. magnatum* mycelial growth in vitro (Graziosi et al. 2024). However, the biochemical exchanges underpinning the symbiosis are unknown. Other bacterial species that co-occur with *T. melanosporum* include *Nannocistis excedens*, *Sporosarcina globispora* and *Singulisphaera limicola* (Herrero de Aza et al. 2022). *Pseudomonas fluorescens* increased the frequency of colonisation of *T. melanosporum* on both gymnosperm and angiosperm host species (Giorgi et al. 2024; Piñuela et al. 2020). Some *Bradyrhizobiaceae* found in *T. magnatum* truffles can fix nitrogen and change the abundance of fibrous roots compared to pioneer roots in seedlings but do not affect the rate of mycelial colonisation of seedling roots (Giorgi et al. 2024). Bacterial

species also participate in producing complex aromas and volatile organic compounds that make truffles so valuable (Splivallo et al. 2019). It is unknown to what extent the subtle difference in flavour and aroma of truffles produced in different regions are due to bacterial metabolism or metabolism of the *Tuber* mycelium. Adding specific strains of bacteria to enhance ascocarp size and quality is an attractive possibility, but experiments to assess the effects of MHB are complicated as some MHB cannot be cultured aseptically, bacteria are impossible to remove from spore suspensions of ground ascocarps (making control, ectomycorrhizal plants without bacteria difficult to produce), mycelial inoculants usually grow poorly without bacterial associates, and any bacteria inoculated onto seedlings will eventually have to compete with species present in orchard soil. However, even though many soil bacteria are ubiquitous, the effect of the addition of MHB may be more evident in orchards on land where cultivated *Tuber* species are not native. Given the present state of knowledge, another reason for preferring spore inoculation over mycelial inoculation is the co-occurrence of MHB in the inoculum.

Conclusion

Increasing the yield of cultivated organisms can be achieved through improvements in cultivation practices and selective breeding. Additional information on the habit, life cycle and genetic population structure of truffles recently gained through the availability of molecular techniques provides possibilities for improvements using both strategies. It is now possible to survey the entire soil microbiota before selecting land for the establishment of new truffle orchards and avoid areas with known disease organisms. In established orchards, monitoring the distribution and abundance of undesirable *Tuber* spp. and other competitive ectomycorrhizal species that will decrease productivity will allow early implementation of control measures. Fruiting bodies used to produce spore slurries for inoculation of seedlings can also be assessed for the absence of deleterious organisms, and the identity of the mycorrhizas formed can be analysed accurately. Molecular tools have also identified the most beneficial mulches and provided insight into the competition between genotypes and mating types on the host plant roots. Exciting possibilities exist concerning using beneficial bacteria for improved *Tuber* growth or developing particular flavours or aromas. It appears possible that the effectiveness of some cultivation techniques may differ in orchards in native and non-native habitats.

In relation to selective breeding, information on eliminating one mating type from the host roots needs confirmation and determination of how many genotypes of a particular mating type are retained on a single host and whether this applies to all commercial *Tuber* species. Knowing that trees normally host a single mating type increases the value of selecting elite female genotypes, particularly if mycelial inoculation techniques can be perfected. Truffle orchards on new lands, in regions where *Tuber* species are not native, offer good opportunities for experimental plantings to compare the productivity of different female genotypes without competition and possibly replacement by naturally occurring *Tuber* genotypes. It is unlikely that the selection of elite genotypes for the production of male gametes will be possible or worthwhile as, usually, each male gamete genotype forms only one or very few ascocarps and is usually present for only one year (De la Varga et al. 2017). Questions remain in relation to the habit of the mycelium producing the male gametes and, thus, the best way to ensure

that a low abundance of male gametes does not reduce productivity. It remains unknown whether *Tuber* species are genetically dioecious, or hermaphrodite with the sex of the gametes produced being environmentally determined. The early stages of ascocarp development are also unknown, including the length of time and volume of tissue produced at the dikaryotic and diploid phases. Reductions in the cost of genetic analyses will allow more comprehensive application of molecular techniques for the benefit of truffle growers and the resolution of the remaining gaps in our knowledge of truffle species.

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Acknowledgement

We would like to thank Federico Paci of Great Southern Truffle, Adam Wilson and Nimesha M. Patabendige of Silver Place, Julia McHenry of Mt. Lindesay farm for valuable discussions.

Author contribution

Writing the first draft and revisions following coauthor's comments, MCAG; supervision and writing, JM; editing and supervision, TIB, GEStJH, WAD, AZ and MMcH. All authors have read and agreed to publish the manuscript.

Funding

This work was supported by Future food systems CRC (Project - Australian Truffle: Truffle cultivation for industry (IRMA 20966). Future Food Systems CRC, Murdoch University and Silver Place Pty Ltd provided financial support for Mahesh C.A. Galappathithi.

Declarations

The authors declare no competing interests.