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

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19

20 **Abstract**

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

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

34

35 Introduction

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

42 The first record of cultivation of a mycorrhizal fungus was early in the 19th century (Hall et al. 2009). In 1800,
43 Joseph Talon discovered that oak seedlings growing under trees that produced Périgord black truffles (*T.*
44 *melanosporum*) could be transplanted, and trees would yield truffles after 5-10 years (Chevalier and Frochot
45 1997; Dupont et al. 2017; Zambonelli et al. 2015). Then, in 1847, Auguste Rousseau of Carpentras, Vaucluse
46 successfully introduced Périgord black truffle to a *Quercus ilex* plantation, but exactly how he inoculated the
47 trees is not known (Dupont et al. 2017; Zambonelli and Bonito 2012). The development of modern culture
48 techniques was initiated by Palenzona (1969), who successfully inoculated *Corylus avellana* with *T. brumale*, *T.*
49 *aestivum* and *T. melanosporum*. Clean seedlings were inoculated with spores and raised in a shade house or
50 greenhouse for a few months before planting in the field, and this method is still used today (Zambonelli et al.
51 2017). Trees inoculated in this way may produce truffles usually 5–7 years after inoculation, but the time may
52 range from 3–10 years (Bonet et al. 2006; Zampieri et al. 2012), with yield slowly rising to a maximum after
53 15–20 years (Eslick 2017). Truffle plantations, in addition to yielding valuable truffles, also offer the additional
54 benefits that the host species may produce nuts, timber, carbon credits, promote biodiversity conservation
55 (Therville et al. 2013; Zambonelli and Bonito 2012), and provide habitat for invertebrate and vertebrate fauna,
56 shade and a range of other ecosystem services and functions (Kotze et al. 2022).

57 Knowledge of successful inoculation techniques launched modern truffle cultivation in the 1970s, firstly in
58 countries where *Tuber* species are native (France and Italy) (Reyna and Garcia-Barreda 2014; Wang 2012), but
59 cultivation soon spread to other European and extra-European countries with appropriate climates such as Spain,
60 South Africa (Dullstroom and Western Cape), Chile (Panguipulli, Duao, Chufquen, Quepe and Traiguén) and
61 Argentina (Lobería) (Hall et al. 2017). In New Zealand (NZ) and Australia, the first truffle orchards were
62 established in the mid-1980s; the first harvests were *T. melanosporum* from Gisborne NZ in 1993 and Tasmania
63 in 1999. The primary hosts utilised were initially *C. avellana* and *Quercus robur*, but these days, a combination
64 of *Quercus suber* and *Q. ilex* is usual. The most commonly cultivated truffle species is *T. melanosporum*, but
65 the industry in several countries has recently been expanded to include species such as *T. aestivum* and *T.*
66 *borchii* (Hall et al. 2017), while *T. himalayense* and *T. indicum* cultivation is limited to China (Huang et al.
67 2021; Li et al. 2018; Lu et al. 2021). The first reports of successful ascocarp formation from cultivation of the
68 most expensive truffle, *T. magnatum*, have recently come from France following 4.5 years of growth of
69 inoculated seedlings of *Q. pubescens* (Bach et al. 2021).

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

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

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

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

92 **Life Cycle of the Truffles**

93 **Habitat and asexual reproduction**

94 It has long been known that *Tuber* species can grow symbiotically and form ectomycorrhiza on certain perennial
95 host species such as *Quercus* spp. and *Pinus* spp. (Fischer et al. 2017). Molecular techniques have recently
96 revealed that for several *Tuber* spp., hyphae from the mycorrhiza of the main host can also occur as loose
97 masses on the root surface or as an endophyte in non-ectomycorrhizal species (Schneider-Maunoury et al.
98 2020). For example, *T. aestivum* and *T. melanosporum* mycelium were detected in the roots of species
99 distributed in 29 families of Angiosperm (Gryndler et al. 2014; Schneider-Maunoury et al. 2018; Schneider-
100 Maunoury et al. 2020). Asexual reproduction is largely through mycelial growth, and field observations have
101 shown conidia (mitospores) to occur in *T. oligospermum* and *T. borchii* (Urban et al. 2004). Mitospores have
102 been observed under in-vitro conditions of *T. japonicum* (Nakano et al. 2022), but mitospores have not been
103 recorded for *T. melanosporum* (De la Varga et al. 2017). The distribution of *T. melanosporum* genotypes in
104 roots and soil may thus rely on only ascospore dispersal (from sexual reproduction), and the species displays
105 narrow spore dispersal and low heterozygosity in populations (De la Varga et al. 2017).

106

107 **Sexual reproduction**

108 Most ascomycetes are heterothallic and hermaphrodite, reproducing sexually by producing antheridia and
 109 ascogonia from haploid mycelium (Bennett and Turgeon 2016; Le Tacon et al. 2016; Pöggeler et al. 2006).
 110 However, there are rare examples of Ascomycete species showing trioecy (hermaphrodite, male and female
 111 genotypes) (Benjamin 1986; Bennett and Turgeon 2016; Leslie 1995; Pöggeler et al. 2006). Until recently, it
 112 was considered that truffle species such as *T. melanosporum* were homothallic or self-fertile (Bertault et al.
 113 1998; Linde and Selmes 2012), but it is now known they are heterothallic, and self-fertilisation does not occur.
 114 Molecular analysis of natural and orchard populations indicates that some *Tuber* species have partially evolved
 115 from the ancestral hermaphrodite condition to trioecy (Selosse et al. 2017). For example, *T. melanosporum* is
 116 mainly dioecious (female and male gametes produced from separate genotypes) with occasional hermaphrodites
 117 (De la Varga et al. 2017). The genetic makeup of the gleba and the spores of an ascocarp enables the
 118 identification of the genotypes of the female and male partners involved. Hyphae from the mycorrhiza in the
 119 host tree contribute to the female gamete and forms the gleba. The spores are a mixture of genotypes, that of the
 120 male gamete, the female gamete, and recombinants. Once the female genotype has been determined, analysing
 121 spore tissue using simple sequence repeat (SSR) based fingerprinting enables subtraction of the female genotype
 122 to deduce the male genotype (Rubini et al. 2011a). In a *T. melanosporum* orchard in France, De la Varga et al.
 123 (2017) recorded only 1.5% hermaphrodite genotypes out of a total of 219, while in an Italian field study of *T.*
 124 *melanosporum*, 13.3% of genotypes were hermaphrodite (Rubini et al. 2011b). In *T. aestivum*, hermaphrodites
 125 may also be rare. Staubli et al. (2022) reported 3.2% and 3.9% hermaphrodite genotypes in two districts of
 126 southern Germany. In *T. borchii*, they may be more common, as in an experimental mycelial inoculation trial
 127 using five genotypes; one was shown to be hermaphrodite (Leonardi et al. 2020).

128 **Table 1** Data illustrating the difference in numbers of male, female and hermaphrodite genotypes and their
 129 mating type detected in late spring 2009 and winter 2009–2010 in natural *T. melanosporum* grounds under
 130 *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 +				

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

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

145 There are two mating types (idiotypes), and mating occurs only between gametes with different mating types
146 (Rubini et al. 2014). The mating types can be distinguished using direct PCR methods as they can be amplified
147 using mating type (MAT) specific primers (Rubini et al. 2011a). Different authors use various abbreviations to
148 designate the mating types: MAT 1-1 and MAT 1-2 (Qin and Feng 2022), MAT1-1-1 and MAT1-2-1 (Linde
149 and Selmes 2012) and MAT(+) and MAT(-) (Rubini et al. 2011a), MAT 1-1 and MAT 1-2-1 (Selosse et al.
150 2017). This paper will use MAT(+) and MAT(-). Female gametes can carry MAT(+) or MAT(-), as can male
151 gametes, and MAT(+) and MAT(-) occur equally frequently in female and male gametes (Rubini et al. 2011a).
152 Female and male gametes from a hermaphrodite will have the same mating type precluding self-fertilisation
153 (Rubini et al. 2011a). It appears that certain genotypes may be more prevalent as maternal gametes and others as
154 paternal ones. For example, from 15 genotypes analysed by Rubini et al. (2011a), 60% formed only paternal
155 gametes, 26.6 % only maternal and 13.3% formed maternal and paternal gametes (hermaphrodites).

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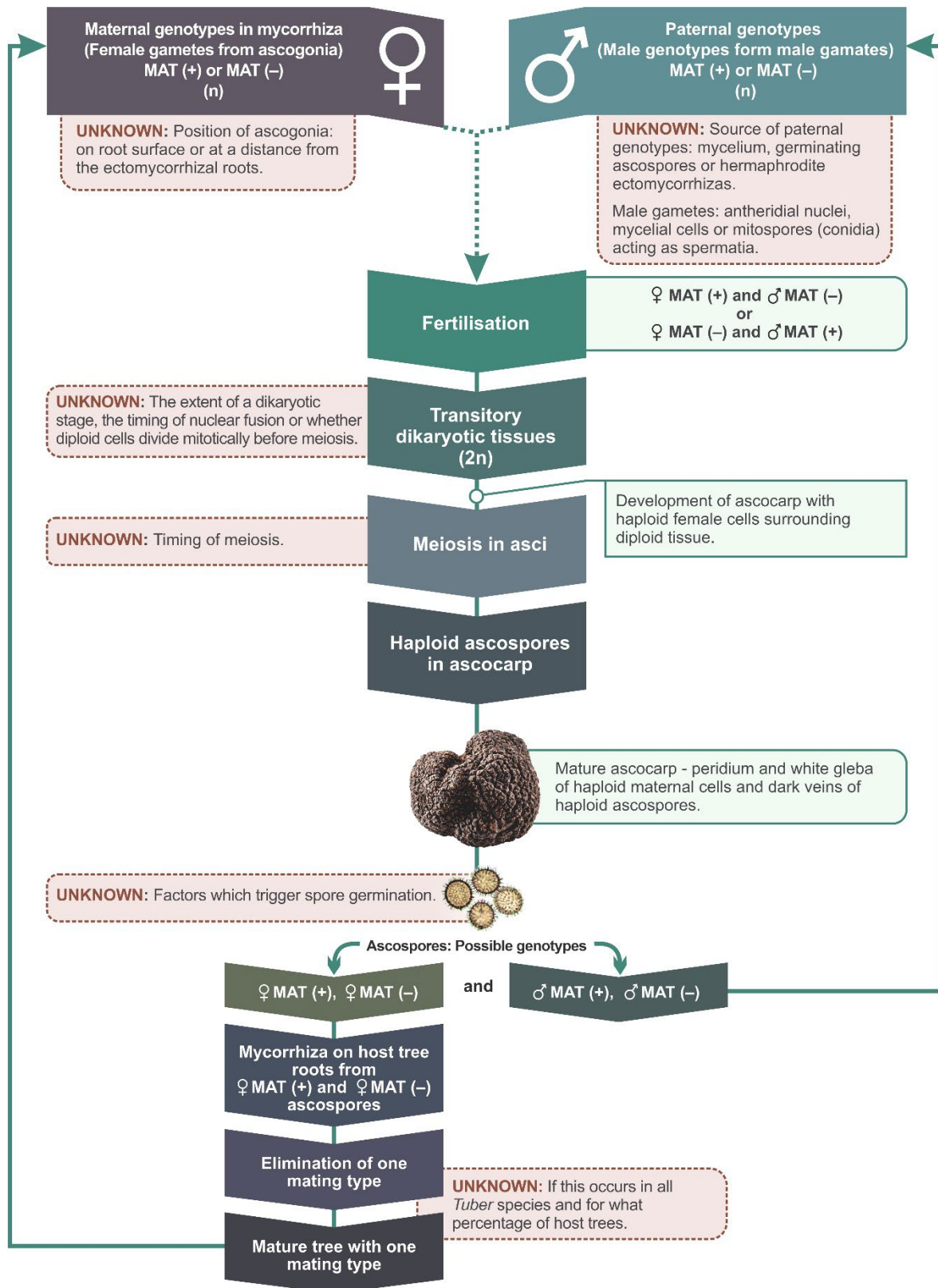
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166 **Fig. 1** Life cycle of true truffles. (Only dioecy is illustrated here, but hermaphrodites occur in low frequency in
 167 several species) (Artwork by Jodi Burgess (<http://jajographics.com.au>))



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169

170 **Ectomycorrhiza and production of female gametes**

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

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

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

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

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

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

222 **Production of male gametes**

223 A critical gap in knowledge is the source of the male gametes and whether a deficit of male gametes is
224 responsible for some low yields. It has been suggested that male gametes could derive from germinating
225 ascospores, mycelium in the soil, mitospores or associations with understory species (De la Varga et al. 2017).
226 As mentioned above, molecular analyses have found a wide diversity of male genotypes in ascocarps. As most
227 male genotypes are detected in only one ascocarp in one year (De la Varga et al. 2017), they are probably
228 present in the soil for only a short period and do not persist after producing male gametes. This indicates that
229 most male gametes are unlikely to come from a persistent endophyte or saprophytic colony, but likely arise from
230 short-lived mycelium from germinating ascospores in the soil. Vilanova et al. (2017) made two key
231 observations; (1) inoculated trees in the nursery and two-year-old trees in the field have viable ungerminated
232 spores near their roots, (2) following the application of spore material in the field, peak fructification does not
233 occur until two years later. The time of germination and length of viability may differ for spores that produce
234 ectomycorrhiza and those that produce male gametes. Spores that produce mycelium developing male gametes
235 may have a longer period of dormancy than those that develop into maternal ectomycorrhizas. Taschen et al.
236 (2022) suggest that a comparison of the effect of adding spores of different ages to truffle traps and assessing
237 the genotypes in the ensuing ascocarps may provide some insight into the importance of spore dormancy in the
238 production of male gametes.

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

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

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

258 **Ascocarp formation**

259 One area of uncertainty is what triggers the initiation of the production of sexual gametes in truffles. Le Tacon
260 et al. (2016) suggest climatic factors such as rainfall and low temperatures, as they noted a positive correlation
261 between winter rainfall and truffle production in France. The dispersal of the male gamete to the female
262 gametangium and the gamete fusion process for truffles are unknown. There are two possibilities. The most
263 likely is that hyphae from the mycorrhizal roots extend into the soil until they encounter hyphae of the opposite
264 mating type, and this contact stimulates gametogenesis. *Tuber* species produce non-motile male gametes (Le
265 Tacon et al. 2016), as do all Ascomycetes, so the formation of male gametes at any distance from the female
266 gametangium would require passive dispersal, which would be a weak point in the reproductive cycle. After
267 fertilisation, the fruiting body forms over a period of weeks (Le Tacon et al. 2016). Within the fruiting body, the
268 peridium and the sterile white veins are haploid and of the maternal genotype. The dark gleba is a mass of
269 haploid spores derived from meiosis and possibly subsequent mitotic proliferation (Selosse et al. 2017).
270 Whether or not there is a dikaryotic stage after fertilisation, whether the diploid zygote undergoes divisions
271 before meiosis, the exact timing of meiosis and whether haploid spores undergo mitotic divisions before
272 maturity is unknown. Although the first paper indicating outcrossing in truffles was for *T. magnatum* (Paolucci
273 et al. 2006), most life cycle studies have been carried out with *T. melanosporum* (De la Varga et al. 2017; Le
274 Tacon et al. 2016; Rubini et al. 2014; Selosse et al. 2017) and *T. aestivum* (Staubli et al. 2022) and details for
275 other species need to be confirmed. Ascocarp growth depends mainly on organic nutrients obtained directly
276 from the host roots rather than the soil organic matter or dead plant tissues (Le Tacon et al. 2016; Le Tacon et al.
277 2013; Mello et al. 2017).

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

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

288 **Cultivation techniques to improve yield**

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

295 A more recent technique is the addition of substrates and generally spores in pits dug in the root zone of well-
296 established host plants. These are known as truffle nests, Spanish wells, Catalan holes, spore traps, truffle wells,
297 and truffle traps (Fischer et al. 2017; Murat et al. 2016; Taschen et al. 2022) and will be referred to here as
298 truffle traps. The techniques evolved from traditional practices used by wild truffle hunters who used spot
299 application of charcoal hearth soils or decomposed organic matter to improve truffle yield (Garcia-Barreda et al.
300 2020).

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

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

325 There is frequently no undergrowth or only stunted plants under the canopy of host trees of truffle species such
326 as *T. melanosporum*, *T. aestivum* and *T. indicum* - a phenomenon known as brûlé. The area of brûlé grows larger
327 each year (Innangi et al. 2020; Streiblova et al. 2012), which is a sign that the inoculated mycorrhiza is well
328 established in host roots. The exact mechanism of plant growth suppression remains unresolved, but fungal
329 exudates are likely to be involved. Some of the volatile organic compounds produced by *T. melanosporum*, and
330 possibly other *Tuber* species are allelopathic (Napoli et al. 2010; Pacioni 1991; Schneider-Maunoury et al.
331 2020). In addition, some truffle farmers deliberately remove all undergrowth chemically or mechanically. In
332 contrast, other growers selectively maintain certain species, such as *Festuca ovina*, which is considered to have
333 a positive impact on *T. melanosporum* yield (Taschen et al. 2020). Molecular techniques have proven that roots
334 of non-ectomycorrhizal plants from a broad taxonomic range can form associations with hyphae derived from
335 ectomycorrhizas of *T. melanosporum* or *T. aestivum* (Schneider-Maunoury et al. 2020). Evidence from both the
336 field and experiments in rhizotrons shows that the presence of other vascular plant species can increase the
337 abundance of truffle mycelium in the soil (Taschen et al. 2020). De la Varga et al. (2017) determined that while
338 most male genotypes were found in just one ascocarp in a single year, 11.6% appeared to be from a perennial
339 mycelium, which persisted for as long as four years. As mentioned above, it is possible that these perennial
340 genotypes were growing as endophytes or at least in association with the roots of understorey species. More
341 studies are required on the most beneficial understorey species and the effect of the brûlé on the presence of
342 *Tuber* mycelium and other microbes. Mello et al. (2013) reported differences in the occurrence and abundance
343 of bacteria groups, such as *Firmicutes*, *Actinobacteria* and *Cyanobacteria*, in and outside the brûlé and Liu et al.
344 (2023) found that understorey species affect the abundance of soil microbial groups. The availability of
345 molecular techniques has opened up new possibilities for managing truffle orchards to increase productivity
346 through manipulating plant and microbial species in the understorey, but current evidence suggests that complete
347 elimination of undergrowth is not desirable.

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

355

356 **Table 2** Composition of truffle traps and their effect

Host relationship	Truffle trap composition	Comments	Reference
<i>T. melanosporum</i> , <i>Q. pubescens</i>	<i>T. melanosporum</i> 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)
<i>T. melanosporum</i> <i>Q. ilex</i> , <i>Q. faginea</i>	European sphagnum peat-based substrate (Turbatruf® from Projar: black peat - white peat - coir - perlite mix 11:5:3:1) with <i>T. melanosporum</i> 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)
<i>T. melanosporum</i> , <i>Quercus</i> 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)

357 **Inoculation methods**

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

377

378 **Evaluation of the success of root inoculation**

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

392 **Mulching**

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

406 **Pest species and competitors**

407 Contamination of orchard soil by unwanted ectomycorrhizal fungi, including the undesirable *Tuber* species,
408 which outcompete the commercial species, is an ongoing problem for truffle producers. For example, *T.*
409 *brumale*, which outcompetes *T. melanosporum* in host roots, has been introduced into Eastern Australia (DPIRD
410 2021). Other ectomycorrhizal species are likely in truffle orchards, including *Tomentella* spp., *Suillus* spp.,
411 *Helvella* spp., *Scleroderma* spp., and *Inocybe* spp. (Ori et al. 2023). Control of diseases of the host species is
412 also essential. For example, *Armillaria* root rot, present in many truffle-growing areas of the world, causes
413 dieback of oaks (Davari and Askari 2005; Kelley et al. 2009; Kim et al. 2022). Molecular analysis of the soil of

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

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

428 It cannot be assumed that all other soil and endophytic fungi will be disadvantageous for truffle production.
429 Both endophytes and ectomycorrhizas utilise the carbon source of host plants (Martin et al. 1987; Wang and Dai
430 2011), and thus there will be competition between other endophytes and *Tuber* spp. for the host root habitat. For
431 example, the abundance of arbuscular mycorrhizal fungi on the understory plants of *T. melanosporum* truffle
432 brûlé decreases in the presence of *T. melanosporum* (Taschen et al. 2020). However, some endophytes may be
433 desirable or essential for maximum host health and growth as endophytes may increase the fitness of host plants
434 by improving tolerance to biotic and abiotic stress and decreasing water consumption (Rodriguez et al. 2009).
435 Investigating the endophytic species of highly productive host trees may elucidate the importance of other
436 endophytic species.

437 **Mycorrhizal helper bacteria that enhance truffle production or quality**

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

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

463

464 **Conclusion**

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

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

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

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847 **Declarations**

848 The authors declare no competing interests.