
Sex: a welcome frontier in truffle cultivation

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Truffles are one of the most highly prized of all fungi. Recent advances and genetic studies have furthered our understanding of the mode by which these fungi reproduce. The understanding that some truffle species require a mating partner in order to produce fruiting bodies leads to many questions and opportunities in truffle cultivation. Here, a summary and analysis of the most recent work is presented. The potential applications of this understanding are discussed along with proposals for in-field modifications to improve the truffle yields of plantations.

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Tuber, a fungal genus in demand

Hypogeous fungi belonging to the genus *Tuber* have long been held in high esteem. They are valued so highly by many cultures, primarily for their culinary properties, that just one kilo of *Tuber spp.* (truffles) can command market prices in the thousands of Euros. This reverence of the subterranean fungi is not a modern phenomena, historical evidence for this appreciation also spans many cultures from representations in the Islamic religion (Hadith, sahih muslim book 023, hadith number 5084) to accounts of consumption throughout the roman empire (Hunt 2010).

Throughout history, the origin and growth of truffles and other hypogeous fungi has been subject of many myths and legends. In arid regions, its often been claimed that when lightning hits the ground it causes truffles to form. The Bedouins of the Negev still refer to desert truffles as ‘the thunder fungus’ (Shavit 2007). Other ideas abound, for example the greek physician Pedanius

Dioscorides believed truffles to be a form of tuberous root (Dioscorides, 1st century).

The road to understanding the biology of *Tuber species* has been a long one. Despite their previously unknown biology, there were some early truffle cultivation attempts with varying levels of success. One of the best documented cases was that of Joseph Talon in France. Talon would transplant young oak trees growing under truffle producing parents to new fields. Some lifted seedlings would have *Tuber spp* established on their root systems and where suitability of natural soils allowed, fruiting bodies would be produced.

However, it was not until the 1970s and with the work of a number of dedicated researchers, that a more informed approach was employed. Work pioneered by Gerard Chevalier allowed the production of trees whose roots had been artificially inoculated with *Tuber species*, which were then planted out into the field. This approach was based on our current understanding that *Tuber species*

depend upon a symbiotic association with the host plant for their survival, categorising these fungi as mycorrhizal. These early experiments kick-started what is, today, a global agricultural movement to produce truffles. This has been so successful that in some regions, such as France, that over 95% of the annual truffle production is from cultivated orchards. Despite these technology-led successes, there have also been many failures. Although our understanding of the ecological parameters needed for truffle cultivation, such as climatic conditions, soil structure (Wedén et al. 2004) and soil pH (Thomas 2012) for many *Tuber species* is good, there is little consensus on the correct management methods for different on-site conditions.

A genetic leap, sex and a new understanding

Despite the traditional focus by truffle cultivation researchers and practitioners on site selection and in-field methodologies, it is a new genetic understanding that is currently creating much interest. Recent research is finally answering important questions in truffle cultivation. In 2010 the genome of *Tuber melanosporum*, the world's most commercially valuable cultivatable truffle species, was sequenced for the first time (Martin et al. 2010). This research created an opportunity to investigate in greater depth than ever before the biology of these expensive fungi.

To those involved in the industry of truffle cultivation, the mode by which truffles reproduce had become one of the most important topics and the sequencing of *T. melanosporum* opened the door for investigation.

Truffles belong to a group of fungi whose majority have spores that develop sexually and in a small sack, known as an ascus (termed ascomycetes). This group of fungi can be further sub-divided into those that produce spores where mating is enabled through contact solely with the same fungi clone (homothallic) and those where interaction with genetically distinct colonies is essential (heterothallic).

Previously, it was believed that *T. melanosporum* was homothallic (Bertault et al. 1998). If this was indeed the case, meaning that truffles could be produced without the need for a mating partner, then only environmental

variables need to be considered for its cultivation. However, as researchers began to look at *Tuber species* at a genetic level, more and more evidence emerged for an ability to out-cross (Rubini et al. 2005; Paolocci et al. 2006; Riccioni et al. 2008). In fact, the groundbreaking unravelling of the *T. melanosporum* genome eventually led to clear evidence that sexual reproduction is actually common place and that there are two key mating types involved (Martin et al. 2010; Rubini et al. 2011). We have therefore arrived at a point where *T. melanosporum* is now acknowledged as heterothallic rather than homothallic. Further, the two mating types are identified as MAT1-1 idiomorph and MAT1-2 idiomorph.

So we now know that *T. melanosporum* requires a mating partner. Previous to this discovery, it was known that some heterothallic ascomycetes employ chemical-gradient attraction and different mating type strains have been recognised (Bistis 1981; Bölker and Kahmann 1993; Dohleman and Thorner 2001). Then, Martin et al. (2010) demonstrated that such components are also present in *T. melanosporum*. Through this leap in the understanding of *T. melanosporum* sexual reproduction we begin to build a picture in which *T. melanosporum* is not a lonely fungus, reproducing by selfing, but instead demands, seeks out, and recognizes opposing mating types. The challenge now is to apply this new understanding to truffle cultivation.

Practically applied to truffle cultivation

Truffle cultivators and truffle researchers are often divided as to the ideal plantation management regimes, with wide ranging and often conflicting ideas. Our new understanding of the sexual nature of *T. melanosporum* may facilitate a greater understanding as to the merits, or not, of many of these systems. For example, a concept often employed in areas of France is that of 'competitive space' (Sourzat 2011). In the practical application of this approach, an attempt is made to manage the plantation so that the extent of truffle mycorrhiza from one tree is not allowed to reach that of another tree. However, now we know that *T. melanosporum* needs another mating type, the merit of this approach is

justifiably questioned.

Mature trees in a natural environment are often host to a whole community of mycorrhizal fungi. As well as a range of species, there may also be a range of 'colonies' of individual species. It is rare for a host plant root system to be colonized by just one genetic individual of a particular species. When we consider truffle cultivation, young truffle-inoculated trees may be produced by fungi that have been cultured artificially, for example by root contact with established mycorrhiza or produced by using spore derived material. In the latter process, the large number of spores being applied means that the inoculation is unlikely to occur by just one genetic individual. In such an example, it may be that trees will form associations with a number of genetically different individuals of both mating types. Applied to our question of root interaction, in this situation it could be hypothesized that the tree does not need to interact with another host tree in order to produce truffles. Rubini et al. (2011) presented an investigation into this intriguing prospect. Rubini and colleagues have eloquently shown that, when using spore-based inoculums, a host tree is indeed colonized by a range of genetic individuals of both mating types. Crucially, however, the conclusion by Rubini and colleagues is that over a period of 19 months one individual mating type begins to dominate resulting in a tree eventually playing host to just one mating type. However, when we look at the presented data in greater detail, the accuracy of this conclusion becomes questionable. Trees were treated with spore-based inoculums and monitored at months 6 and 19 from treatment. The analysis method involved sampling 10-15 *T. melanosporum* colonised root tips (TMC) in each plant tested. This number of TMC is a small sample size that becomes proportionally less representative as the number of TMC substantially grows between 6 and 19 months. We know that *Tuber species* established on a root system spread by colonising newly emerging and proximal root tips. Therefore, if sampling was confined to one small specific area of the root zone, it can be justifiably hypothesized that sequencing of those TMC tips will show a greater dominance of a mating type as the tree ages and the established fungi colonise new root tips. Even

with this potential bias, out of 12 trees tested only 3 showed only one mating type in TMC and soil-based amplification. However, although statistics are not provided there does appear to be mating type dominance in many of the tested trees and taking the Rubini et al. (2011), conclusion as accurate, the results may go some way to explaining why production is lower than expected in many truffle orchards.

The work described above clearly presents a hypothesis of truffle reproduction and development within host plant roots. If the conclusions are correct, then the challenge is to apply this knowledge practically. Using spore-inoculated trees to create a truffle plantation should, assuming no mating-type bias, produce a plantation with a spread of mating types throughout. This would allow the interaction of different mating types and sexual reproduction. However, following the conclusions of Rubini et al. (2011), there may be inadvertent and significant groupings of individual mating types, leaving some colonies on host trees sexually isolated. It has been suggested that this could be one of the leading causes of disappointing yields within orchards. Indeed, Rubini and colleagues go further by looking at mating types in a natural truffle area and conclude that the minimum distance between sites where different mating types were detected was 50m, indicating that mating-type dominance may be a potential yield-lowering issue. Assuming the conclusions are accurate and mating-type dominance occurs and leads to unsatisfactory yields, then it would seem logical that there are principally two areas where procedures can be altered to help improve production; The production of trees and in-field modification.

Production of trees

The understanding of mating types and the development of type dominance within young inoculated trees raises the prospect of producing trees with identified mating types. These can then be strategically planted within an orchard to produce the greatest chance of sexual reproduction and hence, truffle production. However, producing such trees from spore based inoculums would mean holding trees in pots for a minimum of 19 months and probably far longer before an

identified mating type dominance has occurred on a satisfactory number of trees (Rubini et al. 2011). By this stage, the tree is a poor candidate for a truffle plantation due to reduced vigour and root-development considerations (Halter and Chanway 1993). As production by tree-to-tree mycorrhizal transfer is unviable on a commercial scale, the alternative is to produce trees from mycelia and mating-type identified material produced in vitro.

Producing trees from cultured mycelia material is a previously utilized technique (Zambonelli and Branzanti 1990) that can be adapted to produce a mating-type identified and colonised host plant. However, this process creates two practical problems. Firstly, using only a few cloned individuals to produce a large number of trees for truffle plantations brings problems of diseases susceptibility, unknown long-term performance of individuals and decreased plantation resilience (Pinkerton et al. 1993). Additionally, criteria for clone selection would need to be broad and detailed. Indeed, inappropriate selection of cloned material could be problematic. The alternative, producing a broad range of cloned genetic individuals to inoculate from would be costly, time consuming and challenging. Using such a system could financially negate any production advantage.

The drawbacks of producing mating-type identified, cultured mycelia colonised plants to establish an orchard means that this system may not be an economically viable approach. If this is indeed the case then the application of mating-type knowledge to improve production becomes an in-field prospect.

In-field modification

There are two options to correct mating type dominance in existing orchards. The first is to plant trees of opposing mating types within areas dominated by a single mating type (or replace existing trees). The second option is to use spore or cultured inoculums in an attempt to introduce other mating types onto the existing host trees. Both these proposed methodologies have uncertainties and are potentially problematic.

First, let us consider the introduction of an opposing mating type dominated host tree. Based on the research into pot-grown trees, we

know that it can take several years for a mating type to become dominant. Additionally, in all but the most closely monitored orchards, signs of a potential yield-problem may not become evident until expected harvests do not materialize. In this case, we can assume a time frame of at least seven years and usually longer. At this stage, the existing trees within the plantation will have an established root system and any introduced trees hosting opposing mating types will have a far smaller root system with the extent at planting being determined by the pot size of the introduced trees. This would create a situation where in order for the mating types to quickly come in contact with each other, high numbers of the opposing mating type trees will need to be established. Even then, we do not know if this will be beneficial for just the portion of the established mycorrhiza that are in direct contact with the newly planted host-tree or if the positive impact is likely to spread quickly throughout a larger area of the plantation. Even when planting at a high density it will take a number of years for significant root interaction to occur. However, another unknown of this approach is whether the opposing mating type will survive in such a situation or whether the existing, locally dominant mating type will colonize and take over the new host, as has been shown on individual trees roots in pot-based experiments. Even in the unlikely scenario whereby introducing the 'helper-trees' has a significant and quick impact, the introduction of more trees alter the environmental conditions of the plantation by, for example, introducing higher levels of shading and water demand. Such factors, along with the cost implications of creating mating-type identified suitable trees, would need to be considered. It is clear that this approach requires significant research, spanning a number of growing seasons.

The second option, of attempting to introduce an opposing mating type without a host plant has a similar set of challenges. The first obstacle in this situation is to establish the opposing mating type onto the existing root system. We know from mature-tree inoculation work (Thomas 2007) that it is possible to use spore derived inoculums to establish *Tuber species* onto the root system of mature existing

trees, although the application of cultured and identified mating types would likely be a more dependable approach. However, if the conclusions of Rubini et al., (2011) from pot-based experimentation are accepted and are directly applicable to more mature trees in the field, it is possible that any success in establishing an opposing mating type will be short-lived as the locally prevalent mating type continues to dominate and eliminate the introduced type. Anecdotal evidence from a number of truffle cultivators have highlighted a belief in the benefits of ploughing old fruiting bodies back into the plantation soil. If, as claimed, this is beneficial, it may be an indication as to the likely benefits of spore-introduction to counter-act mating type dominance.

It can be logically argued that the most suitable in-field approach is the establishment of host plants with opposing mating types within areas of a plantation dominated by a single mating type. However, long-term investigation is needed to establish whether this is a viable method and also to trial the introduction of spore and cultured mycelia inoculums. Other methodologies also warrant investigation.

A red herring

The discussions in prior sections of this paper are primarily based on conclusions in one recently published paper. However, the conclusion of mating-type host dominance in the field has not been supported independently, so we must remain open to the idea that such dominance may not be common-place. A recent article from Australia has indeed disputed the findings of Rubini et al. (2011). Linde and Selmes (2012) sought to investigate the occurrence of the mating type dominance within the truffle orchards of Australia. In doing so they found that 50% of host trees producing *T. melanosporum* fruiting bodies and 42.9% of host trees not currently producing *T. melanosporum* fruiting bodies had both mating types present. Further, Linde and Selmes hypothesize that the reason why both mating types were not found on all in-field trees, was due to insufficient sampling. It is important to note that Rubini et al. also had a very small sampling size and this may have contributed to

their findings. Additionally, Linde and Selmes cast doubt on the dominance of a mating type occurring in inoculated seedlings at 19 months from inoculation. In Linde and Selmes report, they show that in all 10 saplings analysed, from two different commercial nurseries and at 24 months of age, both mating types occurred on the mycorrhized root-tips.

It is clear that in order to elucidate the importance of mating type, we need further data collection to incorporate higher sampling rates as well as the incorporation of abiotic and biotic variables. In this way we will learn more about the existence and relative importance of local mating-type dominance.

Conclusion

The ideal conditions and methodologies for truffle cultivation have long divided many researchers and practitioners. Recent advances in the identification of mating types may bring some unity. However, the identification of mating types raises the need for investigation not only of the existence and importance of mating type dominance but also how in-field mating-type dominance can be corrected, if necessary. There are several potential corrective methodologies and perhaps the most promising is the introduction of host plants colonized with opposing mating types. However, there is a strong need for further in-depth investigation in order to assess these practical in-field methodologies. Finally, it should be remembered that the identification of mating types is a great leap forward but there are still many other extremely important aspects of truffle cultivation that warrant further study.

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