What makes a good truffle infected tree?

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Abstract

Modern truffle cultivation is based on planting *Tuber* infected plants in suitable sites. The first step is the production of truffle infected plants in greenhouses, generally using spore inoculation techniques. Other less common methods include the inoculating plants with pure cultures and the mother plant techniques.

Regardless of the inoculation technique used, it is advisable to check the geographic origin of the inoculum, and selecting the most suitable one for the climatic conditions of the site where the plants are to be planted. It is also important to verify the quality of the inoculum before inoculating the plants. In particular, when using spore inoculation techniques, the ascomata used for making the spore suspension should undergo morphological observation and molecular testing to verify the absence of less valuable *Tuber* species. It is also advisable to assess the abundance of mature spores and their vitality particularly when the inoculum is not produced from fresh truffle. In contrast when mycelial inoculation or mother plant techniques is used it is important to ensure that the plants are carrying two compatible mating types and that the mother plants are free of contamination with other ectomycorrhizal fungi.

Résumé

Key words: inoculum, quality control, Flouricene-diacetate, vital stain
Introduction

Modern truffle (Tuber spp.) cultivation is based on planting adequately infected plants raised in controlled conditions in greenhouses and planting them in suitable locations. The methods used are based on those devised by French and Italian scientists in the 1960s where the inoculum was spores, cultures, or sections of infected root. Although the general methods that can be used are well documented in scientific articles or chapters of books, nurserymen often fail to closely follow these techniques, so commercially produced plants, for example, may be poorly infected with the inoculated species of Tuber or are contaminated with other ectomycorrhizal fungi including the wrong species of truffle (Zambonelli & Salomoni, 1993; Amicucci & al., 1998).

Here we provide advice aimed at nurserymen for improving the production of Tuber aestivum infected plants and measures that should be taken by growers after outplanting. Although this advice is aimed specifically at T. aestivum they are equally applicable to the cultivation of other species of Tuber spp.

Geographic origin of the inoculum

The spore inoculation technique is still the most commonly used method for producing Tuber infected plants because of its simplicity. The spore inoculum is obtained from fresh, chilled, dried or frozen ascocarps and used to inoculate sterile seedlings or cuttings of suitable host plants (Zambonelli, 1990). Generally $10^6$ to $10^7$ spores are used for each seedling which corresponds to about 1 g of mature truffle, more if the truffles are not fully mature (Hall & al., 2007). Consequently, to inoculate say 10,000 plants about 10 kg of mature truffles are required. If far fewer than this are used then there is an ever present danger that the plants will be poorly infected, if at all, and prone to contamination by opportunistic Ascomycetes such as Sphaerosporella in the nursery and other ectomycorrhizal fungi that may be present at outplant sites (Hall & al., 2007).

The quantities of truffle required to inoculate the hundreds of thousands of truffle trees produced in Europe annually means that nurseries will accept whatever truffles are available from wholesalers regardless of their origin. Consequently, plants might be mycorrhized with fungal strains ill adapted to climatic and edaphic conditions at outplant sites so that a grower in the coolest part of geographic range may finish up growing plants that had been mycorrhized with truffles collected from the hottest part of Europe (Hall & al., 2010, Rubini & al., 2007). This is even more important for T. aestivum than for T. melanosporum. This is because T. aestivum has the widest distribution of any of the edible truffles (Chevalier & Frochot, 2007) and a wider genetic diversity (Mello & al., 2002) and found from Spain to eastern Europe and from Gotland, Sweden, to North Africa. The island of Gotland, off the east coast of Sweden, is probably the coolest part of Europe where the Burgundy truffle is found with a mean daily temperature in July of 15.9 °C and –1.1 °C in January whereas centres such as Clermont-Ferrand, Paris, and Perugia represent the warmer zones with a mean daily temperature of 24 °C in July and 5 °C in January (Hall & al., 2008).

The mycelial inoculation technique which our research group is perfecting (Zambonelli & Iotti, 2006) opens up the possibility of genetically selecting the optimum fungal strains for infectivity, affinity for host plant and ecological conditions. It consists of isolating the mycelium, freeing it of any contaminants, and then using the bulked up mycelia to inoculate seedlings or cuttings under controlled conditions. However, if it is shown that ecological strains are present in truffles, as suggested by Hall & al. (2010), then it will be necessary to isolate suitable strains from each unique environment so creating a Tuber aestivum germplasm bank and then raising plants ideally
suited for each area to be planted. This would be a mammoth task and present a real challenge for those establishing truffières in countries where *T. aestivum* is not naturally present.

**Inoculum quality**

Inoculum quality is one of the most important requirements when *Tuber* infected plants are produced. When large quantities of fruit bodies are used to prepare inocula, operators may accidentally incorporate less valuable species of truffles which can then become established on the host plants. Twenty-five species of hypogeous fungi belonging to Ascomycetes and Basidiomycetes were found to be commercialised together with *T. aestivum* including the similar *T. mesentericum* Vittad. and some rare truffles such as *Tuber panniferum* Tul. and *Tuber malenconii* Donadini, Riousset, G. Riousset & G. Chev. (Paci & al., 2008). Consequently these less valuable hypogeous fungi can contaminate entire batches of plants. Moreover, the mycorrhizae of some contaminants, are very similar to those of *Tuber aestivum*, difficult to identify using morphological methods, and so can go unnoticed during quality control prior to outplanting. For example, the dense woolly mycorrhizal mantle surface and pseudoparechimateus angular mantle cells (fig. 3) formed by *Tuber mesentericum* (Zambonelli & al., 1993; Zambonelli & al., 1995, Granetti, 1995; Granetti & al., 2005) and *m*,(Cooke & W. Phillips) Arnould also called the AD type (Giraud, 1988) or *Quercirhiza quadratum* (Águeda & al., 2008) are not dissimilar to mantles of *T. aestivum*. While it is possible to distinguish the mycorrhizas on the characteristics of the cystidia, which are never ramified in *Tuber aestivum* mycorrhizae, molecular methods of identification (Mello & al., 2002) can be used to give unequivocal identification of both the identity of the mycorrhizae and the ascomata used for inoculum.

A major problem is the frequent use of early season truffles for inoculum. These are much cheaper but often they are very immature. Obviously the effectiveness of such inocula is likely to be reduced either because of the small number of mature spores present or because of an incapacity to germinate (fig. 1).

Another aspect not often taken into consideration is the viability of spores in stored truffles. Usually truffles are stratified in humid sand in a refrigerator at 2-5°C before use. This method seems to improve spore germination probably through the effect of microorganisms and root exudates, and dispenses with the need to release the spores from the ascus (Hall & al., 2007) However, holding truffles in a refrigerator for more than 2 years progressively reduces the ability of spores to germinate (fig. 2). The decrease of inoculum potential of *T. aestivum* spores over 5 years contrasts with the results obtained for *Rhizopogon sp*. where there was no deterioration in viability over 4 years (Bruns & al., 2009). Another technique used to store truffles is freeze drying or simple drying (Zambonelli, 1990) although their effects on long term spore viability are unknown. In an attempt to resolve some of these issues we have carried out some work to find a staining technique to assess spore viability. Initial studies have shown that vital Flouricene-diacetate (FDA) (Rotmanand & Papermaster, 1966) can be used despite some auto-florescence masking. The vital dye FDA can also be used in combination with non vital ones such as Evans blue (Gaff & Okong’o-Ogola, 1971; Turner & Novacky, 1974) (fig. 3).

**Mating types**

In heterothallic Ascomycetes, the capacity to form ascomata is dependent on the presence of two compatible mating strains (Pöggeler & al., 2006). Recently, Paolocci & al. (2006) demonstrated that *Tuber* is also heterothallic and the *T. melanosporum* genome sequencing has recently revealed
the presence of mating-type genes (MURAT & MARTIN, 2008). Hence, within a truffle there should be equal numbers of haploid ascospores of each of two mating types and so when plants are inoculated with $10^7$ spores two mating types will be present. In contrast, mycelial cultures are usually obtained by growing hyphae derived from haploid glebal tissue (IOTTI & al., 2002) of maternal origin, and so carries only one mating type. Consequently, plants need to be inoculated with mycelia of two compatible mating types. Similarly, where mother plant techniques are used it is essential that two mating types are also present.

**Conclusion**

During the production of *Tuber* infected plants, and regardless of the methods used, it is critical that great care is taken over the quality of the inoculum to prevent contamination with other ectomycorrhizal fungi and achieve the production of ascoma in the field.

The mycelial inoculation technique currently being developed by our research group shows great promise because it opens up the possibility of genetically selecting the optimum fungal strains for ecological conditions and truffle production. It also has the added advantage that fruiting bodies do not need to be purchased and there is no risk of introducing contaminants with the inoculum. However, with our technique it is essential that plants are inoculated with compatible mating types if there is to be any chance of ascoma production in the field. The *Tuber melanosporum* genome sequence project and the subsequent identification of mating type genes opens up the possibility for selecting strains of opposite mating types for inoculation purposes and then verifying their presence on the inoculated plants prior to outplanting (MURAT & MARTIN, 2008).

**References**

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Legend of figures

Fig. 1 - Immature empty ascus (a) and ascus containing mature spores of T. aestivum (b). Bar = 10 µm.

Fig. 2 – Percentage of ectomycorrhizal infection (number of tips colonized with T. aestivum) total number of tips x 100 counted after ZAMBONELLI & GOVI, 1990) with T. aestivum obtained inoculating 1 month old seedlings of Quercus pubescens Willd. with puréed T. aestivum ascomata.
(3 g for plant) 1 year after inoculation. The ascomata were preserved stratified in moistened sand in fridge at 5°C for six months, 2 yr, 5 yr 10 yr and 15 yr.

**Fig. 3** – Unramified cystidia of *T. aestivum* mycorrhizae (a) and right angle branched cystidia of *T. woolhopeia* (b) mycorrhizae. Bar = 10 μm.

**Fig 4** Vital spores from a fresh ascoma (a) stained with Flouricene-diacetate (COLGAN & CLARIDGE, 2002) (excitation filter 450-490 nm, barrier filter 515-555 nm, Nikon Eclipse TE 2000-E microscope) giving a greenish fluorescence (b) Non vital spores form an autoclaved ascoma (120°C/60’) (c) stained with Evans Blue in water (excitation filter 330-380 nm, barrier filter 420 nm, Nikon Eclipse TE 2000-E microscope) giving a reddish fluorescence (d). Bar = 100 μm.