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 to select 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 are 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.

Zusammenfassung: Moderne Trüffelkultur basiert auf der Pflanzung von *Tuber*-infizierten Wirtsbäumen in geeigneten Standorten. Der erste Schritt ist die Herstellung der Trüffel-infizierten Pflanzen in Gewächshäusern, üblicherweise durch Sporeninokulation. Andere, weniger bekannte Methoden sind die Inokulation der Pflanzen mit Reinkulturen und die Mutterpflanzentechnik.

Ungeachtet der verwendeten Inokulationstechnik ist es anzuraten, den geographischen Ursprung des Inokulums zu überprüfen und das für die klimatischen Bedingungen des vorgesehenen Auspflanzungsortes am besten passende auszuwählen. Es ist auch wichtig, die Qualität des Inokulums vor der Inokulierung zu verifizieren. Besonders bei der Sporeninokulationstechnik sollten die für die Sporensuspension vorgesehenen Ascomata morphologisch untersucht und molekular getestet werden, um die Anwesenheit weniger wertvoller *Tuber*-Arten auszuschließen. Es ist weiters anzuraten, die Menge und Vitalität reifer Sporen abzuschätzen, vor allem wenn das Inokulat nicht von frischer Trüffel hergestellt wird. Hingegen, wenn Myzelinokulation oder die Mutterpflanzentechnik angewandt werden, sollte sichergestellt sein, dass die Mutterpflanzen zwei kompatible Kreuzungstypen tragen und dass die Mutterpflanzen nicht von anderen Ektomykorrhizen kontaminiert sind.

Résumé: La trufficulture moderne est basée sur le plantage d'arbres mycorrhizées par la *Tuber* dans un lieu idéal. La première étape c'est la production d'arbres mycorhizées sous serre, généralement en utilisant la méthode de l'inoculation sporale. Il existe d'autres méthodes moins connues, comme la méthode de la culture pure ou celle des "plantes-mères".

Quoi qu'il en soit de la méthode utilisée, il est conseillé toujours de contrôler l'origine géographique de l'inoculum et de choisir celui qui s'adapte le mieux aux conditions climatiques de la zone où les arbres mycorrhizées doivent être plantés. Il est aussi important de vérifier la qualité de l'inoculum, avant son utilisation. En particulier, surtout quand on adopte la méthode de l'inoculation sporale, la truffe utilisée pour la suspension doit être soumis à des observations morphologiques et à des tests moléculaires, pour exclure le risque de contamination de spores de *Tuber* indésirables. Il est aussi conseillé de vérifier la maturité des spores et leur vitalité, surtout quand l'inoculum n'est pas produit à partir de truffe frais. Par contre, quand on utilise la méthode de l'inoculation sporale ou celle des plants-mères, il est très important de s'assurer que les arbres mycorhizées aient les deux types séxuels et que les plantes-mères ne soient pas contaminées par des champignons mycorrhiziens indésirables.

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).

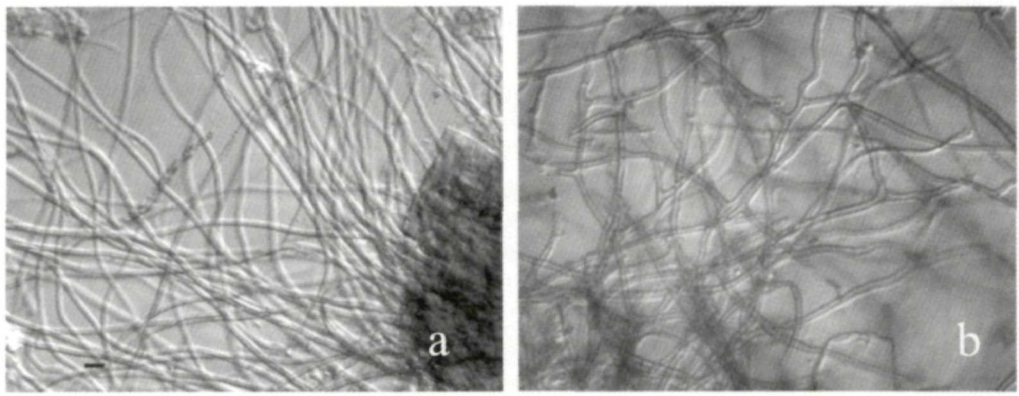
Here we provide advice aimed at nurserymen for improving the production of *Tuber aestivum* infected plants and measures that should be taken by growers after outplanting. Although this advice is specifically for *T. aestivum* it is equally applicable to the cultivation of other *Tuber* species.

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 10000 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 brunnea* (ALB. & SCHWEIN.) SVRČEK & KUBIČKA 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 is 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 of 15.9 °C in July 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).



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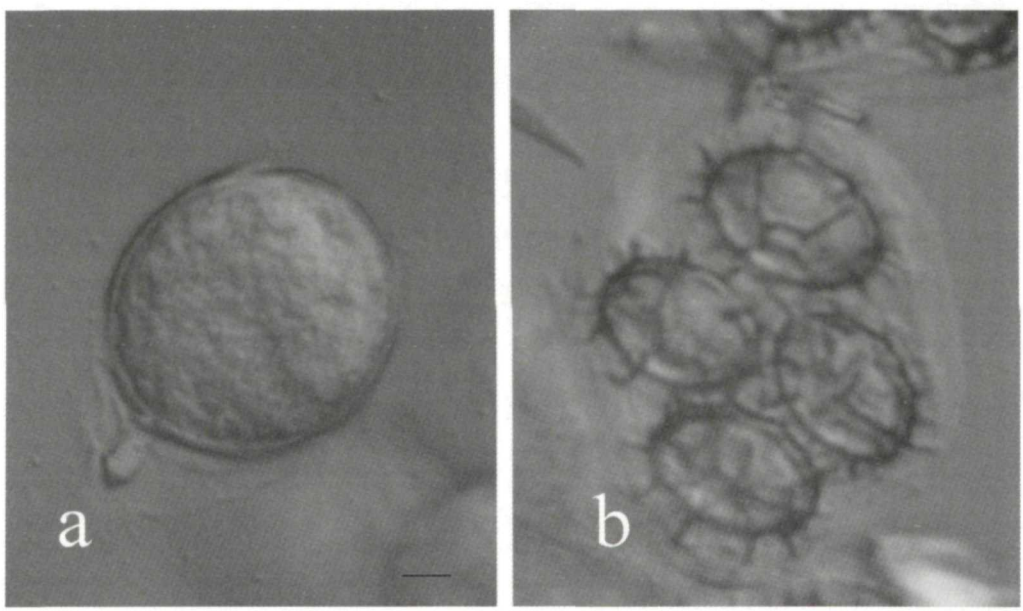

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Fig. 1. *a* Unramified cystidia of *Tuber aestivum* mycorrhizae, *b* right angle branched cystidia of *Trichophaea woolhopeia* mycorrhizae. – Bar: 10 µm. – Fig. 2. *Tuber aestivum. a* immature empty ascus, *b* ascus containing mature spores. – Bar: 10 µm.

The mycelial inoculation technique which our research group is perfecting (ZAMBONELLI & IOTTI 2006) opens up the possibility of genetically selecting the op-

timum 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 *T. panniferum* TUL. and *T. malençonii* DONADINI, RIOUSSET, G. RIOUSSET & G. CHEV. (PACI & al. 2010). Consequently these less valuable hypogeous fungi can contaminate entire batches of plants. Moreover, the mycorrhizae of some contaminants, are very similar to those of *T. 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 pseudoparenchymatous angular mantle cells formed by *T. mesentericum* (ZAMBONELLI & al. 1993, 1995; GRANETTI 1995; GRANETTI & al. 2005) and *Trichophaea woolhopeia* (COOKE & W. PHILLIPS) ARNOULD, also called the AD type (GIRAUD 1988) or *Quercirhiza quadratum (*ÁGUEDA & al. 2008), are similar to mantles of *Tuber aestivum* (Fig. 1). While it is possible to distinguish the mycorrhizas on the characteristics of the cystidia, which are never ramified in *T. 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. 2).

Another aspect rarely 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 two years progressively reduces the ability of spores to germinate (Fig. 3). The decrease of inoculum potential of *T. aestivum* spores over five years contrasts with the results obtained for *Rhizopogon* spec. where there was no deterioration in viability over four years (BRUNS & al. 2009). Another technique used to store truffles is freezing or 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

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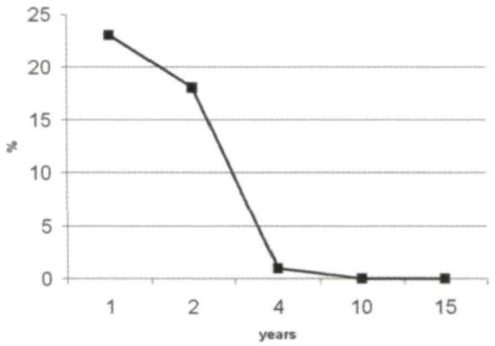

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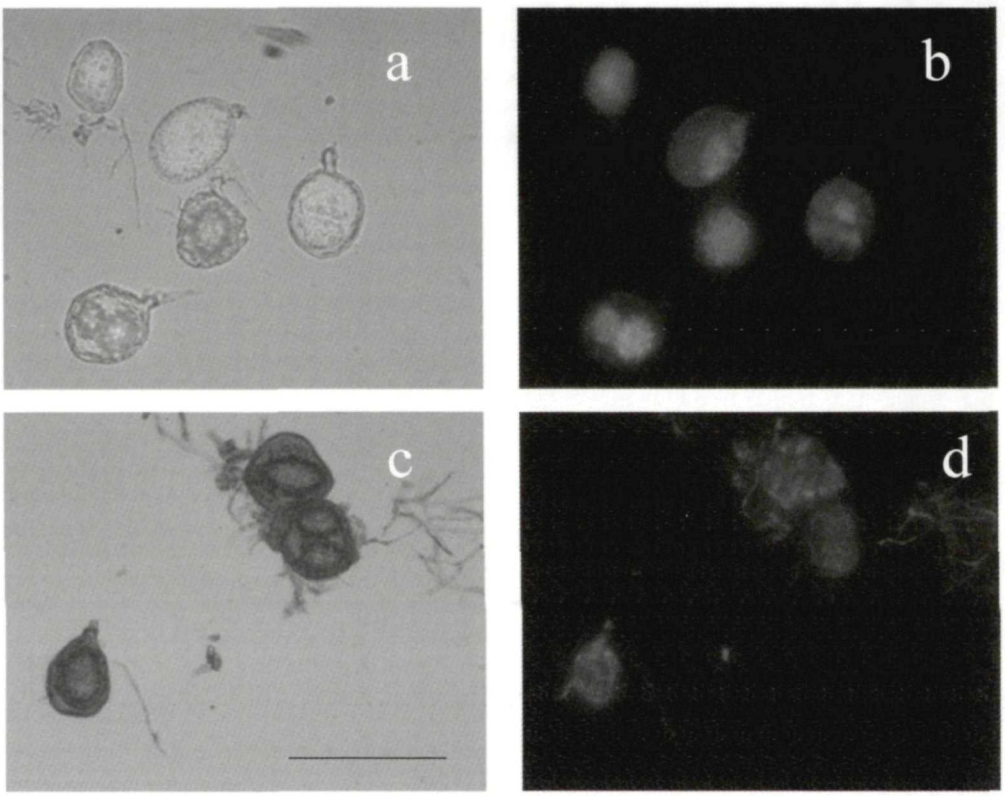

Fig. 3. Percentage of ectomycorrhizal infection (number of tips colonized with *Tuber aestivum* / total number of tips × 100) counted after ZAMBONELLI & GOVI (1990) with *T. aestivum* obtained inoculating 1 month old seedlings of *Quercus pubescens* with puréed *T. aestivum* ascomata (3 g for plant) 1 year after inoculation. The ascomata were preserved stratified in moistened sand in refrigerator at 5 °C for six months, 2, 5, 10, and 15 years. – Fig. 4. *Tuber aestivum. a* vital spores from a fresh ascoma, *b* stained with Fluorescein-diacetate (COLGAN & CLARIDGE 2002) (excitation filter 450-490 nm, barrier filter 515-555 nm, Nikon Eclipse TE 2000-E microscope), giving a greenish fluorescence, *c* non vital spores from an autoclaved ascoma (120 °C/60'), *d* 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. – Bar: 100 µm.

work to find a staining technique to assess spore viability.

Initial studies have shown that vital Fluorescein-diacetate (FDA) (ROTMAN & 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. 4).

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 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).

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