DOI: 10.6092/issn.2465-311X/5593

Current status of truffle cultivation: recent results and future perspectives

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Abstract

In this review the current status of truffle cultivation in Europe and outside Europe is reported. While the cultivation of *Tuber melanosporum* (Périgord black truffle), *Tuber aestivum* (summer or Burgundy truffle) and *Tuber borchii* (bianchetto truffle) gave good results, only the Italian white truffle (*Tuber magnatum)*, which is the most expensive, has yet to be successfully cultivated.
In future a revolutionary approach to truffle cultivation would be the application of mycelial inoculation techniques for producing *Tuber* infected plants which will allow to select the fungal strains adapted to specific climatic, edaphic conditions and hosts. The new insights which will be gained by the extensive *Tuber* genome sequencing programme will also help to improve truffle cultivation techniques.

Keywords: *Tuber melanosporum; Tuber magnatum; Tuber borchii; Tuber aestivum;* cultivation; mycelial inoculation

Riassunto

I tartufi sono funghi ascomiceti appartenenti all'ordine delle Pezizales anche se molti ricercatori considerano "veri tartufi" solo le specie apparteneti al genere *Tuber*, che comprende le specie di maggiore interesse gastronomico e commerciale quali *Tuber melanosporum* (tartufo nero pregiato), *Tuber magnatum* (tartufo bianco pregiato), *Tuber aestivum* (tartufo estivo o uncinato) e *Tuber borchii* (tartufo bianchetto). L'elevato valore economico di questi tartufi ha suscitato grande interesse riguardo la loro coltivazione fin dal lontano rinascimento. La nascita della tartuficoltura moderna, tuttavia, risale agli anni 70 in seguito alla scoperta della naura ectomicorrizica del tartufo.
Oggi la tartuficoltura è diventata un'importante attività agricola in Europa e nel mondo. In Europa la produzione di *T. melanosporum* nelle tartufaie coltivate supera addirittura quella delle tartufaie naturali, mentre in Australia, dove il tartufo non cresce naturalmente, si ritiene che la produzione nel 2015 abbia raggiunto le 8 tonnellate. Oltre al tartufo nero pregiato sono coltivati con successo anche *T. aestivum* e *T. borchii*, mentre la coltivazione del tartufo bianco pregiato è ancora problematica. Comunque, nonostante i significativi successi raggiunti, alcune tartufaie restano improduttive o producono pochi ascomi. Ciò è conseguenza anche della mancanza di un comune regolamento europeo di certificazione delle piantine tartufigene che garantisca ai tartuficoltori la presenza di un prodotto vivaistico di qualità in tutti i mercati europei. Attualmente le piantine micorrizate sono prodotte esclusivamente mediante inoculazione sporale; l'applicazione dell'inoculazione miceliare potrebbe migliorare le tecniche di coltivazione, rendendo possibile l'impiego di ceppi geneticamente selezionati per le loro caratteristiche ecologiche e/o produttive. Il sequenziamento del genoma del tartufo nero

DOI: 10.6092/issn.2465-311X/5593

pregiato (*T. melanosporum*) ha permesso di chiarire alcune fasi del ciclo biologico del tartufo; in particolare, è stato scoperto che è un fungo eterotallico e, perciò, la produzione dei corpi fruttiferi è subordinata dall'incontro di due ceppi appartenenti a "mating type" (tipo sessuale) diverso. Il sequenziamento del genoma di altre specie di tartufo potrà fornirci altre informazioni sulla loro biologia contribuendo a migliorare le tecniche di coltivazione. La genetica potrà inoltre fornirci indicazioni utili a capire il ruolo dei microrganismi associati al tartufo e magari a suggerirci approcci biotecnologici alternativi per il loro utilizzo in tartuficoltura.

Parole chiave: *Tuber melanosporum, Tuber magnatum, Tuber borchii, Tuber aestivum*, coltivazione, inoculazione miceliare

Introduction

Truffles are ascomyceteous fungi belonging to several families in the Pezizales that have independently evolved a subterranean mode of existence (Læssøe & Hansen, 2007). While some basidiomycetes have also evolved underground fruiting bodies, in *sensu stricto*, only species in the genus *Tuber*, Tuberaceae, are considered the true truffles (Jeandroz et al., 2008). The genus has been estimated to contain 180 to 230 species (Bonito et al., 2010) distributed worldwide. Most species produce strong aromas to attract their dispersal agents and a few have considerable economic value because of their unique aromas and flavors. All the truffles live in mycorrhizal symbiosis with the roots of suitable host plants (Bonito et al., 2013). *Tuber* were thought to only form ectomycorrhizas but recently it was found that they are also able to form arbutoid mycorrhizas (Lancellotti et al., 2014) and endomycorrhizas with orchids (Selosse et al., 2004).

Fig. 1 - Ascomata of *T. magnatum* (a), *T. borchii* (b), *T. melanosporum* (c) and *T. aestivum* (d)
Fig. 1 - Ascomi di *T. magnatum* (a), *T. borchii* (b), *T. melanosporum* (c) e *T. aestivum* (d)

The most valuable truffles are the European species *Tuber melanosporum* Vittad. (Périgord black truffle), *Tuber magnatum* Pico (Italian white truffle), *Tuber aestivum* Vittad. (summer or Burgundy truffle) and *Tuber borchii* Vittad. (bianchetto truffle) (Fig. 1) (Hall et al., 2007). Other species with a limited market in Europe are *Tuber brumale*, *Tuber macrosporum* Vittad. and *Tuber mesentericum* Vittad. In the U.S.A. several species of truffle are harvested but only the Oregon white truffles (*Tuber oregonense* and *Tuber gibbosum*) and the pecan truffle (*Tuber lyonii*) have a limited local market (Trappe, 2009; Lefevre, 2012).

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There are many species of truffle in China. Some resemble European species and some are collected and exported to Europe. The Chinese black truffle, *Tuber indicum* Cooke & Massee, is quite similar to *T. melanosporum* but with a relatively poor flavor (Riousset et al., 2001) and *Tuber sinoaestivum* which is similar to the European *T. aestivum* (Zambonelli et al., 2012; Zhang et al., 2012). *Tuber oligosperum* (Tul. & Tul.) Trappe is harvested in North Africa, in particular Morocco, and illegally sold in Italy as *T. magnatum* (Boutahir et al., 2013).

The high economic value of truffles has stimulated researchers to find the most efficient methods for cultivating them. This has not been a simple task because of their complex life cycle involves a symbiotic relationship with suitable host trees and it is entirely completed underground where complex relationships with soil microorganisms are involved.

Here, we briefly report on the current status of truffle cultivation, the most recent research information and the future prospects for these diamonds of cuisine.

Early methods

The first Italian and French attempts at cultivating truffles date back to the Renaissance, but successful and commercial cultivation of truffles did not start until the early 1800s when Josef Talon in France developed a crude but effective method for cultivating the Périgord black truffle (Hall et al., 2007). This technique involved sowing acorns collected under oaks that were producing truffles. It was very successful in France in areas where truffle spores were already present in soil but when applied in Italy the method failed (Mannozzi Torini, 1984). Modern truffle cultivation was introduced into France and Italy in the 1970's after the discovery of the mycorrhizal nature of truffles.

Initially three methods for inoculating plants were tried: spore inoculation, mother plant technique and mycelial inoculation. The mother plant technique involved planting seedlings into the rooting zone of a plant know to be mycorrhized with the required truffle. While mycelial inocula also showed promise (Chevalier, 1973) spore inoculation soon became the method of choice for the commercial production of plants.

Current status of truffle cultivation

In the second middle of 1900s truffle cultivation became popular had a great impulse not only in Europe but also in non-European countries. *T. melanosporum* is the truffle which has been the most successfully cultivated around the world but the cultivation of *T. aestivum* and *T. borchii* has also been successful in many countries.

T. melanosporum cultivation

Most of the black truffles produced in Italy, France and Spain (Fig. 2) are now harvested from cultivated truffières and in France only 10% are now harvested from natural areas (Reyna & Garcia-Barreda, 2014). Truffle cultivation gave also very good results in countries like New Zealand, USA and Australia where truffles were introduced by cultivation. In these countries most of the soils are acidic and in their native state unsuited for truffle cultivation. However, Ian Hall showed that some soils could be modified first by intensive liming and then correcting the almost inevitable trace element deficiencies (Hall et al., 2007). This method soon spread to Australia which produced 3 tonnes in 2011 (Hall & Haslam, 2012), which is projected to grow to 8 tonnes in 2015 (Duell, 2012), and could soon overtake French truffle production at least in a poor fruiting year (Hall & Zambonelli, 2012). Cultivated black truffle have also been produced in the USA, Canada, Morocco, Chile and South Africa (Reyna & Garcia-Barreda, 2014; Berch & Bonito, 2014; Zambonelli unpublished data). *T. melanosporum* plantations have also been established in Israel and in several European countries but to the best of our knowledge production has yet to begin.

DOI: 10.6092/issn.2465-311X/5593

Fig. 2 - *T. melanosporum* plantation in Spain
Fig. 2 - Tartufaia coltivata di *T. melanosporum* in Spagna

T. magnatum cultivation

Of the main European truffles only the Italian white truffle, which is the most expensive, has yet to be successfully cultivated. Only few truffières have produced in Italy. Despite extensive plantings of inoculated trees in Italy from the early 1980s to the late 1990s few truffières have produced and then only a few kilograms per hectare (Gregori et al., 2010). The main reason for this seems to be the difficulties in obtaining *Tuber* infected plants in the nursery and significant contamination problems. Before the introduction of molecular methods for the identification of the mycorrhizas, *T. magnatum* inoculated plants were often sold contaminated with other less valuable *Tuber* species such as *Tuber maculatum* Vittad. and *T. borchii*. In fact these highly infective truffles, which are morphologically similar to *T. magnatum*, when accidentally incorporated in the inoculum colonized the entire root systems (Hall et al., 2007). Another factor limitating the cultivation of *T. magnatum* is that its biology and soil ecology is still a mystery. Its mycorrhizas can be obtained in the nursery (Mello et al., 2001; Rubini et al., 2001), albeit with difficulties, but then they seem to disappear in the field (Hall et al., 2007). *T. magnatum* mycorrhizas are also absent in natural productive areas (Leonardi et al., 2013). Recent molecular techniques like q-PCR are now able to follow the development of *T. magnatum* mycelium in the soil (Iotti et al., 2012a) and are beginning to give new insights into its soil spatio-temporal development (Iotti et al., 2014). The possible role of associate bacteria, and in particular of rhizobia, has also been hypothesized in the growth, development, nutrition and fructification of *T. magnatum* (Barbieri et al., 2007; Barbieri et al., 2010). Clearly an in depth study of the strange ecology of this truffle will be needed before routine methods for its cultivation can be established.

T. borchii cultivation

The first publication reporting the successful cultivation of bianchetto truffle was by Zambonelli and colleagues in 2000 in Marina di Ravenna, Italy. Since then its cultivation has become widespread in Italy and more recently in New Zealand where this truffle is particularly appreciated by gourmets and in particular the Chinese community (Hall et al., 2007; Wong personal communication). The first successful cultivation of *T. borchii* in Australia by Peter Stahle was reported in March 2015 (https://trufflefarming.wordpress.com/2015/03/14/first-tuber-borchii-ever-harvested-in-australia/). Recently, *T. borchii* cultivation was successfully introduced in USA (Isikhuemhen personal communication).

DOI: 10.6092/issn.2465-311X/5593

T. borchii cultivation has a great potential because of its broad ecological adaptability, its wide range of angiosperm hosts such as oaks, hazel, and linden, and conifers including pines and cedars (Hall et al., 2007; Zambonelli et al., 2002). Recently it was found to produce mycorrhizas with the pecan tree *Carya illinoinensis* (Wangenh.) K.Koch, and to fruit when associated with *Arbutus unedo* L. opening up the possibility of combining both the cultivation of this precious truffle and the production of edible fruits of these plants (Benucci et al., 2012; Lancellotti et al., 2014). However, there will be difficulties because pecan nuts and *A. unedo* fruits are ready for picking just as the truffles are forming in the soil. Also some rather toxic insecticides with residual activity have been used to control pecan pests.

T. aestivum cultivation

T. aestivum cultivation in Italy and in France began a little later than the cultivation of *T. melanosporum* but has grown rapidly and is now widespread. In France the cultivation of *T. aestivum* is concentrated within its natural boundaries: Auvergne, Champagne, Lorraine, Bourgogne, and Franche-Comte (Chevalier and Frochot, 1997). In Italy out of around 120,000 truffle trees planted per year only 15% are inoculated with *T. aestivum* (Bencivenga et al., 2009). *T. aestivum* has also been successfully cultivated in Sweden by Christina Weden (Weden et al., 2009) on the island of Gotland which represents the northernmost boundary of its natural distribution in Europe. Like *T. borchii*, *T. aestivum* has a great potential because of its adaption to a wide range of soils, climate and host plants. Recent inoculation experiments revealed that mycorrhization of pecan tree *C. illinoinensis* with *T. aestivum* is also possible which makes truffle–nut coproduction a possibility (Benucci et al., 2012).

The problems

Despite the successful cultivation of *T. melanosporum*, *T. borchii and T. aestivum* there are still some significant problems with their cultivation. Some truffières are not productive or produce few truffles of poor quality. This is often due to the poor quality of plants produced by some companies. In Europe morphological and molecular certification methods for *Tuber* infected plants are locally applied but an European law and a unique certification protocol is needed to protect truffle farmers from failures (Andrés-Alpuente et al., 2015; Murat, 2014). What also makes truffle cultivation uncertain are the unpredictable genetic characteristics of the truffle strains when plants are inoculated with genetically highly variable spores, which may or may not be the most suited to the edaphic and climatic conditions on a particular site.
The sequencing of the *T. melanosporum* genome has revealed that it is heterothallic so that strains carrying different mating types have to cross for fruiting body production (Martin et al., 2010). Both the mating type genes (MAT1-1-1 and MAT1-2-1) were identified and then characterized (Rubini et al., 2011a). After this discovery, studies were carried out in order to verify if the lack of productivity in some truffières was due to the absence of one of the mating types. However, mating type analyses of seedlings planted in Australia showed that both mating types are present suggesting that there are more factors involved in ascocarp production than just the presence of both mating types on host trees (Linde & Selmes, 2012). Recent studies carried out in Europe showed that even in natural productive areas mating type distribution is patchy and often unbalanced (Rubini et al., 2014). In fact, genetically different truffle strains compete with each other and with few genets of the same mating type colonize distinct patches of the truffières (Rubini et al., 2011b; Murat et al., 2013). Some Authors have hypothesized that such spatial segregation may be due to competitive exclusion, with use of the MAT locus as a marker for self-recognition, probably in addition to other polymorphic loci (Selosse et al., 2013). The presence of a vegetative incompatibility system associated with the mating type genes was demonstrated in *Neurospora crassa* Shear & B.O. Dodge (Shiu & Glass 1999). In this fungal species the *tol* gene, which is turned off during the sexual cycle, interact with MAT A-1 and MAT a-1 to form a heterocomplex that mediates vegetative incompatibility. However, a similar homologous

DOI: 10.6092/issn.2465-311X/5593

gene has not been found in *T. melanosporum* (Iotti et al., 2012c). In addition the other het domain genes, which in other ascomycetes trigger a programmed cell death after hyphal fusion between incompatible strains (Saupe 2000), have not been found in *T. melanosporum* (Iotti et al., 2012c). Instead hyphal anastomoses between strains seem to be prevented by other unknown pre-fusion mechanisms (Iotti et al., 2012c) (Fig. 3).

How sexually compatible strains meet and how fertilization occurs is also still an unknown. The discovery of a mitosporic stage in several *Tuber* species suggests the possible role of the conidia in fertilization process like in numerous other ascomycetes (Healy et al., 2012; Carris et al., 2015). Many truffle farmers broadcast low quality truffles onto their truffières in the hope of increasing production. Where this does prove fruitful it might be through introducing new mating strains. However, this practice comes with risks. Often the farmers will spread rotting pieces of truffle which may introduce pathogens. It might also induce a huge genetic load for future generations (Selosse et al., 2013). In nature the role of the farmer spreading mating strains might also be played by mycophagic animals. These are attracted by truffle aromas, eat the truffles and then spread the spores in their feces which remain viable even after passage through the gut (Piattoni et al., 2012; Piattoni et al., 2014).

The future

A revolutionary approach to truffle cultivation would be the use selected mycelial inoculum adapted to specific climatic, edaphic conditions and hosts. This technique was explored at the beginning of modern

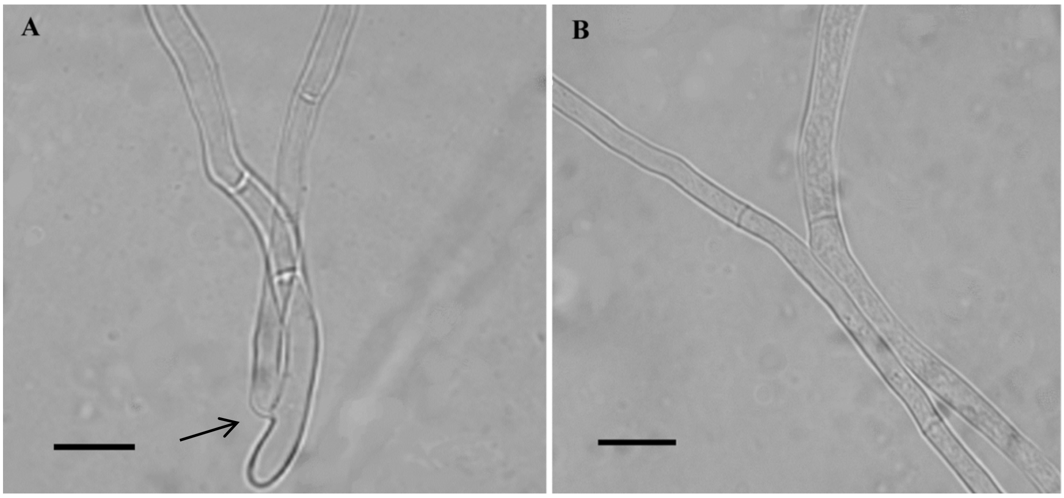

Fig. 3 – Interaction between hyphae of *T. melanosporum*: A) hyphal anastomosis between hyphae of the same strain, arrow indicates the fusion point; B) interaction between hyphae of different strains: no fusion occurs. Bars = 10 µm
Fig. 3 – Interazione fra ife di *T. melanosporum :* A) anastomosi ifale fra ife appartenenti allo stesso ceppo, la freccia indica il punto di fusione; B) interazione fra ife appartenenti a ceppi diversi: nonostante le ife siano a stretto contatto non c'è fusione. Barra = 10 µm

truffle cultivation by Gerard Chevalier (1973) but it has only been used for research exploring the interrelationships the fungi have with their host (Giomaro et al., 2005). One of the biggest limitations to the commercial application of this technique will be the difficulty in isolating and maintaining *Tuber* mycelia in pure culture as well as producing mycelial biomass on a large scale (Iotti et al., 2002; Iotti et al., 2012b). Moreover long-time subculturing in axenic conditions in the absence of host roots results in the loss of isolate infectivity (Boutahir, 2013) and the development of adequate cryopreservation protocols must be used to maintain culture viability (Iotti et al., 2012b). In the past another limit in using mycelial inoculants was the

DOI: 10.6092/issn.2465-311X/5593

lack of knowledge about truffle sexuality resulting in the possibility of producing plants incapable of producing truffles. This is because pure cultures of mycelia are isolated from the gleba of fruiting bodies and hence only carry the maternal mating type. However, thanks to the characterization of the mating type genes of *T. melanosporum* and of the other edible truffles (Paolocci personal communication) we are now able to produce plants inoculated with strains of both mating types.

Inoculating plants with cultures of *Tuber* spp. adapted to a specific set of conditions would facilitate the selection of better-performing fungal genotypes using the same principles that govern the selection of plant cultivars with improved performance. The extensive *Tuber* genome sequencing program will help to determine the genes controlling the qualitative and quantitative characters of truffles but also their adaptability to different environmental conditions. Recent studies carried out by Zambonelli's research group has shown that different strains of *T. borchii* have a different resistance to high temperature (unpublished data). This aspect is particularly important when truffle cultivation is introduced in countries having different climatic conditions to Europe. It could also help to choose strains having the capacity to adapt to global climatic changes. It is also known that plant genotype can also influence truffle production and so some nurseries now use clonal plants selected for truffle production (Robin & Cammalletti, 2001).

Conclusions

Truffle cultivation is an important agricultural activity in many parts of Europe and is growing in popularity worldwide particularly in Southern Hemisphere countries that aim to produce truffles counter season to the Northern Hemisphere.

However problems still exist and need to be addressed. Basic research is clearly needed to better understand the biology of truffles and in particular to unravel the mystery around the sexual stages in the lifecycles. Studies are also needed to elucidate the mysteries surrounding the soil ecology of *T. magnatum* in order to make its cultivation feasible.

Future challenges for truffle cultivation also include: adapting modern mycelial inoculation technology to large scale production of mycorrhized plants, the creation and identification of elite fungal and plant cultivars; maintaining germplasm collections of these cultivars, and the selection of the best cultivars for different ecological conditions. The possibility to selectively introducing mycorrhizal helper bacteria and perhaps other fungi, which could directly or indirectly affect ectomycorrhizal development and fruit body formation, could be another perspective for future truffle cultivation.

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