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Quality assessment of truffle-inoculated seedlings in Italy: proposing revised parameters for certification

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Abstract

Aim of study: The main aims of this study were to evaluate the quality of truffle-inoculated seedlings produced by commercial nurseries in Italy and to identify their minimum requisites in terms of plant age, health, homogeneity, and cut-off percentage of inoculated *Tuber* and non-*Tuber* ectomycorrhizae, based on the analysis of an extensive sample of seedlings subjected to quality control and certification.

Area of study: Truffle-inoculated seedlings produced by Italian commercial nurseries.

Material and methods: Analysis of truffle-inoculated seedlings for health and quality standards; recording of presence of inoculated *Tuber* spp. and other concurrent fungi according to the official Italian method for certification; selective amplification of ectomycorrhizal DNA by PCR species-specific primers.

Main results: We showed that mycorrhization levels in truffle-inoculated seedlings increased with time after truffle-spore inoculation. The highest mean percentage of the inoculated *Tuber* spp., but also the highest presence of contaminants, were recorded after three years. The mycorrhization level of *Tuber melanosporum* and *T. aestivum* was higher in *Corylus* and *Ostrya* seedlings than in *Q. ilex* and *Q. pubescens*, but the latter two host species showed the lowest presence of other ectomycorrhizal fungi. Mycorrhization level distribution in truffle-inoculated seedlings of suitable batches differed very little from the distribution in only all suitable seedlings. Truffle seedlings with other *Tuber* spp. were very few and even absent after three years. The general quality of Italian truffle-inoculated seedlings is high but can be improved even further by revising the parameters used for their certification.

Research highlights: Mycorrhization assessment in truffle-inoculated seedlings produced by commercial nurseries and a revision of the parameters of quality standards following several years of certification in Italy.

Key words: truffle cultivation; truffle seedlings; mycorrhization level; *Tuber*; commercial nursery; certification methods.

Introduction

Hypogeous fungi of the genus *Tuber* (Pezizales) are globally considered delicacies, sold for high prices in markets, and served in the most prestigious restaurants, especially in Italy and France (Hall *et al.*, 2007). In one of the first attempts at cultivation, Frank (2005) discovered that these fungi are ectomycorrhizal (EM) and live in close association with trees. Today, many *Tuber* spp. are successfully cultivated worldwide by the establishment of new plantations using mycorrhized seedlings, previously infected with *Tuber*-spore inocula by commercial nurseries. Truffles are a real economic resource particularly for people living in rural areas characterized by marginal economies mostly based on agricultural products (Benucci *et al.*, 2012b).

The selection of plantation sites and the quality of the truffle-inoculated seedlings are two fundamental prerequisites to allow these fungi to complete their life cycle and produce truffles. The good quality of truffle-inoculated seedlings alone does not guarantee the production of truffles, because there are many factors that affect the truffle life cycle (Granetti *et al.*, 2005; Bencivenga and Baciarelli Falini, 2012). When soil and climate conditions are ideal for the specific truffle-plant combination, a high mycorrhization level becomes critical to guarantee the advantage of the inoculated truffle over other soil-resident EM fungal competitors (Kennedy, 2010).

Many research centers and universities have developed methods for the analysis and certification of

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truffle-inoculated seedlings based on both morphological and molecular analyses (Govi *et al.*, 1995; Fischer and Colinas, 1996; Reyna *et al.*, 2002; Miko and GaÏo, 2007; Bach *et al.*, 2010; Alvarado and Manjon, 2013). Each of these methods has prioritized specific issues, making them all different from each other. None of the existing methods establish quality standards specific to the host partners or to the *Tuber* spp. spore inocula, even though this inoculum can unexpectedly represent a source of contamination due to the unintentional inclusion of other *Tuber* species.

In Italy, as a result of the high economic importance of truffle-inoculated seedlings and their cultivation, some regional governments created specific certifications of mycorrhization levels and plant quality for marketing, out-planting and the establishment of truffle plantations (DGR n. 13429 of 24 May 1996). Since the late 1980s, the research group of the Laboratory of Applied Mycology at the University of Perugia, has carried out analysis and certification of truffle-inoculated seedlings following the official national method (Govi *et al.*, 1995). On the basis of acquired knowledge and experience after several years of truffle-inoculated seedling quality control, this method was revised to take into account not only mycorrhization levels but also the plant host and inoculum quality (Donnini, 2005).

The analysis of a large amount of truffle-inoculated seedlings allowed us to: i) have an overview of the overall quality and mycorrhization levels of the seedlings produced by Italian nurseries; ii) evaluate the mycorrhization levels of the inoculated *Tuber* and different mycorrhizal contaminants (including other *Tuber* spp.) of suitable and unsuitable seedlings and homogeneous batches following the current methodology; iii) propose new and improved parameters for a fast, standardized and reproducible method for seedling quality certification.

Material and methods

Sample collection

In seven Italian commercial nurseries truffle-inoculated seedlings were sampled from 2006 to 2012 among different batches for quality control and mycorrhization level evaluation according to Donnini (2005). Seedling samples belonged to different plant species: *Quercus pubescens* Willd., *Quercus ilex* L., *Quercus cerris* L., *Quercus robur* L., *Corylus avellana* L., *Tilia cordata* Mill., *Ostrya carpinifolia* Scop., *Pinus pinea* L., *Pinus halepensis* L., *Cistus incanus* L. and were mycorrhized with *Tuber melanospourm* Vittad., *Tuber magnatum* Pico, *Tuber aestivum* Vittad., *Tuber brumale* Vittad., *Tuber macrosporum* Vittad., and *Tuber borchii* Vittad. A total of 4,737 truffle-seedlings (2,986 being one year old, 1,371 two years, and 380 three years) were sampled and included in the analyses. Truffle-inoculated seedling samples differed according to plant species and inoculated truffles as well as pot type and dimension, potting mixes and watering regime. Detailed data on the mycorrhization level of the inoculated *Tuber* species and concurrent fungi were analyzed only for the most important truffle/host combinations. In particular *C. avellana*, *O. carpinifolia*, *Q. ilex* and *Q. pubescens* in association with *T. melanosporum* (2,189 truffle-seedlings) and *T. aestivum* (1,662 truffle-seedlings) respectively were included. Since a certification method must be standard, collected data were also analyzed all together regardless of the truffle/plant combination and the growth and cultivation conditions.

Truffle-seedling health and quality

The health and quality of truffle-seedlings were evaluated: the stem height, diameter, number of shot branches were measured, while lignification was assessed visually. Root system characteristics (*e.g.,* length and abundance of fine roots, presence of circling roots), foliar health (*e.g.,* leaf color and damage) and presence of pathogens or pests were observed according to the most recent European rules (Council Directive 1999/105/EC of 22 December 1999).

Morphological and molecular analyses of *Tuber* spp. ectomycorrhizae

Ectomycorrhizae were identified morphologically following the key of Agerer (1987-2012) and the online key DEEMY (http://www.deemy.de) as guide references. Samples of ectomycorrhizae were randomly collected from the root system of each seedling analyzed, placed in 1.5-mL microfuge tubes containing deionized water, and stored at –20°C for subsequent molecular analysis.

From a pool of 60-100 ectomycorrhizae (at various stages of development, with different colors and mantel thicknesses, with/without peritrophic elements *e.g.,* cystidia and emanating hyphae) selected randomly from each truffle-inoculated seedling within the batch under analysis, genomic DNA was extracted with the Extract-N-Amp kit (Sigma) following the manufacturers' advice. Each DNA extract was then amplified with the following species-specific primer pair: UNCI/UNCII for *T. aestivum* (Mello *et al.*, 2002), ITSML and ITSB and ITSCHCH/ITS4 for *T. melanosporum* , *T. brumale* and *T. indicum* Cooke & Massee respectively (Rubini *et al.*, 1998), TBA/TBB for *T. borchii* (Mello *et al.*, 1999), TMAGN/TBACK3 for *T. magnatum* (Rubini *et al.*, 2001) and Tmacr for/Tmacr rev for *T. macrosporum* (Benucci *et al.*, 2011).

The amplifications were carried out in a final volume of 50-µL containing 10 × PCR buffer (Invitrogen), 200 µM dNTPs, 20 mg of bovine serum albumin, 10 pmol of forward and reverse primer, and 2 U of Taq DNA polymerase (Invitrogen). PCR amplifications were carried out with a first denaturation of 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 60°C annealing for 30 s for UNCI/UNCII, TBA/TBB and TMAGN/TBACK3 (62°C for ITSML and ITSB and ITSCHCH/ITS4) with an extension of 1 min, and with a final extension step at 72°C for 10 min. Negative (no genomic DNA added) and positive (Genomic DNA extracted from different *Tuber* spp.) controls were added to PCR reactions. Ten Ìl of PCR products were run on 1.8% (w/v) agarose gel stained with ethidium bromide for evaluation of the presence/absence of bands.

Evaluation of mycorrhization levels, suitable and unsuitable truffle seedlings and batches

Differences in the percentage of mycorrhization were evaluated in 1) inoculated *Tuber* species, 2) other EM fungi (including other *Tuber* spp.) and 3) other *Tuber* spp. only, in batches of seedlings 1 year, 2 years and 3 years after truffle-spore inoculation. The analyses were carried out using the official method (DGR n. 13429 of 24 May 1996) and subsequent modifications (Donnini, 2005) which included biomolecular analysis. This procedure abandons the classical counts of ectomycorrhizae in the root system or portions of it and relies on assessment through a visual evaluation of the mycorrhization level in the whole root system, using a stereomicroscope. The estimated percentage of mycorrhization is evaluated with intervals of 5%. The distribution of mycorrhization levels was analyzed combining: 1) all truffle-inoculated seedling samples; 2) only suitable and unsuitable truffle-inoculated seedling samples (A truffle seedling is suitable when it has ≥30% *Tuber* mycorrhization, ≤ 5% of other EM fungi mycorrhization and the difference between these two percents is ≥20); 3) only truffle-inoculated seedling samples of suitable and unsuitable batches, one, two and three years after spore inoculation. A batch of seedlings is suitable when it has ≥ 80% of suitable samples. The frequency of suitability/unsuitability was calculated by dividing the number of suitable or unsuitable batches by the total number of seedlings. Frequency was also calculated in the following three types of batches: 1) batches with only suitable seedlings, 2) batches with 1 unsuitable seedling, and 3) batches with 2 unsuitable seedlings. Mean mycorrhization values and the frequency of the inoculated *Tuber* spp., concurrent fungi (including other *Tuber* spp.), and other *Tuber* spp., were also calculated for seedlings belonging to suitable and unsuitable batches. No relation between the number of ectomycorrhizae and total number of tips was recorded.

Results

Forest quality

Truffle-inoculated seedling samples belonging to *Quercus pubescens* Willd., *Quercus ilex* L., *Quercus cerris* L., *Quercus robur* L., *Corylus avellana* L., *Tilia cordata* Mill., *Ostrya carpinifolia* Scop., *Pinus pinea* L., *Pinus halepensis* L., *Cistus incanus* L. species were of good quality with regard to health, stem height and diameter, lignification and number of shots and branches. Detailed data of *Q. pubescens* × *T. melanosporum* measurements are reported as a reference in Table 1. In some samples the root system was coiled, but only in those analyzed three years after truffle-spore inoculation (Fig. 1).

Molecular analyses of the inoculated *Tuber* spp. ectomycorrhizae

None of the DNA analyses performed on the ectomycorrhizae of truffle-inoculated seedling samples showed any inconsistency with the inoculated *Tuber*

Table 1. Measurements data of *Q. pubescens* × *T. melanosporum, Q. ilex* × *T. melanosporum, Q. cerris* × *T. melanosporum, Q. robur* × *T. aestivum, C. avellana* × *T. melanosporum, T. cordata* × *T. melanosporum, O. carpinifolia* × *T. melanosporum, P. pinea* × *T. aestivum, P. halepensis* × *T. aestivum, C. incanus* × *T. melanosporum* inoculated seedling samples after one and two years. Values are mean ± SE

	1 year			2 years		
	Diameter	**Heigh**	**Shoots**	**Diameter**	**Heigh**	**Shoots**
Q. pubescens	4.7 ± 0.22	24.8 ± 1.40	1.1 ± 0.31	7.2 ± 0.64	45.8 ± 2.60	2.5 ± 0.56
Q. ilex	2.6 ± 0.15	22.4 ± 2.33	0.3 ± 0.15	4.8 ± 0.13	40.8 ± 1.6	0.5 ± 0.17
Q. cerris	4.3 ± 0.19	19.0 ± 1.45	1.1 ± 0.35	4.9 ± 0.23	26.4 ± 1.23	2.4 ± 0.76
Q. robur	3.8 ± 0.23	28.3 ± 1.7	0.7 ± 0.26	5.8 ± 0.17	58.4 ± 2.73	1.4 ± 0.16
C. avellana	4.1 ± 0.13	33.4 ± 1.30	0.4 ± 0.22	4.9 ± 0.26	45.5 ± 1.19	2.1 ± 0.23
T. cordata	2.7 ± 0.14	4.7 ± 0.56	0	3.6 ± 0.26	10.5 ± 1.23	0.8 ± 0.35
O. carpinifolia	4.1 ± 0.29	32.0 ± 1.54	1.0 ± 0.52	4.7 ± 0.38	41.6 ± 2.14	1.4 ± 0.48
P. pinea	3.8 ± 0.31	24.2 ± 2.02	5.0 ± 1.07	4.9 ± 0.34	36.0 ± 3.09	5.9 ± 1.12
P. halepensis	2.5 ± 0.18	16.6 ± 2.05	6.1 ± 0.49	3.1 ± 0.16	27.7 ± 1.80	7.2 ± 0.78
C. incanus	2.9 ± 0.07	15.1 ± 1.55	0	3.2 ± 0.24	20.0 ± 1.78	1.7 ± 0.47

spp. used for the mycorrhization declared by commercial nurseries. A single case of contamination with *T. brumale* on seedlings mycorrhized by *T. melanosporum* was detected, the entire batch was therefore rejected and declared unsuitable for commercialization.

Mycorrhization standards of all the analyzed truffle-inoculated seedling samples

For 50% of all the truffle-inoculated seedling samples, the mycorrhization level distribution falls between 20% and 50% after one year (Y1) from inoculation, while it falls between 35% and 60% after two (Y2) and three (Y3) years from truffle-spore inoculation, respectively (Fig. 2). Fig. 3 shows that three years after spore inoculation there was a substantial increment of contamination by concurrent fungi in comparison to after only one and two years.

Figure 1. A *Quercus pubescens* Willd. × *Tuber aestivum* Vittad. seedling with huge circling roots at the bottom and few lateral small ones.

The mycorrhization level of the truffle-inoculated seedling samples with *T. melanosporum* was between 20% and 50% in *C. avellana* and between 35% and 60% in *O. carpinifolia*. For *T. aestivum,* the mycorrhization level was between 35% and 65% in *C. avellana*, and between 30% and 65% in *O. carpinifolia*. The distribution was therefore similar in *Q. ilex* and *Q. pubescens*, both with *T. melanosporum* and *T. aestivum* (Fig. 4). Observation of mycorrhization levels in truffle-inoculated seedling samples with concurrent fungi showed that *C. avellana* and *O. carpinifolia* were the most susceptible host species. In contrast, only outliers were detected in *Q. ilex* and *Q. pubescens* (Fig. 4).

Mycorrhization standards of suitable and unsuitable truffle-inoculated seedling samples

In half of the suitable truffle-inoculated seedling samples, the mycorrhization level falls between 35% and 55% after one year (1YS) from truffle-spore inoculation, while it varies from 40% to 60% and 45% to 65% after two (2YS) and three (3YS) years, respectively (Fig. 2). In half of the unsuitable truffle seedling

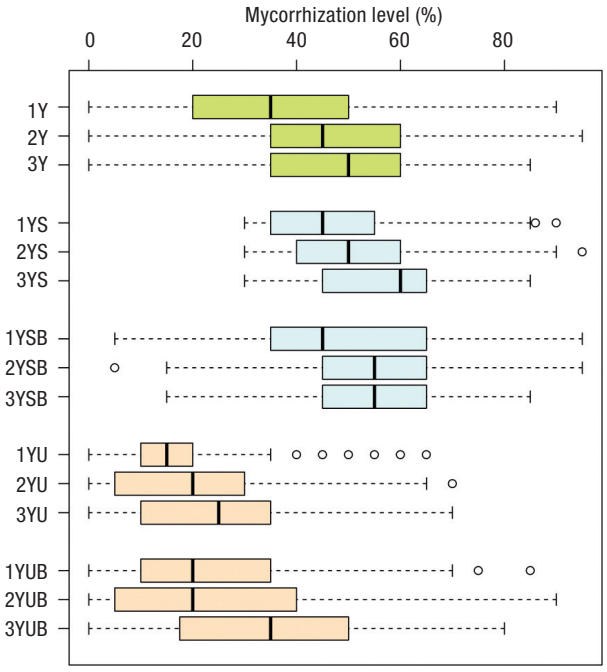

Figure 2. Box plots illustrate the medians, upper and lower quartiles, the range and extreme values of the mycorrhization level distribution in: all the truffle-inoculated seedling samples collected in the commercial nurseries after one (1Y), two (2Y) and three years (3Y) from spore inoculation; only the suitable truffle-seedling samples after one (1YS), two (2YS), three years (3YS) from spore inoculation; only the truffle-seedling samples of suitable batches after one (1YSB), two (2YSB), three years (3YSB) from spore inoculation; only the unsuitable truffle-seedling samples after one (1YU), two (2YU) and three years (3YU) from spore inoculation, and only the truffle-seedling samples of unsuitable batches after one (1YUB), two (2YUB) and three years (3YUB) from spore inoculation according to the official method of certification in Italy.

samples, the mycorrhization level fell between 10% and 20% after one year (1YU) from spore inoculation, while it varied from 5% to 30% and 10% to 35% after two years (2YU) and three years (3YU), respectively (Fig. 2).

Mycorrhization standards of suitable and unsuitable batches

The 50% of the suitable truffle-inoculated seedling samples after one year (1YSB) from truffle-spore inoculation fell between 35 and 60% (median 45%) of mycorrhization observation distribution while they varied from 40 to 60% (median 40%) and 45 to 65% (median 55%) after two (2YSB) and three years (3YSB), respectively (Fig. 2).

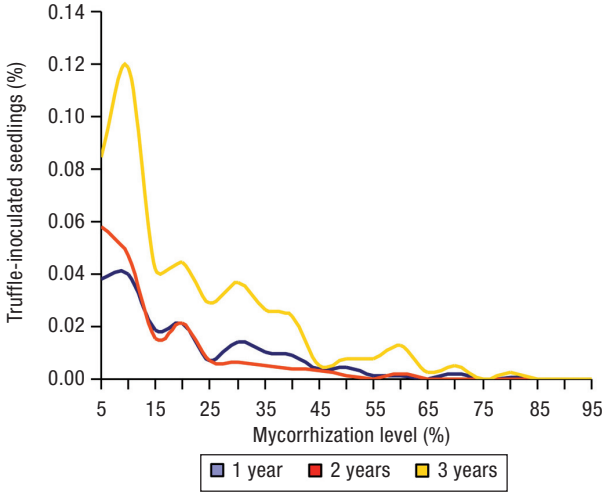

Figure 3. Mycorrhization levels of concurrent fungi detected in all the truffle-inoculated seedling samples after one, two and three years from spore inoculation.

Two quartiles of the distribution in unsuitable truffle-seedling samples after one year (1YUB) from spore inoculation fall between 10 and 35% (median 20%) of mycorrhization observation distribution, whereas they varied between 5 and 40% (median 20%), 20 and 50% (median 35%) after two (2YUB) and three years (3YUB), respectively (Fig. 2).

The batches comparisons showed that suitable batches increased from 56.5% after one year to 82.3% after two and 66% after three years from inoculation. Similarly, batches with all suitable truffle-inoculated seedling samples as well as with one or two unsuitable ones increased (Table 2). The inoculated *Tuber* spp. was always present in the suitable seedling samples regardless the year when they were analyzed.

The frequency of seedlings contaminated by concurrent fungi increased consistently from ≈12% to ≈13% and even to ≈25% after one, two and three years from spore inoculation, respectively (Table 3). Mean levels

Table 2. Percentage of suitable and unsuitable batches of truffle-inoculated seedlings

Batches	1 year	2 years	3 years
Total suitable batches (%)	56.51	82.28	63.73
Total unsuitable batches (%)	43.49	17.72	36.27
Suitable batches with all seedlings suitable (%)	39.93	62.60	53.92
Suitable batches with 1 unsuitable seedling (%)	14.26	17.32	9.81
Suitable batches with 2 unsuitable seedlings (%)	2.32	2.36	0.00

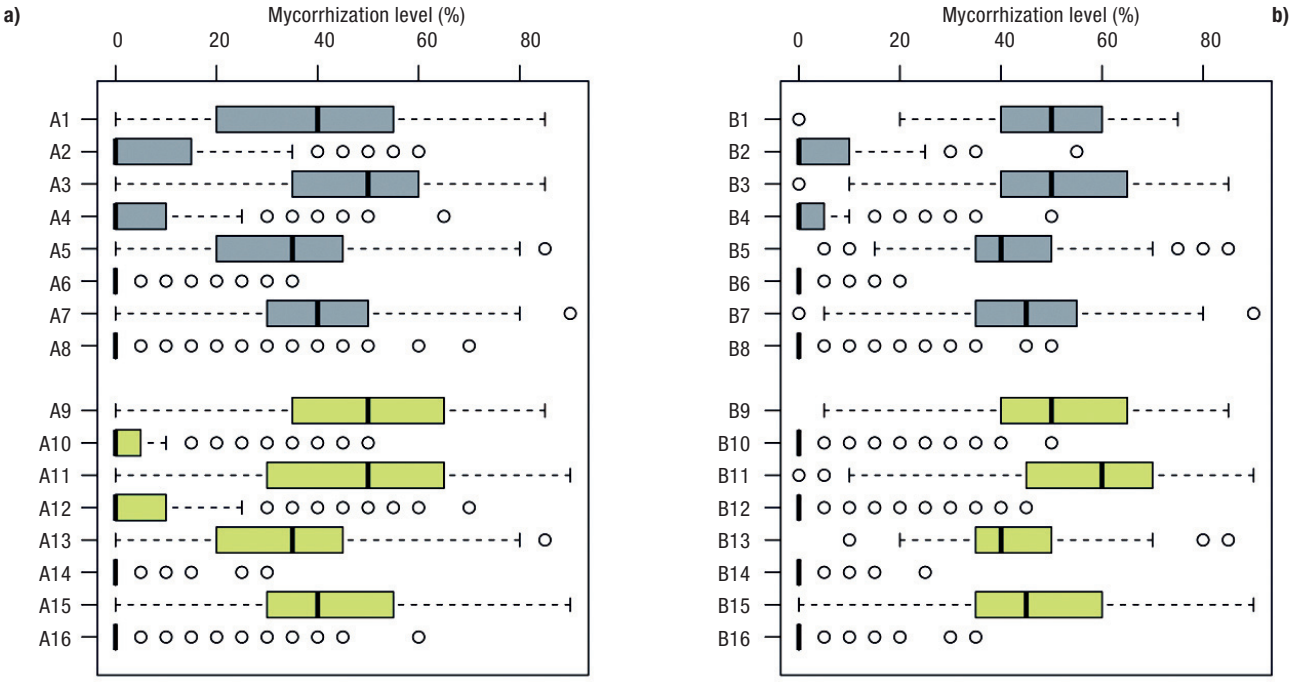

Figure 4. Box plots illustrate the medians, upper and lower quartiles, the range and extreme values of truffle and concurrent fungi mycorrhization level distributions. (A) all the truffle-inoculated seedling samples collected in the commercial nurseries: *C. avellana* × *T. melanosporum* (A1) and concurrent fungi (A2); *O. carpinifolia* × *T. melanosporum* (A3) and concurrent fungi (A4); *Q. ilex* × *T. melanosporum* (A5) and concurrent fungi (A6); *Q. pubescens* × *T. melanosporum* (A7) and concurrent fungi (A8); *C. avellana* × *T. aestivum* (A9) and concurrent fungi (A10); *O. carpinifolia* × *T. aestivum* (A11) and concurrent fungi (A12); *Q. ilex* × *T. aestivum* (A13) and concurrent fungi (A14); *Q. pubescens* × *T. aestivum* (A15) and concurrent fungi (A16). (B) the truffle-inoculated seedling samples of only suitable batches: *C. avellana* × *T. melanosporum* (B1) and concurrent fungi (B2); *O. carpinifolia* × *T. melanosporum* (B3) and concurrent fungi (B4); *Q. ilex* × *T. melanosporum* (B5) and concurrent fungi (B6); *Q. pubescens* × *T. melanosporum* (B7) and concurrent fungi (B8); *C. avellana* × *T. aestivum* (B9) and concurrent fungi (B10); *O. carpinifolia* × *T. aestivum* (B11) and concurrent fungi (B12); *Q. ilex* × *T. aestivum* (B13) and concurrent fungi (B14); *Q. pubescens* × *T. aestivum* (B15) and concurrent fungi (B16).

of mycorrhization in truffle-inoculated seedling samples belonged to suitable batches increased from ≈46% to ≈49% and even ≈54% after one, two and three years from spore inoculation, respectively (Table 3).

The mycorrhization levels of concurrent fungi was in general low, but lowest (≈ 1.5%) after two years from spore inoculation. Moreover, their frequency was similar after one (≈12%) and two (≈13%) years from

Table 3. Frequency and mean ± SE mycorrhization levels of truffle-inoculated seedlings: inoculated *Tuber* spp., all concurrent fungi and other *Tuber* spp. in suitable and unsuitable batches for one, two and three years from spore inoculation

	1 year batches		2 year batches		3 year batches	
	Suitable	**Unsuitable**	**Suitable**	**Unsuitable**	**Suitable**	**Unsuitable**
Truffle seedling frequency (%)						
Inoculated *Tuber* sp.	100	92.32	100	79.52	100	84.35
Concurrent fungi	12.12	24.71	13.35	38.10	24.89	76.87
Other *Tuber* spp.	0.12	2.15	1.89	10.05	0	9.59
Mean Mycorrhization level (%)						
Inoculated *Tuber* sp.	45.68 ± 0.41	24.52 ± 0.44	48.63 ± 0.46	24.79 ± 1.44	54.30 ± 0.90	34.05 ± 1.90
Concurrent fungi	1.82 ± 0.15	5.66 ± 0.35	1.46 ± 0.14	7.95 ± 0.93	2.40 ± 0.33	20.58 ± 1.54
Other *Tuber* spp.	0.05 ± 0.04	0.45 ± 0.11	0.16 ± 0.04	1.98 ± 0.54	0	2.04 ± 0.73

spore inoculation it and doubled after three years (≈25%). It was worth nothing that in unsuitable truffle-inoculated seedling samples mean mycorrhization level with the inoculated *Tuber* spp. was ≈34% after three years from spore inoculation, even higher than the cut-off value of 30% identified for suitable seedlings. Anyway, for the same year, the mean mycorrhization level of concurrent fungi was ≈21% and their frequency ≈77%. In suitable batches other *Tuber* spp. mean mycorrhization level and frequency were very low and even absent after three year from spore inoculation. The highest presence of other *Tuber* spp. in suitable seedlings was recorded after two years from spore inoculation (≈2%) with a mean mycorrhization of 0.16% (Table 3).

Discussion

Concerning the quality standards of truffle-inoculated seedlings produced in Italy

The forest quality of the seedlings was in general very good and in agreement with European law for forest propagation material (Council Directive 1999/105/EC of 22 December 1999). Nevertheless, the main problem detected was the presence of coiled root systems in truffle-seedlings after three years from spore inoculation. Plants with severe circling roots close to the trunk can eventually slow growth and girdle the trunk making the tree unstable to winds (Gilman and Sadowsky, 2007). Truffle-inoculated seedlings with malformed root, even with high mycorrhization level, were therefore discarded like unsuitable ones.

The mycorrhization level of the inoculated *Tuber* spp. increased with time from spore inoculation regardless of whether the truffle-inoculated seedling samples were analyzed altogether, divided in suitable and unsuitable or even if analyzed in suitable and unsuitable batches. Nevertheless, with age, the seedlings also increased their contamination with concurrent fungi: the highest frequency and mean mycorrhization levels were therefore registered after three years from spore inoculation. At this age more seedlings showed higher inoculated *Tuber* spp. mycorrhization level but most of them had also a percentage (even small) of concurrent fungi.

The mycorrhization level of suitable batches was not dramatically lower than that of all suitable truffle-seedlings: less than the 25% of observations of suitable batches was less than the minimum value for the suitable truffle-inoculated seedlings.

Different host species have diverse responses to inoculation treatments in terms of truffle mycorrhization and presence of concurrent fungi. In particular the range of mycorrhization level distribution of *C. avellana* and *O. carpinifolia* is higher than of *Q. ilex* and *Q. pubescens*, but the distribution range of concurrent fungi is narrower in the last host species. This was also more evident if only truffle-inoculated seedling samples of suitable batches were included in the analysis, meaning that the quality standard adopted can upgrade the general truffle-inoculated seedlings quality.

Proposing revised parameters for truffle-inoculated seedling certification

With this work new parameters for truffle-inoculated seedling quality as well as a modification of the current Italian method for their certification were proposed (Table 4). We believe that an ideal method must be fast and simple, and needs to reflect the effective quality of truffle seedling batches. The screening by visual estimation of the entire root system allowed us to identify contaminants that are not always detected by random sampling of root portions. Moreover, visual estimation of mycorrhization levels had already proven its worth: no statistical differences between visual and root tip counting-based assessments had been reported (Benucci *et al.*, 2012a).

The taxonomic identification of truffle-inocula can help to avoid contamination with other *Tuber* spp. accidentally introduced in spore-slurries preparation. Although low percentages of other *Tuber* spp. were also detected in suitable batches, these were discarded to reduce possible co-generic competition after seedling outplanting. The pre-screening of the root-systems of randomly selected young seedlings immediately before inoculation is recommended to reduce the presence of concurrent fungi [*e.g., Sphaerosporella brunnea* (Alb. & Schwein.) Svrček and Kubička, *Pulvinula constellatio* (Berk. & Broome) Boud.] that usually occurs during the first phase of growth in climatically controlled greenhouses (Bencivenga *et al.*, 1995; Amicucci *et al.*, 2001; Benucci *et al.*, 2012c). The minimum limit of 30% mycorrhization with the inoculated truffle is confirmed for truffle-inoculated seedling suitability, as recommended by the official method (DGR n. 13429 of 24 May 1996).

Table 4. Parameters for spore inoculum and evaluation and certification of truffle-inoculated seedling quality

Quality of truffle-spore inocula	
Ripeness	Well ripe truffles have well-formed germinable spores.
Species	The correct taxonomic identification is essential to avoid contamination from other truffles.
Quality of the host plant	
Habitus	Size, stem height, diameter, number of shot branches need to be in proportion to age.
Lignification	Lignified stem and branches to resist the winter after planting.
Health	Good phytosanitary status, absence of pathogens or pests.
Root systems	Well-formed, equipped with numerous lateral roots.
Quality of truffle-inoculated seedlings	
Pre-screening	Inspection of the root system before inoculation to evaluate the absence of concurrent fungi.
Identification of ectomycorrhizae	— Visually under stereomicroscope and light microscope according to their specific morphological characteristics. — Molecular analyses through DNA markers to verify consistency of the inoculated *Tuber* spp. and to detect contamination.
Suitability requirements for truffle-inoculated seedlings	— Visual evaluation of mycorrhization levels in the whole root system (or counting of first 50 root-tips in 6 proximal and 6 distal root portions). — The percentage of *Tuber* ≥ 30%. — Absence of other *Tuber* spp. mycorrhizae. — Percentage of concurrent fungi ≤ 10%. — Difference between percentage of inoculated *Tuber* spp. and concurrent fungi ≥ 30.
Suitability requirements for batches	— Homogeneous (same species, age, truffle inoculum and growth conditions) no more than 500 seedlings. — Samples of 1% plus 5 truffle-inoculated seedlings randomly collected. — Only one unsuitable truffle-inoculated seedling is allowed in the same sample.

The limited incidence of concurrent fungi and their low value of mean mycorrhization in both suitable and unsuitable truffle-inoculated seedling batches found in this study, demonstrated the careful work of the commercial nurseries, and allowed us to reduce their presence from 15% to < 10%.

Concerning batch suitability, in order to validate the batch examined only one unsuitable seedling at most is acceptable in the analyzed sample. Despite the fact that two years after the truffle-spore inoculation we had the best balance between the frequency and the mycorrhization levels of the concurrent fungi and the inoculated *Tuber* spp., no limitations of specific mycorrhization levels or ages were adopted. We believe that only the distinction between suitable and unsuitable truffle-inoculated seedlings is important for truffle cultivation, no concerns regarding higher or lower quality can therefore be contemplated. Similarly, within seedling root systems we did not foresee any minimum number of inoculated *Tuber* spp. mycorrhizae with respect to all the root tips present. Although non-mycorrhizal tips can easily be contaminated by soil-resident fungi, the real connection between the mycorrhization level and truffle production is as yet unknown (Horton and Bruns, 2001).

Finally, the biomolecular analysis for the identification of the inoculated *Tuber* spp. must be the fastest and cheapest possible. Reliable , simple techniques like the use of species-specific PCR primers for selective amplifications are recommended (Rubini *et al.*, 1998; Mello *et al.*, 2002; Benucci *et al.*, 2011). We discourage laborious procedures like cloning previously amplified products and sequencing, as required by other methods of truffle-inoculated seedling certification (Alvarado and Manjon, 2013).

This experience is proposed as a stimulus for discussion on the potential establishment of an official European method for truffle plant certification which, as technicians and professionals have argued for many years, would be a way of protecting truffles and truffle-farmers.

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