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REVIEW

Comparative analysis of different methods for evaluating quality of *Quercus ilex* seedlings inoculated with *Tuber melanosporum*

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Abstract The quality of seedlings colonized by *Tuber melanosporum* is one of the main factors that contributes to the success or failure of a truffle crop. Truffle cultivation has quickly grown in European countries and elsewhere, so a commonly shared seedling evaluation method is needed. Five evaluation methods are currently published in the literature: three are used in Spain and two in France and Italy. Although all estimate the percentage colonization by *T. melanosporum* mycorrhizae, they do it in different ways. Two methods also estimate total number of mycorrhizae per seedling. Most are destructive. In this work, ten batches of holm oak seedlings inoculated with *T. melanosporum* from two different nurseries were evaluated by means of the five methods noted above. Some similarity was detected between the percentages of *T. melanosporum* mycorrhizae estimated by each method but not in their ability to assess the suitability of each batch. We discuss the advantages and disadvantages for each method and suggest approaches to reach consensus within the truffle culture industry for certifying mycorrhizal colonization by *T. melanosporum* and seedling quality.

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Introduction

Nursery production of seedlings inoculated with *Tuber melanosporum* Vittad. is one of the keystones of modern black truffle cultivation. Since the 1960s, joint efforts between the University of Turin and the Institute for Woody Plants and Environment (IPLA, Italy), with the French National Institute for Agricultural Research (INRA) from Clermont-Ferrand (Chevalier and Grente 1973) allowed the establishment of a reliable method for the production of seedlings colonized by this ascomycete. The amount of seedlings produced by nurseries is proportional to the increase of land use for black truffle cultivation. Cocina et al. (2013) report that currently dozens of nurseries specialize on production of seedlings colonized by *T. melanosporum* in Spain, France, and Italy. Furthermore, they note at least 29 additional nurseries in the rest of the world.

Successful truffle cultivation involves a long-term process, so starting with high quality plant material for plantation establishment is crucial. This is especially important regarding the percentage of root tips colonized by the black truffle; a high level of truffle mycorrhizal colonization will limit colonization by other ectomycorrhizal fungi that exist in the soil and, thus, a potential decrease in truffle yields (Pruett et al. 2008; Iotti et al. 2012).

Seedling quality evaluation is performed on a sample of seedlings from each batch. A batch consists of plants that have the same seed provenance, sowing date, truffle inoculum, and matching nursery management practices (Palazón et al. 1999). Nursery seedlings must meet forestry plant quality requirements and achieve a specific level of truffle fungus mycorrhiza colonization as determined by a particular evaluation

method. Colonized seedlings must also be free from contaminant mycorrhizae belonging to nontarget *Tuber* species, and specific levels of mycorrhizal colonization by other competing fungi cannot be exceeded (see Table 1).

Detecting mycorrhizae belonging to *Tuber* species other than the targeted truffle species on inoculated seedlings is unusual. Black truffle mycorrhizae can be confused mainly with those of *Tuber indicum* Cooke and Massee and *Tuber brumale* Vittad. Importation of fresh *T. indicum* fruiting bodies creates a high ecological risk. Mycorrhizae of *T. indicum* have been detected in at least one Italian *T. melanosporum* plantation (Murat et al. 2008) and have produced fruiting bodies in a truffle plantation from the USA (Bonito et al. 2011). The morphological resemblance between *T. indicum* and *T. melanosporum* mycorrhizae makes it difficult to differentiate them without a molecular analysis.

Although the use of well-colonized truffle-inoculated seedlings is critical to plantation success, we lack common criteria for evaluating the quality of *T. melanosporum*-colonized seedlings. Five methods for quality evaluation have been published and commonly used in Europe: Chevalier and Grente (1978) INRA-ANVAR, Govi et al. (1995) University of Perugia, Fischer and Colinas (1996) University of Lérida, Palazón et al. (1999) INIA-Aragón, and Reyna et al. (2000) CEAM-Valencia. Most methods examine the whole root system and then estimate the percentage of root tips colonized by *T. melanosporum*. However, the method used by Reyna et al. (2000) only analyzes a portion of the root system by extracting a cylindrical sample of the potting mix in the seedling container. All methods also examine and estimate the presence or absence of other contaminating ectomycorrhizal fungi and discard those batches with root tips colonized by other *Tuber* species. In Tables 1 and 2, the most important characteristics of each evaluation method are described.

Given the lack of consensus on how best to evaluate the quality of truffle-inoculated seedlings, we tested the five most used methods to address the following objectives: (1) assess their ability to estimate root colonization by *T. melanosporum* in *Quercus ilex* subsp. *ballota* (Desf.) Samp. (holm oak) seedlings and (2) determine the similarity in the ability for the different methods to estimate root colonization by *T. melanosporum*.

Material and methods

Sampling of colonized seedlings in nurseries

In Spain, *Q. ilex* is the most common host seedling inoculated with *T. melanosporum* for truffle plantations. This is explained by its greater hardiness, longevity, and higher truffle production compared to other host species under Spanish environmental and edaphic conditions.

Ten batches of holm oak seedlings inoculated with black truffle were examined for mycorrhizal colonization. The batches belonged to two different nurseries and each batch had 1,000 seedlings. A random sample of 12 seedlings was selected from each batch; 12 seedlings are required by the two evaluation methods with the highest number of analyzed samples per batch, Fischer and Colinas (1996) and Reyna et al. (2000). Inoculation in both nurseries took place in April 2010. Sample collection took place in November 2011, thus allowing generous time to achieve an optimum mycorrhizal status for outplanting.

Seedling and batch evaluation

As each seedling had to be evaluated by five methods, the sequence of analyses was arranged such that each method would not affect the following one. First, a cylindrical sample of the root system was extracted from each of the 120 seedlings by means of a punch tool and stored. This procedure follows the method of Reyna et al. (2000) and was done first because it is the least destructive. Next, roots were washed free of potting mix, and forestry plant quality was evaluated by measuring height and root collar diameter (according to Council Directive 1999/105/CE). Additional morphological quality observations related to root health and root system architecture were recorded (Peñuelas 1993). Samples were frozen together with the cylinders at −20 °C until processed. Next, the method of Chevalier and Grente (1978) was performed by rating seedling mycorrhizal quality on a 1 to 5 scale. For statistical purposes, this scale was converted to percentages according to Trouvelot et al. (1986). We followed the procedure by Govi et al. (1995), which extracts root fragments by spreading the root system onto a numbered grid. This procedure was followed by the method of Palazón et al. (1999), which divides the root system into three sections and takes a fragment from each. Finally, the method of Fischer and Colinas (1996) was performed. It requires the root system to be cut into 2−3 cm fragments for mycorrhizal root tip analysis. The time needed for evaluating each seedling by each method was also recorded. Following the guidelines of each method, which are summarized in Table 1, batches were sorted by their suitability. Batch suitability can be understood as the final diagnosis of the quality of the batch given by each method.

Morphological and anatomical identification of *T. melanosporum* ectomycorrhizae were carried out following the descriptions of Zambonelli et al. (1993) and Rauscher et al. (1995). Other nursery contaminants were described according to the studies of Agerer (2006) and Agerer and Rambold (2004–2013).

Table 1 Summary of the methodologies used for mycorrhizal seedling evaluation

Descriptors	INRA-ANVAR (Chevalier and Grente 1978)	University of Perugia (Govi et al. 1995)	Universidad de Lérida (Fischer and Colinas 1996)	INIA-Aragón (Palazón et al. 1999)	CEAM-Valencia (Reyna et al. 2000)
Criteria for single seedlings					
Root tip counting	No counting	400 (50 root tips/root fragment) (4/sector)	250	300 (100/section)	All tips in the sample
Minimum number of fine roots analyzed or estimated/seedling	Not specified (well-balanced root system architecture)	Not specified (well-developed root system)	900	300	Not specified
Minimum percentage of colonization/seedling or its estimation	≥1 (Chevalier et Grente 1978) ≥1 % (Trouvelot et al. 1986)	≥30 %	≥10 %	>10 %	≥10 %
Minimum number of *T. melanosporum* mycorrhizae/seedling	Not specified	≥30 % colonized root tips	90	100	100
Estimates the total number of colonized root tips	No	No	Yes	No	Yes
Maximum percentage of contaminants	<1/5 (20 %) 0 % *Hebeloma* sp.	Gap of 20 points with the *T. melanosporum* percentage but always ≤15 %	<25 % over the number of colonized tips	<30 %	<20 % of the total mycorrhizae in the roots <25 % over the number of
T. melanosporum-colonized tips					
Criteria for batches					
Number of seedlings to analyze/1,000 seedling batch	10 (1–5 %)	11 (1–3 %) Batches under 1,000 units= 10 seedlings	12 (1.2 %)	5 (0.5–0.8 %)	12 (1.2 %)
Batch suitability (after meeting the criteria of forestry plant quality and 0 % of other *Tuber* sp.)	Mean seedling value >2.5 Mean (Trouvelot et al. 1986) >18.5 % All seedlings must be colonized	≥80 % suitable seedlings	Lower limit %*T. melanosporum* >33 % Lower limit fine roots >1,800 Upper limit contaminants <25 % All plants >10 % colonization with *T. melanosporum* and <50 % contaminants	Mean seedling value ≥30 %	≥11 seedlings with more than 100 colonized tips/seedling Mean number >260
Other considerations					
Average evaluation time/seedling (estimated in this work)	6 min	20 min	34 min	15 min	7 min
Country where the method is used	France, Spain	Italy, France, Romania	Spain	Spain	Spain
Main updates	Molecular analysis of *Tuber* spp.	Counting of 600 tips per plant The grid is not used anymore Molecular analysis of *Tuber* spp.	Molecular analysis of *Tuber* spp.	Molecular analysis of *Tuber* spp.	No updates

Table 2 Summary of the protocols used by each tested method for seedling quality evaluation

INRA-ANVAR (Chevalier and Grente 1978)	University of Perugia (Govi et al. 1995)	Universidad de Lérida (Fischer and Colinas 1996)	INIA-Aragón (Palazón et al. 1999)	CEAM-Valencia (Reyna et al. 2000)
Preparatory steps: Removal of potting mix from roots by gentle wash; Forestry quality evaluation (root collar diameter, plant height, root architecture, and plant morphology tests according to Council Directive 1999/105/CE); First observation of the seedling root system covered by water under a stereo microscope (presence of mycorrhizae of the inoculated fungus and possible contaminants, root amount and proportion). Identification and checking by microscopy for the fungi present in the seedling				*Preparatory steps*: Forestry quality criteria (root collar diameter, plant height)
Observation under a stereo microscope of the amount of mycorrhizae and possible contaminants, estimation of seedling suitability on a 0 to 5 scale	The root system is spread onto a grid that divides it into two sectors. At least four root fragments are extracted (through some of the grid slots) from each sector and seedling. 50 root tips are examined under a stereo microscope for each fragment. Colonized root tips, noncolonized fine roots, and possible contaminants are recorded	The root system is cut into 2–3 cm segments and placed in a shallow tray over a grid with squares of four different colors. One color is chosen at random. Under a stereo microscope, colonized root tips, noncolonized fine roots, and possible contaminants present over that color are counted for at least 250 root tips	The root system is cut into three fragments of the same length (sectors). Root fragments are collected randomly from each sector. 100 root tips are counted for each sector under a stereo microscope. Colonized root tips, noncolonized fine roots, and possible contaminants are recorded. The mean value for the three sectors is calculated	A root sample from the central section of the container is extracted by means of a punch tool (2 % of the total container volume). The obtained sample is washed gently and passed through a 1-mm sieve. The roots retained in the sieve are observed under a stereo microscope and colonized root tips, noncolonized fine roots, and possible contaminants are counted

Statistical analysis

Each variable was tested for normality (Kolmogorov–Smirnov test; $p>0.05$), and each analysis checked for homoscedasticity (Levene's test; $p>0.05$) to comply with the premises of parametric analyses. Colonization percentage was transformed by raising it to a positive power, but the means shown are not transformed. A Pearson correlation matrix was constructed to examine the relationship between the colonization percentages obtained by the different methods. The differences in the colonization percentage obtained by the different methods and in both nurseries were tested by ANOVA (general linear model) with two fixed factors (method and nursery) and their interaction. However, for the representation of confidence intervals for colonization percentages obtained by the different methods, a one-factor ANOVA was performed in order to differentiate between them. In both cases, post hoc Tukey's test was used to discriminate between methods.

The time needed for estimating colonization percentage by each method was analyzed using nonparametric methods. Their differences were evaluated by the Kruskall-Wallis test and the Mann-Whitney *U* test was used to compare the means in pairs as independent samples. The design and basic information about the tests used can be found in Montgomery (2001). Their implementation was carried out using SPSS (2012).

Results

The data on batch suitability in relation with colonization levels and in relation to the evaluation method used for each of the ten batches selected are shown in Table 3. The average percentages of colonization by *T. melanosporum* mycorrhizae obtained for each batch by taking the mean of the five methods are also shown in Table 3. Consensus among methods, understood as agreement in evaluating each batch suitability, only happened in the batches with the highest (batches N2B6 and N1B3, with 42.3 and 35.0 % average colonization, respectively) and the lowest (batch N1B2, 17.6 %) colonization levels. In the other seven batches, results differ depending on the evaluation method used. According to our data, the most liberal method seems to be that of Chevalier and Grente (1978), with nine out of ten batches considered acceptable. The most conservative method was that of Govi et al. (1995), which found only three batches acceptable.

In nursery 1, mycorrhizae from *Sphaerosporella brunnea* (Alb. and Schwein.) Svrček and Kubička were found on some seedlings. No ectomycorrhizae belonging to the genus *Tuber* (other than *T. melanosporum* species) were found in any of the two nurseries. Furthermore, forestry plant quality of all seedlings met the basic standards required by the Council Directive 1999/105/CE. Thus, both the presence of contaminant mycorrhizal fungi and plant quality did not lead to the rejection of any batch evaluated by any of the methods.

Table 3 Batch averages and standard deviations of the percentages of colonization by *T. melanosporum* estimated by each method and the grand mean for the methods

Nursery (N) and batch (B)	Grand mean per batch	Reyna et al. (2000)	Chevalier and Grente (1978)	Govi et al. (1995)	Fischer and Colinas (1996)	Palazón et al. (1999)
N1B1	25.2 (±15.5)	38.3 (±24.4) A	27.2 (±10.6) A	17.4 (±11.3) R	19.8 (±8.3) R	23.3 (±8.7) R
N1B2	17.6 (±14.9)	30.0 (±20.5) R	16.8 (±13.9) R	15.6 (±11.5) R	6.1 (±4.8) R	19.3 (±14.0) R
N1B3	35.0 (±16.8)	46.7 (±24.6) A	30.3 (±8.2) A	32.6 (±15.4) A	29.6 (±12.4) A	36.1 (±14.9) A
N1B4	25.1 (±14.4)	31.1 (±15.1) R	32.7 (±9.3) A	12.9 (±11.9) R	17.9 (±11.5) A	30.7 (±13.6) R
N1B5	32.4 (±14.7)	46.4 (±13.5) A	37.0 (±7.2) A	24.3 (±15.1) R	19.2 (±10.8) A	34.9 (±9.5) R
N2B6	42.3 (±18.8)	46.3 (±26.7) A	41.5 (±16.6) A	33.3 (±12.8) A	34.6 (±10.7) A	55.7 (±15.9) A
N2B7	31.2 (±12.7)	26.8 (±11.9) R	28.2 (±10.6) A	30.7 (±13.8) A	26.5 (±9.5) A	43.7 (±10.1) A
N2B8	31.0 (±16.1)	39.1 (±24.0) R	33.3 (±16.3) A	24.3 (±14.1) R	22.9 (±8.1) A	35.4 (±7.5) R
N2B9	33.2 (±12.5)	47.0 (±14.7) A	31.5 (±11.5) A	24.3 (±8.1) R	27.5 (±5.4) A	35.4 (±4.9) A
N2B10	29.8 (±12.7)	29.7 (±11.0) A	36.5 (±13.3) A	26.1 (±18.1) R	21.8 (±3.4) A	34.8 (±5.5) R
Number of batches suitable		6	9	3	8	4

Batch suitability (*A* accepted; *R* rejected) depending on the evaluation method performed. Batch suitability has been obtained by applying the guidelines for each method (see Table 1)

The correlation analysis between evaluation methods concerning the colonization percentage obtained for each seedling (Table 4) shows the existence of a significant ($p<0.05$) and positive correlation between all methods, with the exception of the method of Reyna et al. (2000), which only had a significant correlation with the method of Govi et al. (1995). The highest correlation coefficient (0.662) was obtained between the method of Palazón et al. (1999) and the method of Fischer and Colinas (1996). The lowest coefficient in a significant correlation (0.399) was found between the methods of Chevalier and Grente (1978) and Govi et al. (1995).

The percentage of colonization, averaged for the five methods, significantly differed between nurseries ($p<0.001$) and was highest for seedlings produced by nursery 2 (33 % compared to 27 % in nursery 1). The statistical model, however, only explained 3 % of the variability in the observations. Furthermore, the estimates of colonization percentages obtained by each method differed significantly ($p<0.001$). These estimates can be subdivided into three groups (Fig. 1): Fischer and Colinas (1996) and Govi et al. (1995) with the lowest colonization percentages, Chevalier and Grente (1978) with an intermediate percentage, and Palazón et al. (1999) and Reyna et al. (2000) with the highest colonization percentages. Additionally, each method gave different values for the colonization percentages from each nursery (Fig. 2), with the exception of the method of Reyna et al. (2000) that did not detect differences between the nurseries. The interaction between nurseries and evaluation method was not significant ($p=0.056$). The evaluation methods account for 10.4 % of the colonization percentage variance. On the other hand, if the variation due to nursery is included, the value rises to 14.2 %.

The methods differ significantly (Kruskall-Wallis *K* statistic=536.852; $p<0.001$) in the mean time spent estimating the colonization percentage of individual seedlings (Fig. 3). The method of Chevalier and Grente (1978) was the quickest for plant evaluation with an average of 6 min per seedling. The most time-consuming method is Fischer and Colinas (1996), with about 34 min per seedling.

Discussion

Many variables affect truffle fungus colonization of inoculated seedlings: nursery practices, inoculum (dose, format, pretreatment), potting mix, type of container, and seedling

Table 4 Correlation matrix (Pearson Product Moment) of the colonization percentages by *T. melanosporum* obtained with the five evaluation methods tested. Significant correlations are highlighted in bold

Methods	Govi et al. (1995)	Chevalier and Grente (1978)	Palazón et al. (1999)	Reyna et al. (2000)
Fischer and Colinas (1996)	**0.554** (<0.001)	**0.593** (<0.001)	**0.662** (<0.001)	0.147 (0.108)
Govi et al. (1995)		**0.399** (<0.001)	**0.610** (<0.001)	**0.215** (0.019)
Chevalier and Grente (1978)			**0.594** (<0.001)	0.114 (0.214)
Palazón et al. (1999)				0.162 (0.078)

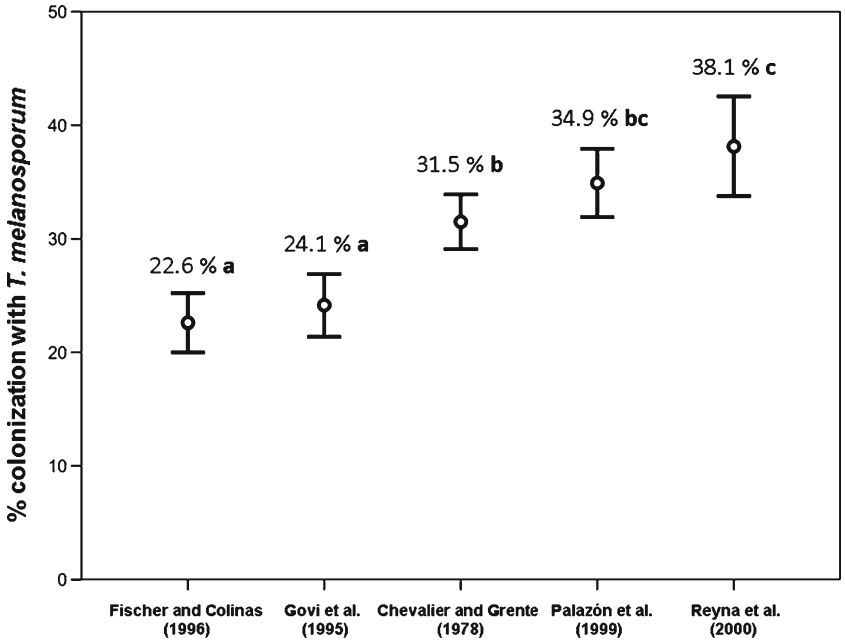

Fig. 1 Least square means and graphical representation of the percentage of colonization by *T. melanosporum* (confidence interval $1-\alpha=95$ %) obtained by the five methods evaluated. The ANOVA analysis was based on a fixed factor method: $R^2_{adj}=0.104$; SEM=2.19; $p<0.001$. *Different letters* show significant differences between the methods contrasted by post hoc Tukey's test

condition at the time of inoculation, among others (Hall et al. 2007). However, we have also observed clear differences between the mycorrhizal colonization evaluation methods used and the resulting batch suitability. One method may find a batch of *T. melanosporum*-colonized *Q. ilex* seedlings acceptable while another method may not. Thus, it is imperative that truffle seedling certifying groups reach agreement on the best certification method. Based on our observations, the

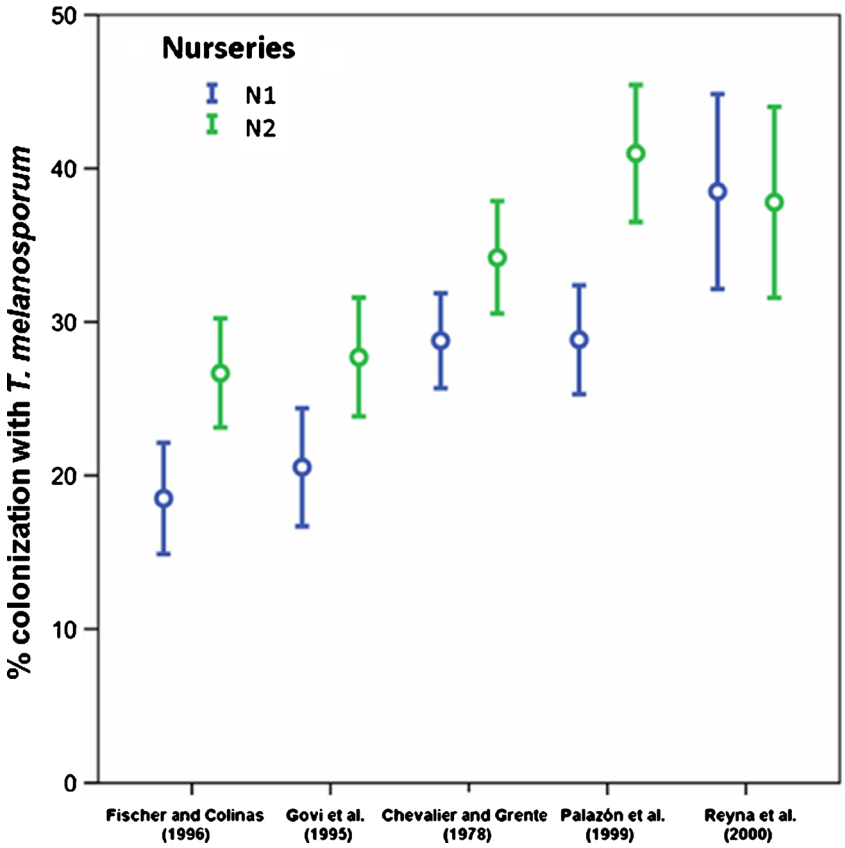

Fig. 2 Graphical representation of the confidence intervals ($1-\alpha=95$ %) of the percentage of colonization by *T. melanosporum* obtained by the different methods for each nursery

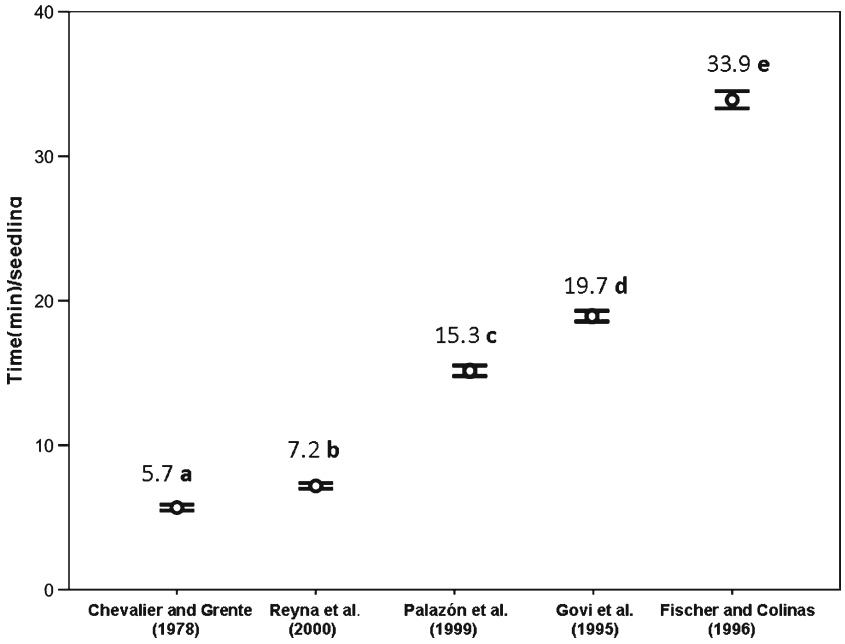

Fig. 3 Means, standard deviations, and graphical representation of the confidence interval ($1-\alpha$=95 %) of the time spent in estimating the percentage of seedling colonization by *T. melanosporum* for one seedling by the different methods for evaluating seedling quality. *Different letters* show significant differences according to Mann-Whitney *U* test ($p<0.05$)

various methods agree to accepting or rejecting a batch when seedlings have either very high or low colonization. However, the strongest lack of consensus among methods for accepting or rejecting batches occurred on batches with intermediate colonization levels, which seems to be the most usual situation.

The methods that achieve the lowest colonization values—Fischer and Colinas (1996) and Govi et al. (1995)—use grids wherein sampling selects random root segments. In the other three methods, estimated colonization levels are higher, either because they are slightly subjective (Chevalier and Grente 1978; Palazón et al. 1999) or because they take a root sample from a highly colonized area (Reyna et al. 2000). In this latter case, the internal variability of each sampling unit should be taken into account (Rocchi et al. 1999). Colonization percentages for each seedling and method are correlated in all cases except in the method of Reyna et al. (2000). It is important to note that only the methods of Reyna et al. (2000) and Fischer and Colinas (1996) estimate the total root tip number for each seedling in addition to colonization percentage. The remaining methods, with the exception of that of Chevalier and Grente (1978), only ensure that suitable seedlings have a minimum number of *T. melanosporum* mycorrhizae. It was noteworthy that the method of Reyna et al. (2000) did not detect the presence of any contaminant fungi while all the other methods did detect the presence of contaminant fungi. The method used by Fischer and Colinas (1996) was particularly efficient at detecting contaminant fungi (data not shown) because it includes a more exhaustive examination of the complete root system.

Sample freezing limits the effect of detecting variation in colonization percentage due to the time elapsed from the first to the last analysis. Freezing and thawing had little effect on the morphology of mycorrhizae and also enabled a single analyst to carry out all evaluations. This uniformity and the single analyst-based analysis allowed us to make a comparative judgment (in terms of advantages and disadvantages) of the different techniques.

The method of Reyna et al. (2000) is fast, easy, and non-destructive. For a given batch, it also enables analysts to estimate the mean number of root tips per plant, as data relate to a specific volume. The main disadvantage of this method is that it assumes a homogeneous mycorrhiza distribution throughout the container volume when actually a considerable amount of mycorrhizae accumulates on the surface of the container inner walls. Thus, it would be necessary to adjust the cylinder extrapolation to volume and surface. Furthermore, some forestry plant quality parameters cannot be assessed when using this method.

The method developed by Chevalier and Grente (1978) is also fast and easy to implement. However, it is highly subjective, so its validity is linked to the observer's experience. Only seedling colonization and contaminant fungi percentages are estimated, while direct and objective quantitative data are not obtained.

The grid sampling method of Govi et al. (1995) might affect the quality of samples, since root extraction through the slots marked in the grid can destroy some root tips. Thus, this grid method is not currently used in Italy. It has been modified by Bencivenga (2013); six fragments each from the upper and bottom sections of the root system are collected and 50 root tips from each counted (a total of 600 root tips). Unfortunately, this modified method could not be included in this work.

The method of Palazón et al. (1999) is similar to that of Govi et al. (1995) as it extracts root fragments but without the use of a grid. Although it is easy and fast, it can involve some subjectivity when choosing which root fragments to evaluate. This may be the reason for the higher colonization percentages observed when using this method, surpassed only by those obtained by the method of Reyna et al. (2000). The number of seedlings sampled (0.5 %) also seems low, so this method is only reliable with very homogeneous or large batches.

The method of Fischer and Colinas (1996) appears to overcome all the disadvantages discussed above. It also uses an analysis with high statistical robustness implemented in an easy-to-use spreadsheet. It provides, among other values, an estimation of the total number of root tips for each seedling. Its major drawback is the time needed for seedling evaluation, which is an important factor as it significantly increases the economic costs of plant certification. However, if the seedlings from a given batch have homogeneous characteristics, good black truffle colonization levels, and few contaminant fungi, the number of replicates can be safely reduced to five instead of the 12 seedlings initially required, thereby reducing the time needed for processing a given batch.

Although there remains a lack of consensus on the best seedling evaluation method, we recommend that the method be objective, systematic, and use a well-defined and clear process as is the case for the method by Fischer and Colinas (1996). It should also be easy to implement, in time and equipment and, if possible, nondestructive. The methods of Bencivenga (2013) and Palazón et al. (1999) are relatively less time consuming. Sensitivity and specificity of subjective methods, such as by Chevalier and Grente (1978), improve as the analysts gain practical experience (Sisti et al. 2010).

With the objective of reaching an agreement between all certification organizations, perhaps the best option would be to combine two correlated methods. For example, analysts could use the method of Chevalier and Grente (1978) when the colonization levels are high and contaminant fungi are clearly absent. However, when colonization is low and contaminant fungi are present, a more accurate method should be used to ensure that the colonization percentage and minimum number of colonized root tips of the batch are correctly estimated.

The assessment of contamination by other ectomycorrhizal fungi needs further development. This is especially important to prevent the introduction of *T. indicum* because this fungus represents a serious ecological risk for European truffle culture (Murat et al. 2008). An ideal new method would also incorporate routine molecular analyses. Molecular techniques that discriminate between morphologically related species within the genus *Tuber* are reported in the literature. These techniques are based on the use of RFLP (Pérez-Collazos et al. 2010), specific PCR primers (Paolocci et al. 1997; Mabru et al. 2001; Suz et al. 2006), or real-time PCR (Sánchez 2012; Parladé et al. 2013) and analyze the DNA of mycorrhizal root tips or ascocarps. Another option would be the analysis of extraradical mycelium present in the potting mix (Suz et al. 2006; Parladé et al. 2013).

There is an obvious need in Europe and elsewhere to reach agreement on unifying the criteria for truffle seedling evaluation. Consideration of compulsory controls by the European Union to limit the potential invasion of troublesome mycorrhizal fungi such as *T. indicum* is also needed to protect the truffle industry. Our results provide an important basis for future decisions concerning the unification of methods to estimate *T. melanosporum* colonization on inoculated *Q. ilex* seedlings.

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