# Quality standard for edible mycorrhizal fungi seedlings in New Zealand

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This standard sets out the quality requirements for seedlings mycorrhized by edible mycorrhizal fungi (EMF) for the establishment of truffle or mushroom orchards. The quality control process is documented in a separate Seedling Testing Protocol.

An EMF seedling is a complex, dynamic, and sensitive living product. Its successful production requires skills, experience, and knowledge. This document sets out the standards expected for EMF seedlings based on current knowledge. The quality of EMF seedlings is improving continuously worldwide as more knowledge and new techniques are acquired. Ongoing research, whether publicly or privately funded, is key to the future development of the industry. Active research is needed to elucidate aspects pertaining to EMF seedling quality that are still unknown, or poorly known, to further improve EMF seedling quality and to develop new products for the NZ industry, e. g. Italian White Truffle, Porcini seedlings etc.

Supporting Information (SI) with more details and references is provided in the Appendix. Both the present standard and the SI have been developed and approved following peer review.

## Plant and fungus material

In New Zealand conditions, the following species have to date been used successfully as host plants for EMF mycorrhizal synthesis:

*Corylus avellana*, *Quercus robur*, *Quercus ilex*, *Quercus cerris*, *Pinus radiata*, *Pinus pinea*, *Pinus pinaster*, *Pinus sylvestris*.

More tree species could be developed in NZ in future (see SI).

In New Zealand conditions, the following high value edible mycorrhizal fungi (EMF) are currently available for cultivation purposes using controlled mycorrhization:

Truffles
*Tuber melanosporum* (The Périgord black truffle), *Tuber borchii* (Bianchetto truffle), *Tuber aestivum* syn. *uncinatum* (Summer or Burgundy Truffle)

Mushrooms
*Lactarius deliciosus* (Saffron milk cap)

More fungal species could be developed in NZ (see SI).

## Quality assurance of the fungal inoculum

Inoculum quality is one of the most important requirements when producing mycorrhizal truffle seedlings. Only truffle spore inoculum is covered here (see SI for other inoculum types).

Only truffles that are true to species AND mature may be used as inoculum. Mature commercial truffle species can be reliably differentiated by an experienced specialist using a combination of characteristics: general aspect, gleba, spores, and aroma. However, there are situations where even a specialist could struggle to discriminate between lookalike species, e. g., *T. melanosporum* and *T. brumale*; *T. borchii* and *T. dryophilum*. Therefore, species-specific DNA testing is compulsory and testing results must be recorded for each batch. Two testing options can be considered:

**Option 1: Each truffle proposed as inoculum is tested individually by DNA to confirm its identity**.

This is the preferred option if truffles are big (> 50g or > 20g each for *T. melanosporum* and *T. borchii*, respectively) and if background information about truffle provenance is totally lacking OR if no pre-selection was done by a specialist. When small truffles are used without background information about their provenance OR if no pre-selection was done by a specialist, option 2 should be used.

OR

**Option 2: Each slurry[1] is tested by DNA to confirm the identity of the species AND to rule out the presence of unwanted contaminant truffle species.** There is always the possibility that a hitherto unsuspected species goes undetected in a slurry (See SI for more information).

This is the preferred option if truffles (of any size) come from orchards devoid of unwanted and very similar species OR have been individually morphologically pre-selected with confidence by a specialist. For truffles coming from orchards with a few years history of no unwanted and visually similar species, a one-off DNA test of a slurry is acceptable, i.e., the test not need to be repeated in each of the following years for truffle coming from the same orchard. However, it would be best practice to carry out a new slurry DNA test every three-five years.

It is preferable to use food grade mature truffles displaying characteristic aroma whenever possible. DNA-validated overripe truffles make good inoculum. SI describes other recommended inoculation practices.

**Definition of a commercial nursery "batch" of EMF seedlings**

A batch is a group of seedlings of the same tree species, same seed source, same age (i.e., sowing date), inoculated at the same time and place by the same staff, using the same materials (e.g., potting mix, type of containers), and same method, with the same source of inoculum (spores or mycelium) and grown under the same conditions after inoculation (greenhouse conditions, watering regime, nutrients).
To ensure consistency a batch should not exceed 5,000 seedlings (G. Chevalier, pers. comm.). Batches of *Tuber borchii* seedlings must be incubated in a separate greenhouse (see SI).

Batches are the nursery's responsibility and should clearly identifiable prior to the visit of the controller.

[1] A slurry is a spore suspension that results from grinding several truffles together in water.

**Quality requirements of the final product, i.e., a tree seedling mycorrhized by a target edible fungal species for the purpose of establishing an orchard to cultivate this species.**

EMF seedlings must be hardened at least 2 months outside prior to testing and planting.

Usually after eight to ten months of incubation, after inoculation, depending on the plant/fungus combination (*T. aestivum* mycorrhization can take longer), EMF seedlings will be subjected to plant and mycorrhiza quality control to assess their suitability for establishing orchards according to the following criteria. This control can also be done whenever the nursery requests it after six to eight months.

## I. Characteristics of the plant.

No single characteristic determines seedling quality. Seedling quality is a combination of height, diameter, plant nutrition, health, root size and shape. Together, these characteristics determine how well the plant will establish itself in the field, and hence the plant's rate of survival. Height alone is often not a good predictor of how a plant will grow in the field. It is good nursery practice to judge seedling quality by multiple traits, and to discard any seedling that has obvious defects.

A seedling should meet the following nursery standards before moving on to evaluation of the level of mycorrhization:

- Seedling age is at least 10 months. Stems of English oaks or hazelnut seedlings should be at least 15 cm high, those of Holm oaks at least 10 cm high.
- A balanced shoot and stem ratio. It is important that the root system appears well developed with a well-balanced architecture.
- Secondary roots should be abundant.
- The collar/stem should be lignified. The diameter of the collar of the stem should not be less than 2mm for pines or 3mm for other species (based on 1 or 2 y old seedlings).
- No malformed root systems: There should be no coiled root system and no circling roots close to the trunk.
- Plant containers should be > 300 mL for oaks and >150 mL for pines
- The seedlings should be devoid of significant diseases and pathogens both in the shoot and the root aspects.

Seedlings that are not satisfying the growth characteristics listed above are not subjected to the mycorrhization specifications detailed below. Seedlings that present no defect but are too small may be grown further prior to be tested for mycorrhization.
For each batch, it is the nursery's responsibility to sort the seedlings that pass or not the growth characteristics prior to the visit of the controller.

## II. Characteristics of the Fungus.

The requirements below cover both individually inspected seedlings or a group of individually tested seedlings representing a random sample of seedlings extracted from a batch (see SI). The whole surface of the root system of seedlings is inspected under a dissecting microscope. Assessment is non-destructive except for mycorrhizae sampled for compound microscopy or DNA testing.

**Requirements for mycorrhization by the target species.**

The presence and abundance of mycorrhizae of the target species on inoculated seedlings is the most important criterion determining their quality and suitability to establish an EMF orchard.

Individual seedlings

The **presence** of mycorrhizae of the target species on a seedling analysed individually can be assessed with 100% efficacy by a trained observer using appropriate tools.

The **abundance** of mycorrhizae of the target species on inoculated seedlings is important but to date no research has definitively clarified this complex aspect (see SI). The more abundant the mycorrhizae of the target species on the inoculated seedling, the higher the chance of persistence and development of the target species after plantation.

The abundance of target mycorrhizae can be assessed qualitatively but its quantification is challenging, time-consuming and unrealistic from an economic/practical point of view (see SI). To address this problem, it is proposed to consider fit for purpose, regarding mycorrhization by the target species, any seedling that shows at least one of the following two crucial, reliable, and **tangible criteria**:

1. The rapid detection of target mycorrhizae - this should take no more than 10-15 s [2] on a seedling with a moderate to high percentage of mycorrhization.
2. The presence of well-developed, bigger, branched ectomycorrhizae, or mycorrhizal clusters (groups of branched mycorrhizae) suggesting that the target fungal species is well-established on the seedling.

Sample of individual seedlings

The smaller the batch, the higher should be the sampling rate (3 to 10%, see SI). The seedlings are randomly collected and form a sample representing a given batch. The distribution, within that sample, of seedlings that show different levels (abundance) of mycorrhization must be taken into consideration to determine the suitability of the sample, and therefore the batch.

At this point of time in New Zealand, it is unrealistic to expect that all seedlings from a batch sample will always show one of the defined criteria. The following threshold for target mycorrhization is therefore proposed for acceptance of a sample of seedlings from a batch:

- 90% or more of sampled seedlings must show the presence of the target edible fungi species.

  AND

- 70% or more of sampled seedlings must show at least one of the two tangible criteria.

[2]Assuming the presence of the target mycorrhizae for the batch the seedling belongs to has been previously confirmed by a double-step microscopy. See Seedling testing protocol.

If more than 10% of seedlings in the sample fail to show the target mycorrhizae, the remaining seedlings from the batch cannot be accepted unless they undergo individual testing to identify and retain only seedlings that show the presence of the target species. Fifty percent or more of the remaining seedlings thus retained should show one of the tangible criteria for the sample to be accepted.

**Requirements for the mycorrhization by non-target species**

The presence of non-target mycorrhizal fungi can be detected with 100% efficacy by a trained observer using appropriate tools.

The weak presence of non-target species on seedlings otherwise well-colonised by the target species is acceptable and acknowledged (SI). The quantification of non-target mycorrhizae meets the same difficulties as those encountered for the target species. The following approach is proposed:

Individual seedlings

- Zero tolerance for undesired truffle species (*Tuber brumale*, *Tuber dryophilum*[3]) and for AD-type mycorrhizae (*Trichophaea woolhopeia* or related species).
- Zero tolerance for other ectomycorrhizal species if the target species is not present.
- Weak tolerance for other ectomycorrhizal species, excluding those mentioned above, if the target species is present. Weak presence is defined as follows: the cumulated area where non-target mycorrhizae are detected should not exceed ≈10% of the total area of the root system. A higher percentage is tolerated for *Sphaerosporella brunnea*, a species frequent in nursery that is poorly competitive in the field and disappear after plantation (G. Chevalier, pers. comm.).

Sample of seedlings
The following threshold is proposed for the acceptance, or not, of mycorrhizae of non-target species on a sample of seedlings, provided the sample has passed the target species requirements:

- Zero tolerance for undesired truffle species (*Tuber brumale*, *Tuber dryophilum*) and for AD-type mycorrhizae (*Trichophaea woolhopeia* or similar species) on any of the sampled seedlings.
- Tolerance for 5% of sampled seedlings showing a strong presence (over 10% of root surface colonised) of ectomycorrhizal species other than those described above.
- Tolerance of 20% of sampled seedlings showing a weak presence (as defined above) of ectomycorrhizal species other than those described above.

NOTE: The final acceptance of the mycorrhization status of seedlings, whether individual or in groups, must meet the requirements described above for both the target and non-target

[3]The mycorrhizae of these two species can only be identified to species level by DNA testing.

species. The distinction by experienced observers between suitable and unsuitable truffle-inoculated seedlings is essential for successful truffle cultivation.

If no tolerance were allowed, the only way to certify seedlings would be to test all of them individually.

So long as the results of sample-based batch testing are disclosed, customers may wish, depending on results, to request individual testing of all seedlings purchased. This would be at their own cost.

To be certified, individual seedlings, or samples of seedlings, must satisfy the characteristics of both the plant and of the fungus described above, with the level of tolerance indicated.

The individual quality labels issued by the controller for each batch tested must correspond to the sum of the plants contained in the said batch.

Nurseries must keep a recording system (they can choose any system) of the results of each batch assessment.

Uncontaminated seedlings with no defects that do not pass the height and mycorrhization criteria the first year after inoculation can be retested similarly the following year. Three-year-old seedlings are not sold unless they present an excellent mycorrhization.

**Recommended DNA tools**

It is recommended to employ QC assays, using the same technology as for COVID virus testing, namely quantitative PCR. These world-leading assays have been developed in NZ and are up to 300-fold more sensitive than other conventional PCR methods in use (e.g., Australia and Europe), especially for the white truffle clade.

# Appendix to the Quality Standard:
# Supporting Information with more details and references.

**Additional tree species**

For the long-term growth and resilience of the industry, it is important to consider additional host tree species, provided they can be sourced in New Zealand. Many tree species are potentially suitable for cultivating EMF species, but an exhaustive listing is beyond the scope of the present standard.

Some examples of potentially interesting host plants for truffles are *Quercus pubescens*, *Quercus faginea*, *Quercus coccifera*, *Carya illinoinensis* (Pecan tree), *Carpinus betulus* (European hornbeam), *Ostrya carpinifolia* (hop-hornbeam), *Tilia cordata*, *Tilia platyphyllos* (Linden or Lime trees), *Fagus sylvatica* (beech), *Castanea sativa* (chestnut), *Cedrus atlantica* (Atlas cedar) etc.

**Additional fungal species**

Truffles

*Tuber magnatum* (The Italian White Truffle or Alba Truffle) has potential in New Zealand.

It is not recommended to produce *Tuber brumale* (Winter black truffle)-inoculated seedlings.

Mushrooms

Other valuable species are available, or importable, for development such as *Rhizopogon roseolus* (Shoro), *Boletus edulis* (Porcini or Cèpe), and *Tricholoma matsutake* (Matsutake or Japanese pine mushroom).

**Other inoculum types**

The viable fungal 'propagules' used to colonise a host plant are called "inoculum". There are three main categories of fungal inoculum (Chevalier & Grente 1973, Chevalier & Grente 1978, Iotti et al. 2012, Guerin-Laguette 2021):

a) Spores, i.e., fungal "seeds" obtained from fruiting-bodies, either truffles or mushrooms

b) Mycelium, i.e., living fungal tissue obtained by the propagation of fungal cells on sterilised, nutrient-rich media.

c) Mycorrhizae (excised or not), from a previously mycorrhized "mother plant" may be used as inoculum.

In theory, any of these inoculum types can produce mycorrhizal seedlings. However, in practice spores work well for some species (e. g. *Tuber*, *Rhizopogon* etc), but not for others

(e. g. *L. deliciosus*), at least under nursery conditions. In case of *L. deliciosus*, mycelium is required (Wang et al. 2019). Mycorrhizae from mother plants are a very efficient inoculum for most species (Pereira et al. 2013).

European scientists have shown the potential of using mycelial pure cultures of *T. borchii* to produce mycorrhizal seedlings (Iotti et al. 2016). This method eliminates the risk of introducing undesired truffle species in the nursery. However, it is expensive to use on a large scale and is not yet available for other commercial truffle species. Furthermore, a given *Tuber* mycelial culture carries only one mating type, so plants would need to be inoculated with mycelia of two compatible mating types (Zambonelli et al. 2010). Additional research and study would be needed to develop this method further.

**Limitations of species-specific DNA tests for slurries in Option 2**

Species-specific DNA tests can only detect the truffle species that they have been designed for. If a hitherto unsuspected truffle species is present in a slurry, it will not be detected by such tests (unless this species was included in the test design).

In practice, it is possible to develop tests that include the detection of species known to be present in New Zealand, e.g., *T. brumale*, *T. dryophilum* etc.

It is important that routine tests also include lookalike species, such as *T. indicum*, that are not known to be present in New Zealand, but whose presence on the international market creates a risk of potential accidental or voluntary introduction.

Beside species-specific tests, next generation sequencing could detect any species in a slurry, but this would be expensive to run and would be overkill as a routine test.

**Other recommended inoculation practices and information**

Plant seedlings are usually obtained from seeds but can be obtained by other methods such as cuttings.

Best practice is to inoculate young seedlings, usually up to 2 to 3 months old.

Seedlings to be inoculated must be completely free from ectomycorrhizal fungi. This can be achieved through the combination of the following measures:

- Cleaning (washing with water) and mild disinfection of seeds.
- Use of a germination mix free of mycorrhizal fungi.
- Germinating seedlings in an environment (tunnel house) free of other ectomycorrhizal plants.

When ready, these seedlings will be used as host plants to engage in a mycorrhizal relationship with a specific fungal species. Random samples of seedlings should be pre-screened, visually or under the dissecting microscope if required, just before inoculation to check for the presence of early competing mycorrhizal fungi (Donnini et al. 2014).

Seedlings should not be grown together (i.e., in the same tunnel house) with other plants potentially harbouring non-truffle, or other ectomycorrhizal fungi.

**Issues relating to *Tuber borchii* seedlings**

Past observations (Urban et al. 2004, Guerin 2015, Guerin-Laguette 2021) reported that *T. borchii* could easily and significantly cross-contaminate batches of seedlings inoculated with other truffle species, i.e., *T. melanosporum* and *T. aestivum*. Therefore, *Tuber borchii* seedlings must be incubated in a separate greenhouse, i.e., physically separated from the greenhouse space where *T. melanosporum* and *T. aestivum*, or other EMF seedlings, are incubated.

Although the cross-contamination of other truffle seedlings by *T. melanosporum* or *T. aestivum* has not yet been strongly evidenced, a cautious approach must be taken, and nurseries are encouraged to grow separately seedlings that have been inoculated with different commercial truffle species.

As of today's knowledge, seedlings mycorrhized by *Lactarius deliciosus* do not present a risk to cross-contaminate other EMF seedlings.

**Random sampling of seedlings**

The person collecting seedlings from a batch must be independent from the nursery or the sampling must be supervised and witnessed by an independent person (e.g., the controller).

**Abundance of target mycorrhizae and its quantification**

To the best of the writer's knowledge, no research has looked, in a robust way, at the importance of the initial abundance of the target mycorrhizae on seedlings with respect to their subsequent persistence and development in the field after plantation. Such studies would be helpful but are also complex. Results would depend on many other factors: biotic and abiotic characteristic of the soil, the species involved, time of planting etc. Therefore, there is no simple answer to this point except to assume that the more abundant the mycorrhizae of the target species on the inoculated seedling, the better. Abundant mycorrhizae of the target species on seedlings are crucial but not sufficient in themselves to ensure mycorrhiza establishment in the field and successful truffle cultivation: a suitable environment and an appropriate orchard management will be equally critical (Murat 2015).

Mycorrhizae are structurally complex: they vary in form and colour according to their stage of development and their age. This variability makes "counting" them difficult and this practice is reserved for research purposes (Murat 2015). For example, a heavily branched mycorrhizal structure is certainly 'worth' many single branch mycorrhizae and therefore could not be counted as one mycorrhiza without creating a bias. Mycorrhizae can also vary in length or depending upon the host plant. Another difficulty lies in the accurate determination of mycorrhizal tips by the observer when "counting" them: if the determination is done carefully, it cannot be a quick process and would be a daunting task which would exhaust the observer, who would rapidly become unreliable or inefficient. Rather than attempting to count these structures, it is recommended that an experienced inspector should assess how frequent or "well-established" the target mycorrhizae are based on the criteria listed in the standard. For this process, well-trained, highly skilled inspectors are key (Murat 2015).

**Sampling rate**

In Europe, usually ≈1% of seedlings in a batch are sampled and tested (Andrés-Alpuente et al. 2014, Corre 2021). However, we recommend adopting the following higher sampling rates:

| Batch size | Sample size: number of randomly collected seedlings to be individually tested | Corresponding sampling rates |
|---|---|---|
| 1-9 | Each seedling | 100% |
| 10-49 | 6 | 60 to 12.24% |
| 50-99 | 11 | 22 to 11.11% |
| 100-199 | 12 | 12 to 6.03% |
| 200-299 | 14 | 7 to 4.68% |
| 300-399 | 16 | 5.33 to 4.01% |
| 400-499 | 18 | 4.50 to 3.61% |
| 500-599 | 20 | 4.00 to 3.34% |
| 600-699 | 22 | 3.67 to 3.15% |
| 700-799 | 24 | 3.43 to 3.00% |
| 800-899 | 26 | 3.25 to 2.89% |
| 900-999 | 28 | 3.11 to 2.80% |
| 1000-1499 | 38 | 3.80 to 2.54% |
| 1500-1999 | 48 | 3.20 to 2.40% |
| 2000-2499 | 58 | 2.90 to 2.32% |
| 2500-2999 | 68 | 2.72 to 2.27% |
| 3000-3499 | 78 | 2.60 to 2.23% |
| 3500-3999 | 88 | 2.51 to 2.20% |
| 4000-4499 | 98 | 2.45 to 2.18% |
| 4500-5000 | 108 | 2.40 to 2.16% |

**Weak presence of non-target species**

In Europe, depending on the testing methodology used, between 15 to 30% of contaminants are tolerated (Andrés-Alpuente et al. 2014), to the exclusion of unwanted species as described in the standard.

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