Final Report

Project title: The development of affordable and reliable DNA-based analysis protocols and sampling procedures for the detection of commercial truffle species used in truffle cultivation

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Summary:

This project addresses a challenge in the truffle industry, specifically the detection and monitoring of truffle fungi during the many-year period of orchard establishment before truffles can be expected to start producing. The successful colonization of the host tree by a commercial truffle fungus and the retention and spread of that fungus on the roots are critical for successful orchard establishment, yet growers have limited tools to monitor and confirm this process. We adapted and developed reliable DNA extraction methods for orchard soil, DNA fingerprinting methods for the major commercial truffle species, and field sampling procedures for the detection and identification of truffle fungi in orchard soil. All procedures were validated using field-collected samples from established truffle orchards in British Columbia (BC) and the eastern United States. These analytical procedures provide truffle growers with affordable and reliable tools to enhance orchard management.

Introduction:

The production of edible truffle fungi is a developing farm sector in North America. The advancement of the truffle industry in Canada and the United States has been supported by the Truffle Association of British Columbia (TABC) and by the North American Truffle Growers Association (NATGA) who provide forums for growers to interact and seek assistance with a range of technical and

commercial solutions to the challenges of truffle production. Technical support is also offered by some of the major suppliers of truffle-inoculated trees, using a variety of different business models.

Research in various truffle-growing areas of the world has demonstrated that the contamination of root systems by the wrong truffle species and the absence of the desired truffle species from seedlings or orchards can occur. Either problem would compromise the production of the intended truffle species. Research using DNA methods has demonstrated that *Tuber indicum* has fruited in an arboretum in Oregon (Bonito et al. 2011). These methods have also shown that non-target *Tuber brumale* can grow in association with host plants in truffle orchards in Canada and Australia (Berch and Bonito 2014; Linde and Selmes 2012). It is believed that these non-target *Tuber* species were accidentally introduced to truffle orchards as inoculum in the tree nursery, prior to the general availability of DNA-based detection methods. Knowing that mistakes can be made in morphological identification, some major North American suppliers of truffle-inoculated trees now check each truffle with DNA-based methods, prior to their use as inoculum. Some suppliers also recommend that, prior to planting, clients have their nursery stock independently assessed for quality (e.g., good seedling growth form, abundant presence of the correct *Tuber* species, and the absence of competing ectomycorrhizal fungi).

Experience tells us that it takes 7 years or more before a truffle orchard begins to produce truffles. Site, soil and climate conditions influence fungal development and the subsequent production of harvestable truffles. To assist in maintaining appropriate growing conditions for truffle fungus development and orchard establishment, growers would benefit from the ability to detect and identify the preferred commercial species of truffle fungi in their orchards (including *Tuber melanosporum*, *Tuber aestivum, Tuber borchii*, *Tuber macrosporum,* and *Tuber lyonii*) and distinguish them from the less desirable cultivated species (e.g. *Tuber brumale* and *Tuber indicum*) and from native species of truffles (also of the genus *Tuber*). Currently the inability to detect and identify truffle fungi during the

establishment and development of truffle orchards is a technical obstacle for many growers that can impair the effective management of their truffle orchards. Useful detection and identification of truffle fungi in truffle orchard soil requires quick, reliable, and affordable analytical methods that minimize the disturbance of trees growing in the orchard.

With the advancement of these DNA-based technologies in recent years, a novel approach has been developed that should provide a solution to this technical obstacle. The method was originally developed in Spain and has been extensively field verified there (Method for the evaluation of plant quality and mycorrhizal status of *Quercus ilex* seedlings inoculated with *Tuber melanosporum*. Fischer and Colinas (1996)[1], revised January 2014). In May, 2015, a few members of TABC and NATGA participated in a training workshop on these quality control methods for truffle-inoculated seedlings and orchard soils (Laboratory and Field Course for Quality Control in the Establishment and Management of Black Truffle Plantations, Lleida, Cataluña, Spain). The course instructors included Christine Fischer, Daniel Oliach and Juan Martínez de Aragón, (Forest Sciences Centre of Catalonia CTFC-CEMFOR, Solsona, Spain), and Carlos Colinas (University of Lleida-Agrotecnio Center (UdL-Agrotecnio), Lleida, Spain and CTFC-CEMFOR, Solsona, Spain).

Because soil types and climatic conditions in North America differ from those found in Spain, it was important to determine whether the soil DNA-based approach used in Spain would work in North American truffle orchards. On returning from this course, TABC collaborated with MycoLogic Inc, a biotechnology company located in Victoria, BC, Canada, to successfully write and submit a request for funding from the Investment Agriculture Foundation of BC to adapt and develop these methods for North American conditions. The NATGA generously provided support for this project and members of both associations volunteered to provide samples to be used in method development and testing.

[1] http://trufflegrowers.com.au/wp-content/uploads/2014/01/Fischer_Colinas_Methodology-for-certification-Spanish-of-Quercus-ilex-seedlings.pdf

The application of these DNA-based technologies would allow truffle growers to directly sample existing truffle orchard soils to determine truffle fungal development without disturbing tree root systems. These methods could also be used to confirm the quality of planting stock. Since the lead time to establish a new truffle orchard is considerable, it is of critical importance to truffle growers to plant new orchards with the best quality of inoculated seedlings available and to have the ability to monitor the status of the truffle species in their orchard. Detection of ectomycorrhizal fungi on roots of trees has traditionally been done by experts with morphological diagnostic skills, but few have the expertise for that kind of work. If proven effective for North American conditions, the direct analysis of soil samples by DNA-based methods can be offered as a service to growers by biotech labs with proven expertise.

Project objectives:

1. The adaptation and development of reliable laboratory protocols and field sampling procedures for the detection of *Tuber* species in soil
2. The validation of these diagnostic procedures with field-collected soil from existing truffle orchards
3. The provision of a diagnostic service to orchard managers for use in improved truffle orchard management

Methods and Results:

Initially, laboratory experiments confirmed the specificity of the published genetic markers by tests with identified collections of truffle species. We amplified both native and cultivated species of *Tuber* and sequenced those products for the internal transcribed spacer (ITS) region of the rDNA gene to verify species identifications (Table 1). These experiments were followed up with tests of species-specific PCR primers that are used to detect *T. melanosporum* (ITS regions including the 5.8S rRNA gene; Suz et al., 2006), *T. aestivum* (ITS; Gryndler et al., 2011), *T. lyonii* (ITS; Bonito et al., 2011b), and all

species of the genus *Tuber*, using genus-specific primers (β-tubulin gene; Zampieri et al., 2009) with all available native and cultivated species (Table 1). All experiments used PCR (Polymerase Chain Reaction) technology to selectively amplify the target DNA sequences.

We carried out trials of sample materials collected from different sources (confirmed fruiting bodies and ectomycorrhizal root tips) to confirm the effectiveness of total DNA extraction procedures using material from each of these sources. The extracted DNA served as the template DNA for all subsequent PCR reactions. These different starting materials were used to optimize the DNA extraction procedure to obtain good quality DNA suitable for PCR use.

Once reliable protocols and consistent results were established using prepared samples, we proceeded to validation of lab protocols using field-collected soil samples from North American truffle orchards: four in BC (members of TABC) and four in the eastern USA (members of NATGA).

With soil samples from the four truffle orchards tested in BC, we evaluated the use of fresh, frozen, ethanol-preserved, CTAB-preserved, or dried soil samples. Each of these storage and transport formats can potentially affect the quality of the fungal DNA present in the soil, the yield of total DNA from extraction protocols, and the presence of inhibitory compounds in the extracted DNA samples. We determined that, with all factors being considered, the order of preference for sample storage method is; fresh soil is best, followed by frozen, then dried, all of which are much better than ethanol-preserved, which is much better than CTAB-preserved samples. Fresh and frozen samples generally provide the best yields of DNA but their shipping is complicated by the need to keep the samples cold and the shipping time to a minimum, as well as by their higher weight and therefore greater cost. Dried samples provide acceptable yields and are likely more cost-effective to ship, as they are less sensitive to conditions and lower in weight. However, careful soil drying is required immediately after sample collection (see Appendix 1 for details).

Four different DNA extraction protocols were tested; they included (i) the Chloroform:Isoamyl alcohol (STE) protocol, (ii) Cetyl trimethylammonium bromide (CTAB) protocol, (iii) PowerSoil DNA extraction kit (MoBio), and (iv) the Nucleospin soil extraction kit (Machery-Nagel). Extraction results varied for extraction method and soil type tested (Figures 1 and 2). In general, the kits (PowerSoil and Nucleospin) out-performed the chemistry-based protocols (STE and CTAB) with regard to yield and purity of the DNA. However, among the chemistry- based protocols, CTAB was more effective than STE, and may represent a low-cost alternative to the kits.

Another factor to consider is the potential for error due to inappropriate collection, storage or shipping of soil samples. To address this, we developed a standardized sampling procedure that, if followed rigorously, provides consistent results and is easily followed by orchard managers (Appendix 1).

When analyzing DNA samples from orchard soils, we used a number of PCR primer sets. The DNA samples were:

1. Tested with **genus-specific primer sets** for *Tuber*. Ten species of *Tuber* (cultivated and native) were tested with the genus-specific primers that amplify the β-tubulin gene. All species showed consistent positive amplification products with the *Tuber* genus-specific primers except *T. melanosporum* (Table 1). *Tuber melanosporum* showed weak or no amplification for some specimens (including both fruiting bodies and soil samples), even though the specimens showed positive amplifications for *T. melanosporum*-specific primers. To increase our confidence in the results, some putative *T. melanosporum* products (positive *T. melanosporum* amplification, negative *Tuber* amplification) were sequenced and these yielded sequences of *T. melanosporum*.
2. We also tested three **species-specific ITS primer sets** that amplified either *T. melanosporum*, *T. aestivum*, or *T. lyonii* template DNA. The species-specific primers were shown to amplify only samples of the target species, with one exception. While the *T. melanosporum*-specific and *T. lyonii*-specific primers amplified only *T. melanosporum* and *T. lyonii*, respectively among 10 species tested,

the *T. aestivum*-specific primers amplified both *T. aestivum* and *T. mesentericum* under normal PCR conditions (Table 1). However, we found that by increasing the annealing temperature in the PCR cycle, this eliminated the amplification of *T. mesentericum* template DNA and maintained primer specificity (Table 1).

In summary, these four primer sets were tested on different soil samples and for *T. melanosporum* fruiting bodies and the amplified products were confirmed by nucleotide sequencing. We confirmed that the genus-specific (*Tuber*-specific) primers amplify a number of cultivated and native *Tuber* species, while the three species-specific primer sets will not amplify non-target *Tuber* species under defined PCR conditions (Table 1). These results support the specificity of genus and species-specific primer sets, when used to analyze environmental soil samples.

Results for TABC orchard samples:

Soil samples were provided by TABC members from four different truffle orchards in BC. Some of these samples were used to help develop and test the protocols; others were used to test the developed protocols on soils from different truffle orchards. All of the results are provided in Table 2.

The DNA template obtained for all soil samples was screened with the *Tuber* genus-specific and the *T. melanosporum*-specific or *T. aestivum*-specific PCR primer sets, depending on the target truffle species. Positive results from some PCR amplifications were sequenced to verify the identity of the amplified product. Some of the samples that were *Tuber* positive but *T. melanosporum* negative were also tested with *T. aestivum*-specific and *T. lyonii*-specific primers.

Tuber melanosporum was detected from one or more soil samples from each truffle orchard where *T. melanosporum* was the target species. *Tuber aestivum* was detected in orchards growing this species. *Tuber lyonii*, a species not known to be native to western North America, was detected in one

truffle orchard. Other *Tuber* species detected in some of the truffle orchards are likely to be the non-commercial native *Tuber* species already detected in some of these orchards, i.e. *Tuber anniae*, *Tuber beyerlei*, and *Tuber menseri* (Berch and Bonito 2014). When sampled for this study, orchard S was in decline due to eastern filbert blight (*Anisogramma anomala*) on the hazelnut trees and was no longer being managed, but *T. melanosporum* was still detected in most of the soil samples.

Two soil samples taken from a native conifer stand adjacent to orchard N served as negative controls; cultivated truffle DNA should not have been found and was not found. In another negative control soil sample taken from a pasture adjacent to truffle orchard N, *Tuber melanosporum* was weakly detected only in one of four replicates, most likely due to cross-contamination in the field.

Results for NATGA orchard samples:

Five soil samples were provided by NATGA members from each of four different truffle orchards in the eastern United States; each of the five soil samples per orchard were composed of 3 soil sub-samples per tree, if the orchardists followed instructions (Appendix 1). These soil samples were provided to allow us to test the protocols and methods we had developed on different soil types from active truffle orchards. All samples were received at the University of Victoria and two independent subsamples (fresh and dried) were used from each soil sample for total DNA extractions. The MoBio Power Soil DNA extraction kit was used to extract and purify DNA for use in PCR reactions. For each soil sample, 250 mg of well-mixed soil was extracted and purified using this kit.

The DNA template obtained for all samples was screened with both the *Tuber* genus-specific and the *T. melanosporum* species-specific PCR primer sets, as described previously. Positive results from some PCR amplifications were sequenced to verify the identity of the amplified product, whether it be a *Tuber* sp., or the species *T. melanosporum*. Samples that were *Tuber* positive but *T. melanosporum*

negative were also tested with *T. aestivum*-specific and *T. lyonii*-specific primers. The results of these analyses are summarized in Table 3.

Tuber melanosporum was detected from at least one soil sample in each of the four NATGA orchards (Table 3). The number of soil samples from an orchard containing *T. melanosporum* ranged from one (orchards A and C) to four (orchard D). Other species of *Tuber* were detected from at least one soil sample in each of the four orchards. Sequences of the ITS or β-tubulin regions or species-specific primers were used to aid in the identification of the *Tuber* species that were not *T. melanosporum*. We detected four additional species of *Tuber* using sequencing; using *T. lyonii* species-specific primers we detected this native truffle species in one of the NATGA orchards.

The percent match of sequences for the four additional *Tuber* species was low when we compared our sequence information to the GenBank database, therefore species identification was not possible. As a general rule, a 97% sequence similarity indicates the same species. The genus-specific primer set for *Tuber* is based on the β-tubulin gene (Zampieri et al. 2009), whereas most studies of *Tuber* species identification have used the ITS-rDNA gene region. The Genbank database against which sequences are compared is therefore deficient in β-tubulin sequences for many *Tuber* species and species level identification is therefore limited. However, we did compare these *Tuber* sequences to *T. melanosporum* β-tubulin gene sequences to confirm that the species were not *T. melanosporum*. It is possible that the four additional unidentified *Tuber* species belong to native truffle species that are not included in the Genbank sequence database.

While one might expect similar results between runs 1 and 2 for each orchard, we had greater success in run 2 for some orchards. Differences between runs 1 and 2 represent different treatment of the soil samples prior to their use for DNA extractions. For run 2, soil samples were dried and ground with a pestle, to break up chunks and allow for more thorough mixing. This improved results for samples where the soil was wet and clumpy, and will be included as part of the regular protocol. Still, we

recommend that at least two independent replicates are tested by DNA analysis from each soil sample, in order to increase confidence in the results.

Soil physical and chemical analyses:

For all tested orchards, select soil physical and chemical analyses were carried out (Table 4) on a bulked soil sample after the DNA extraction work was done. No clear patterns of success or failure of the DNA protocols were detected relative to any of the soil parameters determined.

Discussion and conclusions:

With this project, we have confirmed that soil DNA-based detection methods developed in Spain can be successfully applied under North American conditions. We amplified species of *Tuber* from soil samples for each truffle orchard tested. Some samples that produced positive amplifications for *T. melanosporum* using species-specific primers were secondarily verified with sequencing of the amplified PCR products. Samples that did not produce positive amplifications for *T. melanosporum,* but did amplify the genus *Tuber,* were sequenced or tested with other species-specific primers in order to identify the *Tuber* species present. We were also able to identify or exclude two additional species (*T. aestivum* and *T. lyonii*) using their species-specific primers.

The *Tuber* genus-specific primers amplify a portion of the β-tubulin gene. The Genbank sequence database for this gene is limited, therefore precise identifications could not be made for all the *Tuber* species we detected. However, we were able to rule out that the unknown *Tuber* species were *T. melanosporum* or *T. aestivum.* Future work should consider either building our own database of β-tubulin sequences for all known species of *Tuber* from North America, or developing new *Tuber* genus-specific primers for the ITS-rDNA region, for which a sequence database has already been established.

Furthermore, we are currently testing additional species-specific primers for *T. brumale* (Rubini et al., 1998), *T. borchii* (Mello et al., 1999) and *T. indicum* (Rubini et al., 1998). Preliminary results are ambiguous, but with some adjustments to the protocols, we may be able to add these primers to our suite of markers. This may allow us to rule out the presence of other contaminating species of *Tuber*.

The outcomes of this project will provide a number of benefits to the TABC and NATGA, and thus to all truffle producers of North America. Application of these DNA-based technologies would allow growers to directly sample existing truffle orchard soils to determine the extent of fungal development without disturbing tree root systems. Since the lead time to establish a new truffle orchard is considerable, it will be of critical importance to truffle growers to plant a new orchard with the best quality of trees available and to have the ability to manage the soil conditions of the orchard based upon reliable information gathered from soil surveys of truffle fungus development.

Recommendations:

This study has demonstrated the need for proper soil sampling and storage (preservation) methods. It also highlighted the need for the inclusion of multiple replicates at the testing stage, in order to increase confidence in the results. Specifically, we recommend that:

1. samples are stored in a fridge and shipped fresh (on ice), frozen before shipment, or rapidly dried after sample collection for best yield of DNA;
2. thorough mixing of samples, both during field collections and before DNA extraction, be carried out to get a homogenous sample;
3. thorough cleaning of the soil sample collection tools between each sample be carried out to avoid cross-contamination;

4. because only a small amount of soil is used for the DNA extraction, at least two independent replicates of each sample be tested in the DNA lab to increase the probability of detecting *Tuber* DNA that may be present in small quantities;
5. if the grower wants only to know whether *Tuber melanosporum* is present, amplify with the *T. melanosporum*-specific primer set only;
6. similarly, if the grower only wants to know whether *Tuber aestivum* is present, amplify with the *T. aestivum*-specific primer set only;
7. if the grower wants to know whether the target *Tuber* species is present and whether other *Tuber* species are also present, amplify with *Tuber* genus primer set and with primer sets specific to the target *Tuber* species; if results are positive for the presence of *Tuber* but negative for target *Tuber* species, further investigation may be warranted.

Finally, it is very important to note that there is some possibility of false negatives, specifically, the lab analysis fails to detect a *Tuber* species that is actually present because the fungus and therefore its DNA are present in very small amounts, or due to problems with sampling, shipping or storage. Since there are potentially serious consequences of failing to detect the target *Tuber* species in all soil samples, such results should be followed up with a more intensive study that includes both morphological examination of ectomycorrhizas from the orchard, as well as further DNA-based sampling. The detection of false positives (e.g., analysis detecting a *Tuber* species that is actually not present) could also occur through cross-contamination in the field or lab. Careful adherence to the soil sampling protocol and lab protocols are essential to minimize this risk.

Literature cited:

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Figure 1. Amplification products using *T. melanosporum*-specific primers (ITS1-TM/ITS2-TM and T.mel-for/T.mel-rev) for two soil samples (Tm1, Tm2) for each of three extraction protocols, STE, CTAB and PowerSoil (PS). A positive amplification is indicated by the presence of a dark band of approximately 500 base-pairs, and indicates the presence of *T. melanosporum* DNA. Pos= positive control; neg= negative control. The ladder shows DNA fragments from 100 to 1000 base pairs (bp) in length.

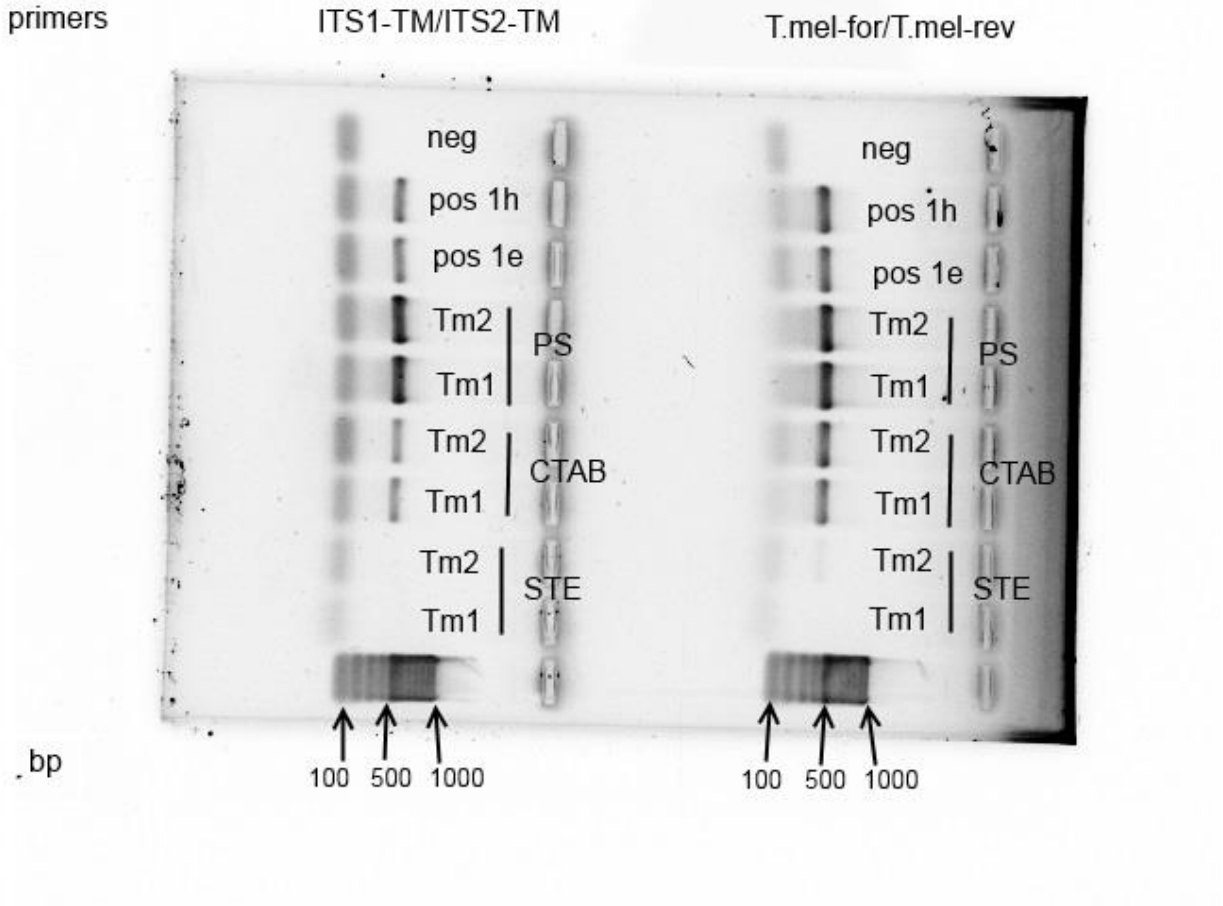

Figure 2. Amplification products using fungal-specific primers (ITS-1F and LRC42) for four soil samples (St5, St6, St14, St17) for three extraction protocols, STE, CTAB and PowerSoil (PS). A positive amplification is indicated by the presence of a dark band of approximately 700 base-pairs, and indicates the presence of fungal DNA. Pos= positive control; neg= negative control. The ladder shows DNA fragments from 100 to 1000 base pairs (bp) in length.

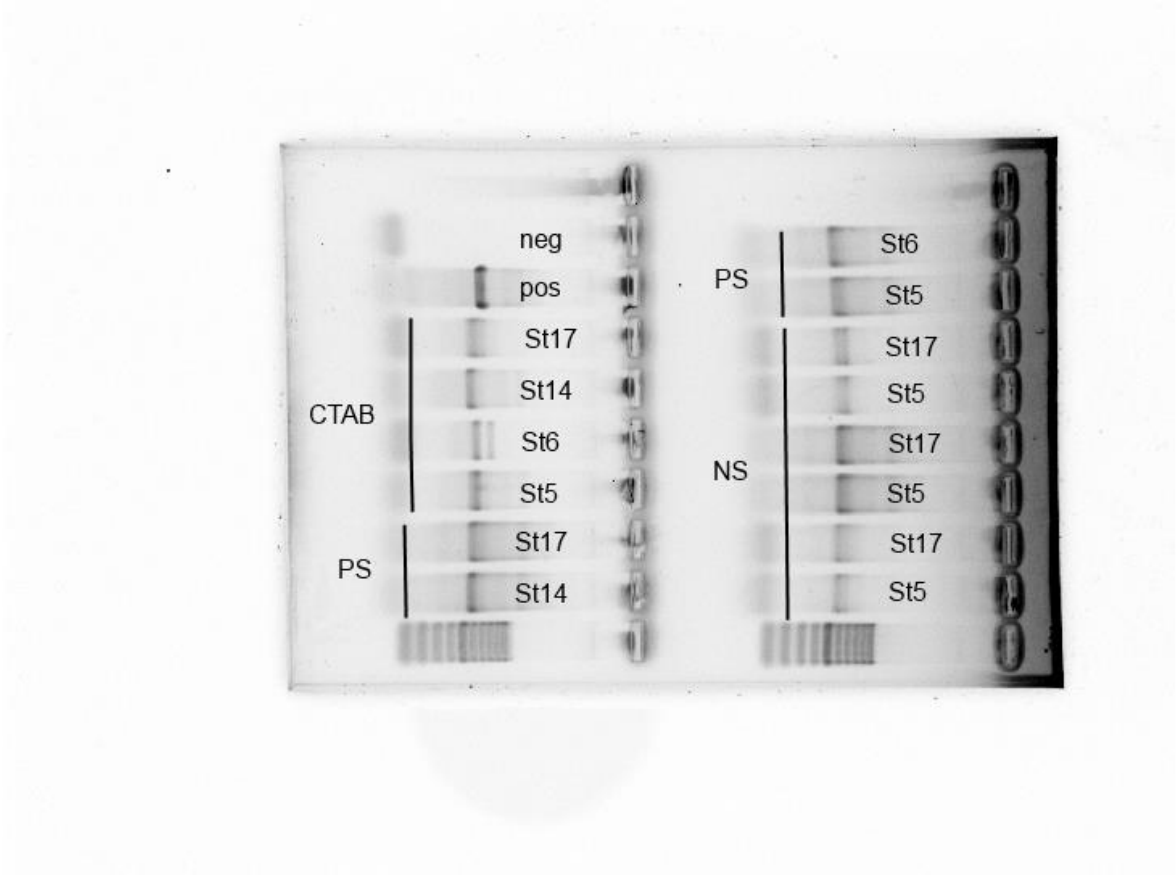

Table 1. Species of *Tuber* for which ITS sequences were analyzed initially to confirm their identity. Each species was then tested with species-specific primers for *T. melanosporum, T. aestivum* and *T. lyonii* and scored for amplification (+), or no amplification (-) of the template DNA.

Species	Amplification of *Tuber*-specific primers	Amplification of *T. melanosporum*-specific primers	Amplification of *T. aestivum*-specific primers	Amplification of *T. lyonii*-specific primers
T. indicum	+	-	-	-
T. anniae	+	-	-	-
T. beyerlei	+	-	-	-
T. oregenense	+	-	-	-
T. gibbosum	+	-	-	-
T. aestivum	+	-	+	-
T. melanosporum	+*	+	-	-
T. lyonii	+	-	-	+
T. maculatum	+	-	-	-
T. mesentericum	+	-	+ **	-

*weak amplification or absent in some specimens

**does not amplify at annealing temp = 65.5°C

Table 2. Summary of TABC truffle orchard soil sample analysis, including four different BC orchards.

Orchard code	Soil sample number	*Tuber melanosporum* – specific primers	*Tuber aestivum* – specific primers	*Tuber lyonii* – specific primers	*Tuber* genus – specific primers	Conclusion	Sequencing results of ITS PCR product (% match)
C Ta	S1	not tested	+	not tested	+	*T. aestivum*	
C Tm	S10	+	not tested	not tested	-	*T. melanosporum*	*T. melanosporum* (100%)
C Tm	S3	+	not tested	not tested	not tested	*T. melanosporum*	*T. melanosporum* (100%)
C Tm	S5	+	not tested	not tested	+	*T. melanosporum*	*T. melanosporum* (100%)
C Ta	S6	not tested	+	not tested	+	*T. aestivum*	
N	B23 inside brulé	weak +	not tested	+	+	*T. melanosporum* + *T. lyonii*	*T. lyonii* (100%)
N	B23 outside brulé	+	not tested	-	+	*T. melanosporum*	
N	E24	+	not tested	-	+	*T. melanosporum*	
N	H7	-	not tested	-	+	*Tuber* sp. present; not *T. melanosporum* or *T. lyonii*	
N	J5	weak +	not tested	-	+	*T. melanosporum*	
N	(-) control - pasture	-	not tested	not tested	-	no *Tuber* present	
N	S7	+	not tested	-	+	*T. melanosporum*	
N	W6	+	not tested	-	+	*T. melanosporum*	
N	(-) control near conifer stand 1	-	not tested	not tested	-	no *Tuber* present	
N	(-) control near conifer stand 2	-	not tested	not tested	-	no *Tuber* present	
S	1		not tested	-	-	no *Tuber* present	
S	4	weak +	not tested	-	-	*T. melanosporum*	
S	5	+	not tested	-	+	*T. melanosporum*	
S	6	+	not tested	-	(+)*	*T. melanosporum*	
S	12	-	not tested	-	weak +	*Tuber* sp. present; not *T. melanosporum*, *T. aestivum*, or *T. lyonii*	
S	14	-	not tested	-	weak +	*Tuber* sp. present; not *T. melanosporum*, *T. aestivum*, or *T. lyonii*	
S	17	+	not tested	-	+	*T. melanosporum*	
S	20	weak +	not tested	-	not tested	*T. melanosporum*	
S	23	weak +	not tested	-	-	*T. melanosporum*	
S	26	-	not tested	-	not tested	not *T. melanosporum* or *T. lyonii*	
S	29	+	not tested	-	-	*T. melanosporum*	
G	3	-	-	not tested	+	*Tuber* sp. present; not *T. melanosporum* or *T. aestivum*	
G	5	+	-	not tested	+	*T. melanosporum*	
G	7	+	-	not tested	+	*T. melanosporum*	
G	13	-	-	not tested	-	no *Tuber* present	
G	16	-	+	not tested	-	*T. aestivum*	

* Band of different size; may be wrong product.

Table 3: Summary of the NATGA truffle orchard soil sample analysis. Four different orchards were randomly designated as A, B, C and D.

Orchard code/sample number	Sample run	*Tuber melanosporum* - specific primers	*Tuber aestivum* - specific primers	*Tuber lyonii* - specific primers	*Tuber* genus - specific primers	Conclusion	Sequencing results of PCR product (percent match)
A1	1	-	not tested	not tested	-	no *Tuber* present	
	2	-	not tested	not tested	-	no *Tuber* present	
A2	1	-	not tested	not tested	+	*Tuber* sp., not *T. melanosporum*	*Tuber* sp. (94%)*
	2	-	not tested	not tested	+	*Tuber* sp., not *T. melanosporum*	
A3	1	+	not tested	not tested	-	*T. melanosporum*	*T. melanosporum* (100%)**
	2	+	not tested	not tested	+	*T. melanosporum*	
A4	1	-	not tested	not tested	+	*Tuber* sp., not *T. melanosporum*	*Tuber sp.* (92%)*
	2	-	-	-	+	*Tuber* sp., not *T. melanosporum, T. aestivum,* or *T. lyonii*	
A5	1	-	not tested	not tested	-	no *Tuber* present	
	2	-	not tested	not tested	-	no *Tuber* present	
B1	1	-	not tested	not tested	+	*Tuber* sp., not *T. melanosporum*	*Tuber sp.* (95%)*
	2	-	-	-	+	*Tuber* sp., not *T. melanosporum, T. aestivum,* or *T. lyonii*	
B2	1	+	not tested	not tested	+	*T. melanosporum*	
	2	+	not tested	not tested	+	*T. melanosporum*	
B3	1	+	not tested	not tested	+	*T. melanosporum*	
	2	+	not tested	not tested	+	*T. melanosporum*	
B4	1	-	not tested	not tested	+	*Tuber* sp., not *T. melanosporum*	*Tuber sp.* (95%)
	2	-	-	-	+	*Tuber* sp., not *T. melanosporum, T. aestivum,* or *T. lyonii*	
B5	1	+	not tested	not tested	+	*T. melanosporum*	
	2	+	not tested	not tested	-	*T. melanosporum*	
C1	1	-	not tested	not tested	-	no *Tuber* present	
	2	-	-	-	+	*Tuber* sp., not *T. melanosporum, T. aestivum,* or *T. lyonii*	
C2	1	-	-	-	+	*Tuber* sp., not *T. melanosporum*	sequencing failed
	2	-	-	-	+	*Tuber* sp., not *T. melanosporum, T. aestivum,* or *T. lyonii*	
C3	1	-	not tested	not tested	-	no *Tuber* present	
	2	-	-	+	+	*Tuber lyonii*	*Tuber lyonii* (100%)**
C4	1	-	not tested	not tested	-	no *Tuber* present	
	2	+	not tested	not tested	+	*T. melanosporum*	
C5	1	-	not tested	not tested	-	no *Tuber* present	
	2	-	not tested	not tested	-	no *Tuber* present	
D1	1	-	not tested	not tested	-	no *Tuber* present	
	2	+	not tested	not tested	+	*T. melanosporum*	
D2	1	-	not tested	not tested	-	no *Tuber* present	
	2	+	not tested	not tested	-	*T. melanosporum*	sequencing failed
D3	1	-	not tested	not tested	-	no *Tuber* present	
	2	+	not tested	not tested	+	*T. melanosporum*	
D4	1	-	not tested	not tested	-	no *Tuber* present	
	2	+	not tested	not tested	-	*T. melanosporum*	sequencing failed
D5	1	-	not tested	not tested	-	no *Tuber* present	
	2	-	not tested	not tested	+	*Tuber* sp., not *T. melanosporum*	*Tuber sp.* (93%)*

Footnotes for Table 3 (previous page)**:**

* β-tubulin gene amplified by *Tuber* genus primer set and sequenced for further identification
** ITS amplified by *Tuber* species specific primer sets and sequenced for further identification
Notes: Two independent DNA extractions were completed for each soil sample and these are presented as sample run 1 and 2. Positive (+) and negative (-) amplifications for either the *Tuber*-specific, or the species-specific (*T. melanosporum, T. aestivum, T. lyonii*) PCR reactions are indicated.

Table 4. Select physical and chemical attributes of tested soils.

Orchard codes	pH (H_2O)*	pH ($CaCl_2$)*	Percent organic matter	Percent sand	Percent silt	Percent clay	Texture class
C Ta	7.81	7.35	7.89	73	20	6	sandy loam
C Tm	7.85	7.36	6.93	71	23	6	sandy loam
N (in orchard)	7.29	6.90	7.94	64	28	8	sandy loam
N (under Douglas-fir)	5.32	4.82	8.10	65	29	5	sandy loam
N (outside of orchard)	6.40	6.05	10.72	57	31	12	sandy loam
S (bulked)	7.13	6.86	10.80	23	59	18	silt loam
S (bulked)	7.15	6.68	11.75	26	56	18	silt loam
G	7.38	7.10	10.34	43	46	10	loam
NATGA A	7.61	7.40	3.86	49	20	30	sandy loam
NATGA B	7.56	7.11	4.78	38	45	18	loam
NATGA C	7.64	7.19	2.04	81	13	6	loamy sand
NATGA D	7.33	6.94	3.66	16	56	28	silty clay loam

* Soil pH has traditionally been measured in deionized water (H_2O). Soil pH measured in water is more changeable (in response to fertilizing, irrigation, liming) than when measured in $CaCl_2$.

Appendix 1

Soil sampling procedures used when evaluating truffle orchards

Objectives:

Surveys of orchard soils using molecular techniques will be conducted:

1) To determine whether the target truffle (*Tuber*) species is associated with truffle orchard trees
2) To determine whether non-target truffle (*Tuber*) species are associated with truffle orchard trees, including native, European and Asian truffle species

Summary of approach:

Traditionally, the presence of *Tuber* species on host trees has been determined by expert examination of the ectomycorrhizas under the microscope. This is time-consuming and expensive and very few experts are available to do this work. DNA-based methods used in Spain and elsewhere can detect the presence of particular truffle species directly from soil samples; this approach should be just as reliable as the morphological approach while being less expensive and more available. Orchardists can do their own soil sample collection, as long as they follow sampling instructions precisely, and ship samples directly to the lab for analysis. Not following sampling and handling instructions precisely will jeopardize the accuracy and usefulness of results. It is essential to avoid contamination between samples and to protect the fungal DNA in the soil samples from degradation.

Equipment required:

- Hand trowel, spade, or soil core sampler (20 cm / 8 inch long)
- One bucket for washing the trowel or spade or core sampler
- Scrub brush

- A supply of soapy water for washing implements
- A spray bottle of ethanol (70%) or 10% (v/v) sodium hypochlorite (bleach)
- A spray bottle of water
- Large and sealable plastic freezer or zip-lock bags for soil samples
- Rubber gloves to wear when sampling and for washing implements
- Some method of labelling each sample bag for sample location and date (permanent markers to write on the bag, or twist ties with labels)

Soil sampling procedure:

Prior to sampling, contact the analytical lab (Paul de la Bastide, Mycologic Inc) to discuss and confirm details.

In general terms, it is best to collect soil samples when soils are not too wet (not after heavy rains), or too dry. The best season to collect soil samples is likely the fall, but sampling in other seasons should also work.

A general sampling procedure includes the following:

1. Remove any surface debris, leaves, stones, moss, or plant material
2. For each tree sampled, collect soil at three equally distributed points within 20 – 40 cm (8 to 16 inches) of the trunk
3. For each sample point, dig to a depth of 15-20 cm (6 to 8 inches) using a hand trowel, spade or soil core sampler
4. For each tree, add the 3 soil samples to the same new plastic bag (2 litre bag) and remove any stones and large organic debris from the soil
5. Collect a total soil volume of about 0.5 litres (about 3 hand trowels full) for each tree sampled

6. Label the bag with appropriate number codes to identify location and date of sampling. Record which trees are sampled with these same unique identifiers
7. Before you sample around the next tree, the sampling implements must be cleaned thoroughly to avoid cross-contamination.
    i. Wash the tool first by vigorously scrubbing with a brush in soapy water to remove all soil.
    ii. Spray tool with either 70% ethanol or 10% bleach.
    iii. Spray tool with clean water to remove soap and bleach or ethanol
8. Sample the number of trees agreed upon. Record which trees are sampled.
9. Keep samples cool (out of direct sun in a cooler) while sampling the orchard
10. Once all trees have been sampled, mix each soil sample thoroughly (5 minutes each) by shaking in the individual bag. Store samples in the fridge until they can be dried.

Preparation of samples for shipping:

After evaluating different methods of soil preservation, we have found that the drying of soil samples is effective in preserving DNA for later analysis. Drying should be done immediately after field collection. Although refrigeration and freezing are effective, for a number of reasons, drying is the preferred method[2].

How to dry soil samples:

Soil samples should be stored at fridge temperature (4 to 8°C) until dried. Ideally, soil drying should be done immediately after field collection. In order to dry soil samples, any oven with a

[2] Fridge temperature storage (4 to 8°C) and freezing of samples are also effective methods, but problematic and more expensive when long-distance shipping is required. If an orchard is located close to the analytical lab, fridge-stored or frozen samples are an option. For shipping of refrigerated or frozen samples, we recommend a styrofoam box with some freezer packs to keep the soil samples cool in transit.

temperature control may be used. Soil should be broken up and spread into a thin layer on a tray to maximize its surface area. At this stage, continue to be aware of the potential for cross-contamination of samples and be careful to clean any implements between samples. Set the oven temperature to 60°C (140°F) but no higher; drying at a higher temperature will destroy the DNA. Dry the soil overnight (10 to 12 hours). If your oven cannot be set as low as this, soil can be dried in a food dehydrator if spread into a thin layer on paper towels.

Once thoroughly dried, the soil should be mixed well and remaining clumps broken up in a new plastic bag. Once mixed, retrieve about three teaspoons of soil from the bag and immediately (to maintain dry state) place into a new, clean, small sealable plastic freezer or zip-lock bag and label clearly (zip-lock sandwich bags work well).

Bulk all of the remaining dried soil from all sampled trees into one, mix thoroughly and retrieve about one cup of soil. Retain this soil for possible future determination of soil parameters such as pH, texture and organic matter content.

Shipping:

Soil samples should be shipped in a sturdy, puncture-proof container to ensure the bags are undamaged and the soil remains dry. Samples may be sent by parcel post (dried soil), or by a major courier (fresh or frozen soil); the latter choice will usually arrive in a few days. Courier shipments should be sent early in the week, since couriers will only deliver to us on a week day.

Please use the following shipping address for both postage and for couriers:

Attn: Paul de la Bastide (phone: 250-721-7145)

Petch Building Rm. 168, University of Victoria

3800 Finnerty Road, Victoria, BC, V8P 5C2 CANADA