From Forest Nursery Notes, Winter 2012

122. Molecular tools for rapid and accurate detection of black truffle (*Tuber melanosporum* Vitt.) in inoculated nursery plants and commercial plantations in Chile. Cordero, C., Caceres, P., Gonzalez, G., and Quiroz, K. Chilean Journal of Agricultural Research 71(3):488-494. 2011.

SCIENTIFIC NOTE

CHILEANJAR
CHILEAN JOURNAL of AGRICULTURAL RESEARCH

MOLECULAR TOOLS FOR RAPID AND ACCURATE DETECTION OF BLACK TRUFFLE (*Tuber melanosporum* Vitt.) IN INOCULATED NURSERY PLANTS AND COMMERCIAL PLANTATIONS IN CHILE

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Truffle (*Tuber melanosporum* Vitt.) culture is an agroforestry sector in Chile of increasing interest due to the high prices that truffles fetch in the national market and the recent evidence that its commercial production is possible in Chilean climatic and soil conditions. In this study, the efficiency of three methods of DNA extraction from a mix of 5 g of soil and roots from both nursery and field plants of *Quercus ilex* L. mycorrhized with *T. melanosporum* were evaluated, and a simple and reproducible protocol was established. Detection of *T. melanosporum* was performed by the technique of cleaved amplified polymorphic sequence (CAPS) from amplicons generated with the primers ADL1 (5´-GTAACGATAAAGGCCATCTATAGG-3´) and ADL3 (5´-CGTTTTTCCTGAACTCTTCATCAC-3`), where a restriction fragment of 160 bp specific for *T. melanosporum* was generated, which allows the discrimination of this species from the rest of the species belonging to the *Tuber* sp. genus. Direct detection of *T. melanosporum* in one step was also obtained by polymerase chain reaction (PCR) from total DNA isolated from mycorrhized roots and with the primers ITSML (5´-TGGCCATGTGTCAGATTTAGTA-3´) and ITSLNG (5´-TGATATGCTTAAGTTCAGCGGG-3´), generating a single amplicon of 440 bp. The molecular detection of *T. melanosporum* by the methods presented here will allow the rapid and accurate detection of mycorrhization of trees, both under nursery and field conditions. This technology will also provide more security to farmers by controlling the quality of the mycorrhized trees they will plant and also by following the mycorrhization status of established orchards.

Key words: *Tuber melanosporum*, ectomycorrhizas, molecular markers, PCR, CAPS.

Truffles are underground, or hypogeum, fungi that belong to the Class of the Ascomicetes. These fungi live associated with roots of certain woody plants, mainly of the *Quercus* genus, such as *Q. rotundifolia* Lam. and *Q. robur* L. as well as *Corylus avellana* L. These fungi have developed reproductive structures that emit a strong aroma and in this way are capable of attracting animals which detect and consume them (Lefevre and Hall, 2001). The black truffle (*Tuber melanosporum* Vitt.) is a product in high demand for "haute cuisine" (Suárez, 2007).

There are about 70 types of truffles and almost half of them are found in Europe. In Spain, France, and Italy, 21 different species of the *Tuber* genus have been found, but only a few are edible (Reyna, 2000). *Tuber melanosporum* is the most appreciated species in Spain and France, where it can fetch high prices in comparison to the rest of the truffle species. Other species of the genus are also commercialized, as in the case of *T. brumale* Vitt., *T. aestivum* Vitt. and *T. magnatum* Pico, whose price in the market, while relatively high, are still lower than those of *T. melanosporum* (Reyna, 2000).

In Chile, the black truffle is one of the few mycorrhizal fungi that can be cultivated artificially (Álvarez, 2004). The first truffles harvested in Chile were obtained in the year 2009 in Los Ríos Region, thanks to a project supported by the Fundación para la Innovación Agraria (FIA) and developed by Agrobiotruf Company (FIA, 2009). At present there are a total of 70 ha, but this is expected to grow up to 100 ha annually, data show that correctly established and adequately managed plantations begin commercial production between the fifth and seventh year. It should also be noted that a truffle plantation has a productive life that can be greater than 50 yr (FIA, 2009).

From 1995 till 1999 there was a strong demand in Europe to obtain experience in the correct identification and classification of truffles that came from exporting countries but the morphological similarity makes it almost impossible to differentiate species. This was reflected in the identification errors produced through macroscopic or

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Received: 30 December 2010.
Accepted: 20 April 2011.

microscopic observations, making it necessary to include a molecular focus (Lanfranco *et al*., 1993; Henrion *et al*., 1994; Amicucci *et al*., 1996; Reyna, 2000).

Molecular methods may be applied in the identification of ascocarps and mycorrhizas, making possible plant certification (Séjalon-Delmas *et al*., 2000). Actually, the traditional way of DNA analysis through mitochondrial markers for the identification of the *Tuber* genus is being used; this has been carried out using specific primers for black truffle (Mello *et al*., 1999; Paolocci *et al*., 1999; Séjalon-Delmas *et al*., 2000; Mabru *et al*., 2001).

In the Chilean conditions, it is highly desirable to develop a fast and precise method that allows the identification of *T. melanosporum* throughout all the technological chain. The early identification of the fungus in nursery plants will generate confidence and security in the farmers who develop plantation projects by reducing the risks, as well as by allowing the monitoring of plant colonization, by the mycorrhiza, during the development and growth of the plantation. On the other hand, the certification by molecular means would also help ensure the quality of truffles produced and would thus open better access to markets that may be restricted to Chile due to the lack of tradition in this kind of biological product.

In this paper, the use of different strategies for rapid, efficient and accurate molecular detection of black truffle from rhizospheric DNA in trees, infected with mycorrhizas under nursery and field conditions in Chile, is established to assist plant growers and farmers in establishing safe and high quality based production systems.

MATERIALS AND METHODS

Biological material

Up to 10 plants of *Q. ilex* mycorrhized with *T. melanosporum* under nursery conditions were supplied by Agrobiotruf S.A. (Talca, Chile) and used as the starting biological material. Mycorrhized roots were conserved at -80 ºC, until the analysis was carried out. DNA extraction from mycorrhized roots was performed using the protocol described by Edwards *et al*. (1991), but including two modifications in the methodology as detailed below. The efficiency of the modified protocols was compared to the Power Soil extraction kit (PS Kit) from MoBio Laboratories Inc. (Carlsbad, California, USA), which was used according to the instructions of the manufacturer. As controls for the experiments, fruiting bodies of *T. melanosporum* and *T. aestivum* were used.

Buffer A (BA) DNA extraction protocols

From mycorrhized root samples, 0.5 g tissue were weighted and powered in liquid nitrogen until obtaining a homogeneous mixture. The pulverized samples were aliquoted in 1.5 mL tubes, which were treated with a solution of 5 mL buffer A (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS). Extracts were centrifuged at 11 000 rpm for 10 min, supernatants were transferred to a new additional tube with the same volume of isopropanol for its precipitation during 30 min at -20 °C. Centrifugation at 11 000 rpm for 10 min was carried out, the precipitate was left to dry at room temperature for 10 min before it was dissolved in 300 μL ultra pure water. The same volume was added of phenol/chlorine and was vigorously mixed; the mixture was centrifuged at 13 000 rpm for 5 min. In order to precipitate the DNA, the supernatant was rescued and added to a new tube, where 2.5 volumes of absolute ethanol were added and it was incubated at -20 °C for 1 h. The mixture was centrifuged at 13 000 rpm for 20 min at 4 °C. The supernatant was eliminated and 500 μL ethanol 70% (v/v/) were added. A last centrifugation was carried out at 13 000 rpm for 10 min, the supernatant was eliminated and the precipitate was dried at room temperature. Finally, the precipitate was diluted in 100 μL ultra pure water. DNA extraction from fruiting bodies of *T. melanosporum* and *T. aestivum* controls was performed following the same procedure as described above.

Modified buffer A (BA Mod) DNA extraction protocols

From mycorrhized roots, a 0.5 g sample was taken and macerated in liquid nitrogen until a homogeneous mixture was obtained. The pulverized samples of mycorrhized roots and the fruiting bodies of the *T. melanosporum* and *T. aestivum* controls were treated with a solution of 5 mL modified buffer A (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% (p/v) of SDS). To the extracts were added 10 μL of Proteinase K at a concentration of 10 mg μL^{-1}. Samples were incubated at 37 °C for 1 h, homogenizing softly. A centrifugation step at 12 000 rpm for 10 min was performed. Supernatant was recovered and transferred to sterile tubes, where the DNA was precipitated with an equal volume of isopropanol (700 μL) and incubation during 1 h at -20 °C. Centrifugation at 12 000 rpm for 15 min was carried out. Supernatant was discarded and the precipitate was diluted in 300 μL water. Following this, the same volume of a mixture of chloroform and isoamilic alcohol (24:1) was added, and the mixture was agitated carefully until it was completely homogenized. The resulting mixture was centrifuged at 12 000 rpm for 10 min, the supernatant was collected in a new tube. Precipitation was performed by adding 600 μL isopropanol and by incubating at -20 °C for 1 h. Centrifugation at 12 000 rpm for 15 min and 4 °C was carried out. Supernatant was eliminated and the precipitate was dissolved in 100 μL ethanol 70% (v/v). A centrifugation step at 13 000 rpm was carried out for 10 min and the precipitate was dried at room temperature. Finally, the precipitate was aliquoted in 100 μL of distilled sterile water.

Electrophoresis in agarose gel and determination of the quality and quantity of obtained DNA

For all the extraction methods, DNA integrity and extraction efficiency was verified through electrophoresis in 1% agarose gel in buffer TAE 1X. DNA quantification was determined through a micro-precision spectrophotometer (NanoDrop 2000/2000c Spectrophotometer, Wilmington, Delaware, USA) at an absorbance of 280 nm.

Experimental design and statistical analysis

To evaluate the best DNA extraction protocol of *T. melanosporum* from roots of mycorrhized plants, 30 plants per treatment with two replicates per plant were taken. DNA concentration from the diluted samples in 100 μL distilled sterile water was selected a quantitative selection criteria. The homogeneity of the variance was determined through the Bartlett test ($p < 0.05$). For the processing and analysis of the results an ANOVA was carried out and Tukey's multiple range test with Honestly Significant Difference ($p < 0.05$), was used for comparing media. All the statistical analyses were carried out with the statistical package Statgraphics Plus 5.0 (Stat Point Inc., Herndon, Northern Virginia, USA).

Detection of *Tuber melanosporum* through Cleaved Amplified Polymorphic Sequences (CAPS)

The detection of *T. melanosporum* from DNA of mycorrhized plants in nurseries was evaluated through the technique of cleaved amplified polymorphic sequences (CAPS), in practice it is a variant of the polymerase chain reaction-restriction fragment length polymorphism technique (PCR-RFLP) from conserved regions of mitochondrial rDNA. The universal primers of the genus *Tuber* sp. ADL1 (5´-GTAACGATAAAGGCCATCTATAGG-3´) and ADL3 (5´-CGTTTTTCCTGAACTCTTCATCAC-3`) (Mabru *et al*., 2004), were used.

The PCR reaction was carried out in a volume of 25 μL and the reaction mixture was prepared with: 20 ng DNA; 12.5 mg mL^{-1} of BSA (BSA Biolabs, New England, USA); 4 mM $MgCl_2$; 0.2 mM dNTPs; 1 U Taq polymerase (Invitrogen, Carlsbad, California, USA); 4 μL Buffer 1X (20 Mm Tris HCl pH 8.4, 50 mM KCl) and 0.4 μM of each primer. The reaction volume was completed with distilled sterile, ultrapure water. The PCR reaction was developed in a PX2 Thermal Cycler (Thermo Electron Corporation, Rockville, Maryland, USA) machine, previously programmed as follows: 3 min denaturation at 94 °C; 40 denaturation cycles at 94 °C, for 30 s, annealing at 66 ºC during 30 s and extension at 66 °C for 30 s; a final extension at 72 °C for 2 min.

For the restriction fragment length polymorphism (RFLP) analysis from the amplicons generated through PCR with the primers ADL1 and ADL3, 8 μL of the PCR products were incubated with 4 U of the restriction enzyme *Alu*I (New England Biolabs, Ipswich, Massachusetts, USA). The digestion was carried out in 20 μL buffer recommended by the manufacturer at 37 ºC for 4 h. The products obtained from the digestion were visualized by electrophoresis in 2% agarose gel.

As a positive control DNA extracted from fruiting bodies of *T. melanospoum* were used, and DNA of *T. aestivum* as a negative control.

Direct detection of *T. melanosporum* through PCR with specific primers

The direct detection of *T. melanosporum* from isolated DNA from the roots of mycorrhized plants was evaluated through the use of primers ITSML (5´-TGGCCATGTGTCAGATTTAGTA-3´) and ITSLNG (5´-TGATATGCTTAAGTTCAGCGGG-3´), which isolate ITS (Internal Transcribed Spacer) from rDNA. The primer 5` ITSML is specific for *T. melanosporum*, while primer 3´ ITS4LNG is specific for the genus *Tuber* sp. in general. Because of this any amplicon obtained as a product in a PCR reaction with these two primers must only come from *T. melanosporum* (Paolocci *et al*., 1999).

The PCR reaction was carried out in a volume of 25 μL and the reaction mixture was prepared with: 5 ng of DNA; 12.5 mg mL^{-1} of BSA (New England Biolabs, Ipswich, Massachusetts, USA); 4 mM $MgCl_2$; 0.2 mM dNTPs; 1 U Taq polymerase (Invitrogen, Carlsbad, California, USA); 4 μL of Buffer 1X (20 Mm Tris Hcl pH 8.4, 50 mM KCl) and 0.4 μM of each primer. The reaction volume was completed with distilled sterile, ultrapure water. The PCR reaction was developed in the automatic thermocycler Maxygen Thermal Cycler (Maxygen, Union City, California, USA) previously programmed, as follows: 3 min of denaturalization at 94 °C; 40 denaturalization cycles at 94 °C, for 30 s, annealing at 63 ºC during 30 s and extension at 66 °C for 30 s; a final extension at 72 °C for 7 min. The reaction was developed in the automatic thermocycler Maxygene Thermal Cycler. The products obtained from the PCR were visualized by electrophoresis in 2% agarose gel.

Cloning and sequencing

The products obtained by PCR were cloned in the pGEM®-T vector (Promega, Madison, Wisconsin, USA). The ligation reaction was carried out at 15 °C for 16 h, placing the insert in 1:1 proportion, in the presence of T4 DNA ligase, afterwards the transformation was carried out using subcloning efficiency *E. coli* DH5α competent cell (Invitrogen, USA). The LB agar culture medium (supplemented with Ampicillin 100 μgmL^{-1}, IPTG 0.5 mM and 80 μg mL^{-1} X-Gal) was used for cloning. Sequence analyses for the generated amplicons were performed with the Basic Local Alignment Search Tool (BLAST 2.2.25, National Center for Biotechnology Information NCBI, U.S. National Library of Medicine, Bethesda, Maryland, USA) and sequence homology was determined against the sequences from GenBank (NCBI), the European Molecular Biology Laboratory (EMBL) Nucleotide Data

Base, the DNA Data Bank of Japan (DDBJ) and Protein Data Bank (PDB).

RESULTS

DNA extraction from *Q. ilex* plants mycorrhized with *T. melanosporum*

In general, the three protocols used generated good quality DNA from roots of *Q. ilex* mycorrhized with *T. melanosporum*, with an absorbance relationship that indicated low concentrations of proteins or other contaminants (Figure 1). The three protocols produced significantly different DNA quantities, with the protocol of Buffer A (BA) extraction as the most efficient (11336.5 ng μL^{-1}) (Table 1).

The biological quality was not influenced by the extraction protocol, because amplification through PCR was obtained for the two detection systems (Primers ADL1 and ADL3; Primers ITSML and ITSLNG) in 100% of the samples prepared with the three protocols.

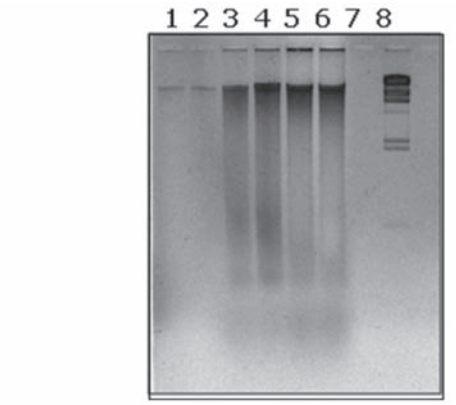
Direct detection of *T. melanosporum* from DNA samples isolated from mycorrhized plants and fruiting bodies

The PCR from DNA extracted from mycorrhized plants and fruiting bodies (positive control) of *T. melanosporum* with the primers 5'ITSML and 3´ITSLNG generated an amplicon of 440 bp, in the concentrated DNA samples as well as in the dilutions 1:10 and 1:1000. Amplification was obtained in 100% of the samples of mycorrhized plants. In the same way, the analysis of the fruiting bodies harvested in Chile from *Q. ilex* in the 2009 season generated an amplicon at the same height as the positive control and the root samples (Figure 2). On the other hand, the negative control (DNA extracted from the fruiting body of *T. aestivum*) did not present any type of amplification in the PCR which was carried out.

Table 1. Effect of different DNA extraction protocols from *Quercus ilex* roots mycorrhized with *Tuber melanosporum*.

Extraction protocols	DNA average concentration (ng μL^{-1})	Relation 260 nm/280 nm
BA	11336.5a	1.7
BA Mod	31363b	1.6
PS Kit	44.3c	2.1

Different letters present differences according to the multiple range test of Tukey HSD ($p \leq 0.05$).
BA: DNA extraction based on the use of Buffer A; BA Mod: DNA extraction based on the use of a modified Buffer A; PS Kit: DNA extraction based on the use the Power Soil commercial kit from MoBio Laboratories Inc.



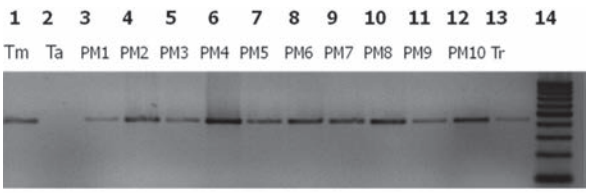
1-2. DNA extracted from roots of *Quercus ilex* mycorrhized with *Tuber melanosporum* using the PS kit; 3-4. DNA extracted from roots of *Q. ilex* mycorrhized with *T. melanosporum* using Buffer A (BA); 5-6. DNA extracted from roots of *Q. ilex* mycorrhized with *T. melanosporum* using modified Buffer A (BA Mod); 8. λ-Hind III marker.

Figure 1. Visualization of total DNA extracted using three different protocols. BA: Buffer A; BA Mod: Buffer A modified; PS Kit: Power Soil kit from MoBio Laboratories Inc.

Detection of *T. melanosporum* through CAPS

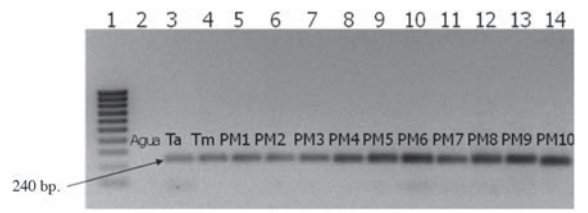
The amplification with the primers ADL1 and ADL3 generated a 240 bp amplicon from DNA isolated from the roots of mycorrhized *Q. ilex*. In the same way, a band of the same size was obtained for the DNA samples of the fruiting bodies *T. aestivum* and *T. melanosporum* (Figure 3).

The digestion of the amplicon obtained with the *Alu*I enzyme allowed the correct discrimination between that generated for *T. melanosporum* and *T. aestivum* (Figure 4). The amplicon that originated from the DNA of the fruiting bodies of *T. melanosporum* and from mycorrhized *Q. ilex* plants generated a restriction fragment of 160 bp and two restriction fragments of 40 bp. Nevertheless, the digestion with the *Alu*I enzyme carried out over the amplicon of the fruiting body of *T. aestivum* only generated two restriction fragments of 200 and 40 bp.



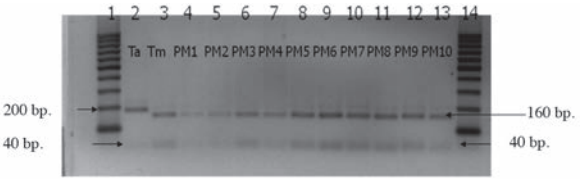
1. Amplicon obtained from DNA isolated from the fruiting body of *Tuber melanosporum* (Tm); 2. Amplification products from DNA extracted from a fruiting body of *Tuber aestivum* (Ta, negative control); 3-12. Amplicons obtained from isolated DNA from 10 plants of *Quercus ilex* mycorrhized with *T. melanosporum*; 13. Amplicon obtained from DNA isolated from a fruiting body of *T. melanosporum* harvested in Chile in the year 2009; and 14. 100 bp marker.

Figure 2. Amplification of ITS fragments with primers 5´ ITSML and 3´ ITSLNG.



1. 100 bp marker; 2. Amplification product without *T. melanosporum* DNA (negative control with water); 3. Amplification product from DNA isolated from a fruiting body of *Tuber aestivum*; 4. Amplification product from DNA isolated from a fruiting body of *T. melanosporum*; 5-14. Amplicons obtained from DNA isolated from the roots of 10 *Q. ilex* plants mycorrhized with *T. melanosporum*.

Figure 3. Amplification products obtained with the ADL1 and ADL3 primers from *Quercus ilex* plants mycorrhized with *Tuber melanosporum*.



1. 100 bp marker; 2. CAPS products generated from amplicon ADL1+ADL3 amplified from DNA originating from a fruiting body of *Tuber aestivum* (200-40 bp); 3. CAPS products generated from amplicon ADL1+ADL3 amplified from DNA originated from a fruiting body of *Tuber melanosporum*; 4-13. CAPS products generated from amplicon ADL1+ADL3 amplified from DNA originating from the roots of 10 *Quercus ilex* plants mycorrhized with *T. melanosporum*;
14. 100 bp marker.

Figure 4. CAPS fragments generated by digestion with the *Alu*I enzyme from amplicons ADL1+ADL3.

Sequencing

The sequencing and sequence analysis from the amplicons obtained with the primers ITSML-ITSLNG (Figure 5A, accession JF807487 in GenBank) from the DNA of mycorrhized plants of *Q. ilex* showed a 98% identity with a query coverage of 92% for accessions GU979083.1 (partial sequence of *T. melanosporum* isolated T99_mel 18s ribosomal RNA gene), FM205581.1 (partial sequence of *T. melanosporum* isolated 15MEL 18s ribosomal RNA gene), FM205580 (partial sequence of *T. melanosporum* isolated 13MEL 18s ribosomal RNA gene). In the same way, the sequence analysis for the amplicons with the primers ADL1-ADL3 (Figure 5B, accession JF807486 in GenBank), showed an identity of 97% with the accession AY294006.1 (partial sequence of *T. indicum* isolated 19 large subunit ribosomal RNA gene) with a "query coverage" of 39%. It also showed 99% homology with accessions AY294007.1 (partial sequence *T. melanosporum* isolated Tm9 large subunit ribosomal RNA gene) and AY294008.1 (partial sequence *T. melanosporum* isolated Tm13 large subunit ribosomal RNA gene) with query coverage of 39% for both of them.

A)
5´TTTTTTTTAAATTAAAAAGGTGGGGCATGTGTCAGATTTAGTAAAGTAA
AAACNNTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGC
AGCGAAATGCGATAGGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA
TCTTTGAACGCACATTGCGCCCTTTGGTATTCCTTAGGGCATGCCTGTTC
GAGCGTCACTACACACCTTATCACAAAGTTTGTGGTCTTGGCAGGAGTG
AATTGCTAGTCTATCAAAATGTTCCAGCTGTACACTCTGCTAAAAATTATG
AGAAGGTTACCAGGCATGAACGACGGACTTTATAAACGGTTATAAGACC
TGGATCAGTCACAAGTCTTGTCTGGTCCTTACCTTAAGGACCCCCATCCT
AGATGAACTATGGGTTGACCTCGAATCNAGTNNNGGAGGGATACCCGCT
GAACTTAAAAAATATCA3´

B)
5´TTTATGAGAACCGAACCCTCCCTACTTACTCGTACAACAAAACCTCCC
ACACGTTAGAAATAAGGGCCGCTTCAAGGGGAACCAAAAAAGGTAATT
GTTTAATATAAAAGCCCATCTTTTCGGGGAACGAATAAGGCCATCGTTTT
TCCTGAAATCCCCATCATTTATTATAGACATTAGGTAATCGTTTTTCCTGAA
CTCTTCATCACTTATTATTTACATCAGCTATTGGGGCGCGATCACTCACTC
CTAAAGAGAGTGAGGGCGTGCGACCCCAGAATGTTTAAAAATTTAAAGG
TTATTACTTTATAAACAAGGTTTTCTCATCGTTTAGTCCTTTAGGTTAATAA
ACTTAGAGGCCGTTACTTTTATCTATACCATAAGCTAAAGGTCGAGGTCA
ACCTATAGATTGGCCTTTATCGTTACAGGTTGGCCTTTATCGTTACCCTATA
GATTGGCCTTTATCGTTACAACATATGATTGGGGTACTTAAGAGTTTTTAT
TTTGCAGAACCAAGCGTGAGTGTTGTTTGAACAATACCTTAAGGATTCCA
TCCTAGGGGAACTATGGGTGGACCTAGAATCAGGGAGTGTTACCCACTT
GTCTTAAGTGTTTTTATT3´

Figure 5. Sequence of the amplicon obtained by using the primers ITSML-ITSLNG (A) ADL1-ADL3 (B). Both sequences were deposited in GenBank and recorded as JF807487 and JF807486, respectively.

DISCUSSION

For the DNA extraction of *Tuber* sp., from fruiting bodies or mycorrhized roots, all the evaluated protocols demonstrated effectiveness, nevertheless the protocol based on the use of Buffer A (BA) was the most convenient one because the samples obtained presented a good absorbance relationship (280 nm/260 nm), a visual observation of DNA with low degradation and a greater DNA yield. The method, at the same time, is relatively simple, reproducible and of low cost.

In the same way, it has been documented that the PCR technique from DNA isolated from the soil may be interfered by the presence of humic acid in the samples, which seems to act as inhibitor of the amplification reaction (Clegg *et al*., 1997). The addition of BSA to the PCR in our trials allowed the generation of the expected amplicons in 100% of the samples, probably through stabilization of the Taq polymerase enzyme, improving its efficiency in the amplification process. This coincides with previously documented reports for isolation of DNA samples obtained from organic matter (La Montagne *et al*., 2002).

The DNA regions that have mostly been used for fungi identification are the internal transcribed spacers (ITS) of the rARN (Seifert, 2008), which have been employed as model regions for the design of species-specific primers to allow the identification of different truffle species (Paolocci *et al*., 1999; Mabru *et al*., 2001; Suz *et al*., 2006; Iotti *et al*., 2007).

The direct detection of *T. melanosporum* from fruiting bodies and mycorrhized plants through the use of species-specific primers was effective in our study, coinciding with the studies documented by (Paolocci *et al*. (1999). This procedure may be effective equally for differentiating samples of *T. melanosporum* from samples of *T. brumale* and *T. indicum* through multiple PCR (Paolocci *et al*., 1999). In contrast to the work carried out by Paolocci *et al*. (1999), where the work was done with fruiting bodies, the detection of *T. melanosporum* DNA was carried out from mycorrhized plants, allowing the creation of a quick and reliable traceability system in the Chilean black truffle production chain.

A 240 bp amplicon was generated using the primers ADL1 and ADL3, both from DNA isolated from mycorrhized *Q. ilex* plants and DNA from fruiting bodies of *T. aestivum* and *T. melanosporum*, as documented previously (Mabru *et al*., 2004). These primers are specific for the *Tuber* sp. genus, where PCR amplification generates a 240 pb band, except for the species *T. magnatum*, which produces an amplicon of more than 1 kb (Mabru *et al*., 2004). Coincidentally, the use of these primers in DNA extracted from the rhizosphere of native species of the hydrophilic forest (*Luma chequen* and *Crinodendron patagua*), did not generate amplification products (data not shown) which corroborated the specificity of the

primers evaluated for the *Tuber* sp. genus. ADL1 and ADL3 primers have demonstrated that they are capable of discriminating the *Tuber* sp. genus from phylogenetically close species of the genus, such as the species *Pezizale*s sp., *Choiromyces meandriformis*, *Picoa juniperi*, *Terfezia claveryi*, *Tirmania pinoyi* and *Morchella vulgaris* (Mabru *et al*., 2004).

The generation of two restriction fragments (160 and 40 bp), by the digestion with the enzyme *Alu*I of the ADL1+ADL3 amplicons generated from DNA of mycorrhized plants or fruiting bodies of *T. melanosporum*, is in concordance with the expectation for the species. These amplicons from *T. melanosporum* harbour two *Alu*I restriction sites, which generate two fragments of 40 bp, each. In the case of the rest of the species of the *Tuber* sp. genus, only some of them present a restriction site for this enzyme that generates only one fragment of 40 bp and another fragment of 200 bp. For this reason, in *T. melanosporum* a contrasting band of 160 pb is obtained, which can be used to differentiate the *Tuber* sp. species present in the roots of mycorrhized trees, from the rest of the *T. melanosporum* species (Mabru *et al*., 2004).

The sequencing analysis allowed the corroboration of the information obtained through the cloning of the fragments obtained in the amplification of the primers ITSML-ITS4LNG and ADL1-ADL3, demonstrating that the results were concordant with the different accessions deposited in the gene bank, belonging to the species *T. melanosporum* (Wang *et al*., 2006). It confirmed that it is possible to develop traceability studies by tracking the genetic fingerprint of *T. melanosporum* using molecular markers through the technological truffle production chain in Chile.

CONCLUSIONS

A fast, reliable and reproducible method for the extraction of DNA from roots and rhizosphere of *Quercus ilex* plants mycorrhized with *Tuber melanosporum* and fruiting bodies of the mycorrhiza was established. The molecular methods performed allow the identification and differentiation in Chile *T. melanosporum* from other ectomycorrhizas and other species of the genus *Tuber* sp., using the CAPS technique with the *Alu*I enzyme, in amplified fragments with the primers ADL1 and ADL3 in rDNA. In the same way, it is possible to identify with high efficiency and sensitivity the species *T. melanosporum* from the rest of the species through PCR with the species-specific primer 5` ITSML in only one step. The availability of these two systems reduces the analysis time and avoids having to carry out sequencing.

ACKNOWLEDGEMENTS

This project was supported partially by private funds coming from the Departamento de Ciencias Forestales at Universidad Católica del Maule and from the company Agrobiotruf S.A. The authors would also like to thank the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) in Chile for partially supporting of Mrs. Cecilia Cordero Alday, a Master's Degree Fellow of the Programa Nacional de Becas de Magíster en Chile, Fondo de Innovación para la Competitividad de la Región del Maule 2009 (2207090021).

Uso de marcadores moleculares para la detección rápida y precisa de trufa negra (*Tuber melanosporum* Vitt.) en plantas de vivero y plantaciones comerciales de Chile. La truficultura es un rubro agroforestal de creciente interés debido a los altos precios que alcanzan las trufas (*Tuber melanosporum* Vitt.) en el mercado nacional y la reciente evidencia que es factible su producción comercial en las condiciones de clima y suelo de Chile. En este estudio se evaluó la eficiencia de tres métodos de extracción de ADN a partir de 5 g de muestra del suelo y raíces de plantas de *Quercus ilex* L. de vivero y de campo micorrizadas con *T. melanosporum*. La detección de *T. melanosporum* se desarrolló mediante la técnica de secuencias de amplificación polimórficas cortadas (CAPS) a partir de amplicones generados con los partidores ADL1 (5´-GTAACGATAAAGGCCATCTATAGG-3´) y ADL3 (5´-CGTTTTTCCTGAACTCTTCATCAC-3`), que generó un fragmento de restricción de 160 pb, específico para *T. melanosporum* que permite discriminar esta especie del resto de las especies del género *Tuber* sp. La detección directa en un solo paso de *T. melanosporum* mediante reacción en cadena de la polimerasa (PCR) con los partidores ITSML (5´-TGGCCATGTGTCAGATTTAGTA-3´) and ITSLNG (5´-TGATATGCTTAAGTTCAGCGGG-3´) generó un amplicón de 440 pb. Los métodos de detección molecular de *T. melanosporum* desarrollados permitirán evaluar la micorrización en árboles de vivero y campo de forma rápida y precisa. La tecnología brindará mayor protección y seguridad a los productores mediante el control de la calidad del material de plantación y mediante el seguimiento de la permanencia del suelo hasta el momento de la cosecha.

Palabras clave: *Tuber melanosporum*, ectomicorrizas, marcadores moleculares, PCR, CAPS.

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