Mycorrhiza (2007) 17:627–632
DOI 10.1007/s00572-007-0140-7

ORIGINAL PAPER

Synthesis and establishment of *Tuber melanosporum* Vitt. ectomycorrhizae on two *Nothofagus* species in Chile

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Received: 21 March 2007 / Accepted: 25 May 2007 / Published online: 28 June 2007

Abstract Axenically germinated seedlings of two species of Southern beech (*Nothofagus obliqua*, *N. glauca*) from Chile were inoculated with spores of the Périgord black truffle (*Tuber melanosporum*). Ectomycorrhizal development was monitored for 6 months in the greenhouse and compared to the performance of the natural host species *Quercus ilex* and *Quercus robur*. Seedling survival and mycorrhization showed major differences in both *Nothofagus* species: *T. melanosporum* readily formed ectomycorrhizae with seedlings of *N. obliqua*, although at a lower rate than with *Q. ilex* but at a proportion very similar to *Q. robur*; survival and colonization rates were high, and seedling growth was not visibly affected by the high soil pH required by *T. melanosporum*. In contrast, more than 50% of *N. glauca* seedlings died after inoculation, and mycorrhiza formation was very sparse. In both species, no colonization by adventive ectomycorrhizal fungi could be observed, whereas both species of *Quercus* showed minor colonization by another fungus, probably *Inocybe* or *Hebeloma*. Our results show that it is possible to infect *N. obliqua* with the Périgord black truffle under greenhouse conditions, which opens up the possibility of cultivating this truffle as a secondary crop during reforestation with *N. obliqua* in Chile.

Keywords *Tuber melanosporum* · *Nothofagus obliqua* · *Nothofagus glauca* · Silviculture · Chile

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Introduction

Over the past 30 years, considerable progress has been made in the controlled infection of forest trees with selected ectomycorrhizal fungi. Of particular interest are those species that form edible fruiting bodies (Danell and Camacho 1997; Olivier et al. 1997). Perhaps the most important in terms of international demand and commercial value is the black truffle or Périgord truffle (*Tuber melanosporum* Vitt.; Lefevre and Hall 2001; Singer 1964), which is now successfully cultivated by raising infected host trees under controlled conditions in greenhouses and then producing truffles in specialised plantations in France, Italy and Spain (Chevalier and Frochot 1997; Reyna 2000). These methods have been used to extend the geographical range of truffle cultivation beyond the limits of natural distribution of this mycorrhizal association (Singer 1964), and attempts have been made in recent years to introduce truffles into other countries such as Australia, New Zealand, USA, Israel and South Africa (Lefevre and Hall 2001). Chile is also suited to the cultivation of truffles with its wide range of climates and soils and its strong forestry sector. Like Australia and New Zealand, it would also be able to take advantage of producing truffles out-of-season to the Northern Hemisphere.

T. melanosporum associates with a broad range of deciduous angiosperm host trees in its natural distribution area; generally, successful establishment of black truffle in the field has been performed on *Quercus* spp. or *Corylus avellana* L. (Hall et al. 2001; Reyna 2000). Both taxa do not grow naturally in Chile, but native Chilean forests are dominated by various species of *Nothofagus* Blume (Nothofagaceae, Fagales; Donoso 1996; Veblen et al. 1996), which include potential native hosts for truffle cultivation.

Nothofagus obliqua, a deciduous species, has its northern distribution limit in Chile at 33°S and is dominant south of 35°, with its southern limit at 41°30′. Although a typical lowland species below 600 m above seal level, it can be found up to 2,500 m above seal level in central Chile. It grows in deep, predominantly moist soils with acidic to neutral pH and annnual precipitations between 1,500 and 3,000 mm (Donoso 1996; Veblen et al. 1996). In central Chile where this study was performed, *N. obliqua* is frequently mixed with another deciduous species, *N. glauca*. *N. glauca* is much more restricted than *N. obliqua*, growing in the Mediterranean climatic zone of central Chile between 33° and 36°50′ (Donoso 1996; Santelices 1997); it is better adapted to thin and rocky soils, low in organic matter, with a pH betwen 5 and 5.8 and grows mainly on slopes and intermediate elevations between 200 and 1,100 m above seal level, typically in discrete patches exposed to summer drought and high radiation with annual precipitation between approx. 300 and 1,100 mm (CONAF 1998; Santelices 1997).

Nothofagus forests of central and Southern Chile have been suffering from severe deforestation over the past 150 years, with no or only marginal efforts invested in their sustainable management. Instead, current mainstream reforestation practice has been to introduce fast growing tree species like *Pinus radiata* D. Don and *Eucalyptus globulus* Labill, which have led to a strong reduction in biodiversity, loss of soil by erosion and sinking groundwater levels. To improve the attraction of using native tree species like *Nothofagus* spp. in silviculture, an alternative, especially for the owners of small- and medium-sized properties, could be to combine long-term timber production with complementary short-term, non-timber forest products like edible fungi, especially truffles. As no native *Tuber* species have been reported so far from Chilean *Nothofagus* forests, an experiment was designed to prove compatibility between the introduced species *T. melanosporum* Vitt. and *N. obliqua* (Mirb.) Oerst. and *N. glauca* (Phil.) Krasser, which are dominant trees of the mediterranean lowland and colline forests in south central Chile, the most adequate climate zone for possible truffle cultivation in Chile.

A critical issue during the experiment, which lasted 6 months, was soil acidity, as cultivation of *T. melanosporum* requires limed soil with a pH above 7, but natural soils in Central and Southern Chile are usually below pH 5. The question was if both *Nothofagus* species, apart from being compatible with *T. melanosporum*, would tolerate limed substrate. As control, the same experiment was performed with seedlings of *Quercus ilex* L. and *Q. robur* L., both natural hosts of *T. melanosporum*. In comparison of their ecological requirements and habitats, *N. glauca* comes close to *Q. ilex* and *N. obliqua* to *Q. robur*.

Materials and methods

Fungal inoculum

The fungal inoculum consisted of certified, air-dried and powdered fruiting bodies of *T. melanosporum* Vitt., mixed with talcum powder (magnesium hydrosilicate) in a 1:4 ratio, obtained from Centro de Estudios Ambientales del Mediterráneo, Valencia, Spain. The fruiting bodies had been collected in a plantation of *Q. ilex* subsp. *ballota* (Desf.) Samp. in the Castellón province. Random samples of the inoculum were microscopically analysed to confirm spore homogeneity and identity. The spore powder was applied directly on the roots with a large salt shaker.

Plant material and inoculation

Seeds of *N. obliqua* and *N. glauca* were obtained from Centro de Semillas de Chillán; their geographical origin was the Linares province (35°50′S, 71°35′W). Seeds of *Q. robur* and *Q. ilex* were commercially acquired at the cities of Talca and Santiago, respectively. Germination, inoculation and cultivation were performed in the laboratory and greenhouse at Universidad Católica del Maule, city of Talca (35°26′10″S, 71°37′05″W). After surface sterilization (10% sodium hypochlorite, 20 min), *Nothofagus* seeds were treated with gibberelic acid (100 ppm, 24 h) and *Quercus* seeds were soaked in cold water during 24 h to induce germination. Once germinated, a total of 54 seedlings per tree species were potted in 18-cm-deep trays in a previously sterilized mixture of bark compost/vermiculite/perlite (3:1:1; approx. pH 7) and grown 6 months in a greenhouse under axenic conditions. For inoculation, seedlings were carefully freed from adhering substrate and rinsed in sterile water to remove remaining substrate and to keep the roots moist; the plants were then spread in portions of 24 at a time over a layer of transplant substrate, and the complete root system of each plant was powdered with 0.5 g of the inoculum mixture. This procedure guaranteed the optimal use of the inoculum, as excessive spore powder fell on the same substrate, which was later used to fill the plant containers. Immediately afterwards, seedlings were trans-

planted in 450-ml containers with the transplant substrate, consisting of limed soil (49%), vermiculite (11%), perlite (25%) and peat (15%), previously steam sterilized for 90 min; the soil had been limed twice with calcium carbonate, first with fine powder, subsequently with coarse particles of 1 mm diameter, adjusting a final pH of 8.1. After inoculation and transplantation, seedlings were kept for a period of 6 months in a greenhouse and watered with chlorine-treated water every 2 days, according to the temperature and substrate condition.

Control of mycorrhization

Six months after inoculation, 18 plants of each group were monitored for mycorrhiza formation. A volumetric, non-destructive method was applied (Reyna et al. 2000), extracting a horizontal cylindric portion of 7 ml of roots and substrate with a cylindric metal corer from the middle of each container. After the extraction, the hole was filled with substrate, and the container was returned to its place. The root core was carefully rinsed with water several times to separate the roots from the substrate and finally poured into a sieve from which all roots were retrieved with fine forceps into a water-filled Petri dish. All fine root tips were screened in water under a stereo microscope (MOTIC SMZ 143) under a halogen light source with fibre optics (ALPHA- 1501) and separated in three categories (non-mycorrhizal tips, tips colonized by *T. melanosporum* and tips colonized by other fungi).

One-way analysis of variance (ANOVA) was applied to test for statistical significant differences in mean values of mycorrhization of the four tree species by *T. melanosporum*. When ANOVA indicated a significant effect of tree species ($P<0.05$), the Duncan a posteriori test was applied.

The entire *Tuber* mycorrhizae and mantle scrapings were microscopically examined and documented by digital colour micrographs (NIKON Coolpix 950) and line drawings (camera lucida, LEITZ) under the stereomicroscope and a compound microscope (LEITZ Dialux, Wetzlar, Germany) according to Agerer (1991) and identified by using keys and reference descriptions by De Miguel and Sáez (1997), Etayo and De Miguel (1998), Rauscher et al. (1995), Sáez and De Miguel 1995 and Zambonelli et al. (1993); for the concise description of synthetized *Nothofagus* mycorrhizae, typical specimens of the most successful combination *T. melanosporum*×*N. obliqua* were chosen; mycorrhizae formed by other fungal species than *T. melanosporum* were identified by reference descriptions in Agerer (1987–2006) and Ingleby et al. (1990); voucher specimens preserved in formaldehyde–acetic acid–alcohol are kept at the Laboratory for Biotechnology, Universidad Católica del Maule, Talca, Chile.

Results

Seedling survival

Whereas all 54 seedlings of the control groups (*Q. ilex*, *Q. robur*) were still alive and only one plant (2%) of *N. obliqua* had died after 2 months, more than half of the *N. glauca* seedlings (30 individuals or 56%) at that time had not survived the inoculation/transplantation procedure after the same period (Table 1).

Mycorrhiza formation

A total of 25,329 root tips was retrieved from 72 core samples of all four tree species and screened for mycorrhiza formation. Table 1 shows that the proportion of seedlings that had been colonized by *T. melanosporum* was high (between 72 and 94%) in all species except *N. glauca* (39%); with 40.8%, the proportion of colonized root tips (Fig. 1) was highest in *Q. ilex*. *N. obliqua* and *Q. robur* showed lower, almost identical values (8,8% and 8,7%), and *N. glauca* had the lowest proportion of *Tuber* mycorrhizae of only 1.5%. the Duncan test showed that *N. obliqua* and *Q. robur* formed a group significantly distinct from *N. glauca* and *Q. ilex* (Table 2).

The highest number of total fine root tips was found in *Q. robur* (14,078) and the lowest in *Q. ilex* (1,514). Colonization by other, adventive ectomycorrhizal fungi was only occasionally observed in both *Quercus* spp. (2.2% of the root tips in *Q. ilex* and 1.1% in *Q. robur*) but not in *Nothofagus* (Fig. 1); the morphological and anatomical details of the colonized root tips (whitish colour, presence of clamp connections, cottony extramatrical mycelium, lacking rhizomorphs and a plectenchymatous mantle) suggest an *Inocybe* or *Hebeloma* sp.

Table 1 Survival, mycorrhization and fine root formation of two *Nothofagus* spp. and two *Quercus* spp. after inoculation with *Tuber melanosporum*

	N. obliqua	*N. glauca*	*Q. ilex*	*Q. robur*
Total seedlings	54	54	54	54
Surviving seedlings after 2 months	53 (98%)	24 (44%)	54 (100%)	54 (100%)
Total sampled seedlings after 6 months	18	18	18	18
Sampled seedlings colonized by *T. melanosporum*	13 (72%)	7 (39%)	17 (94%)	14 (78%)
Total root tips from all cores (total soil volume: 126 ml)	7,748	1,989	1,514	14,078

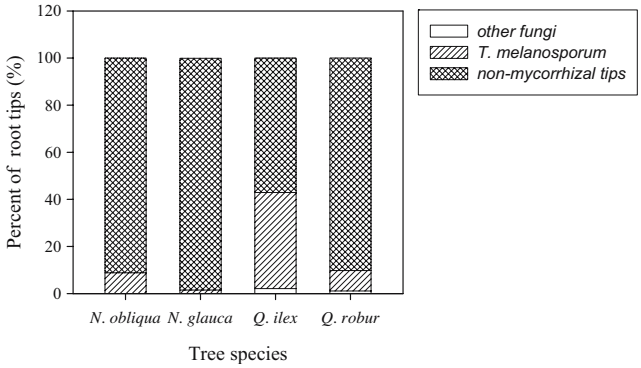

Fig. 1 Colonization rates of *Tuber melanosporum* and adventive fungi on two *Nothofagus* spp. and two *Quercus* spp. 6 months after inoculation

Description of *T. melanosporum*×*N. obliqua* mycorrhizae

General morphology and anatomy of ectomycorrhizae formed by *T. melanosporum* and *N. obliqua* matched all important diagnostic features as described in previous reference descriptions, especially in the detailed description of *T. melanosporum*×*C. avellana* mycorrhizae by Rauscher et al. 1995; consequently, the following concise description includes only diagnostic key features (colour, shape, cystidia, mantle pattern), described according to the criteria established by Agerer (1987–2006, 1991): young mycorrhizal systems short and unramified (Fig. 2a), mature systems monopodial-pyramidal with stout lateral branches (Fig. 2b), length of main axes 0.5–6 mm, length of lateral branches 0.5–2.5 mm, diameter of main axes and lateral branches 0.2–0.4 mm; colour varying with age of individual mycorrhizae and ranging from light brown in young mycorrhizae over reddish brown in mature systems to blackish brown in senescent tips; mantle surface smooth to slightly grainy and often covered with minute, dark brown scales; cystidia-bearing portions with a spiny to woolly appearance (Fig. 2b); cystidia long, branched (Figs. 3b, 4b), patchily distributed and often emerging in distinct bundles, typically close beneath the tip of a main axis or side branch (Fig. 2b); mantle surface, especially in freshly formed portions with a coarse, appressed hyphal net; outer mantle layers pseudoparenchymatous, cell pattern epidermoid (interlocking; Figs. 3a, 4a), in older portions also of angular or polygonal shape, cell walls brown, smooth, occasionally slightly gelatinous; cystidia emerging from "foot cells" on the mantle surface (Fig. 4b), up to 300 (–400) μm long, 2–4 μm in diameter squarrosely branched in approx. 90° angles, cell walls hyaline, smooth, occasionally with isolated warts, septa simple; emanating hyphae and rhizomorphs lacking; structure of Hartig net and rhizodermis not deviating from previously described mycorrhizae on other host trees.

Table 2 Duncan test (5% significance), showing differences in mycorrhization of two *Nothofagus* spp. and two *Quercus* spp. with *T. melanosporum*

Species	Mean[a]	Significance
Quercus ilex	0.695	a
Nothofagus obliqua	0.262	b
Quercus robur	0.236	b
Nothofagus glauca	0.084	c

[a] Proportion of root tips colonized by *T. melanosporum*, compared to the total sample

Discussion

As could be expected, a native host species, *Q. ilex*, showed by far the highest rate of colonization by *T. melanosporum* in our experiment with more than 40% of the root tips colonized; however, the performance of *N. obliqua* was

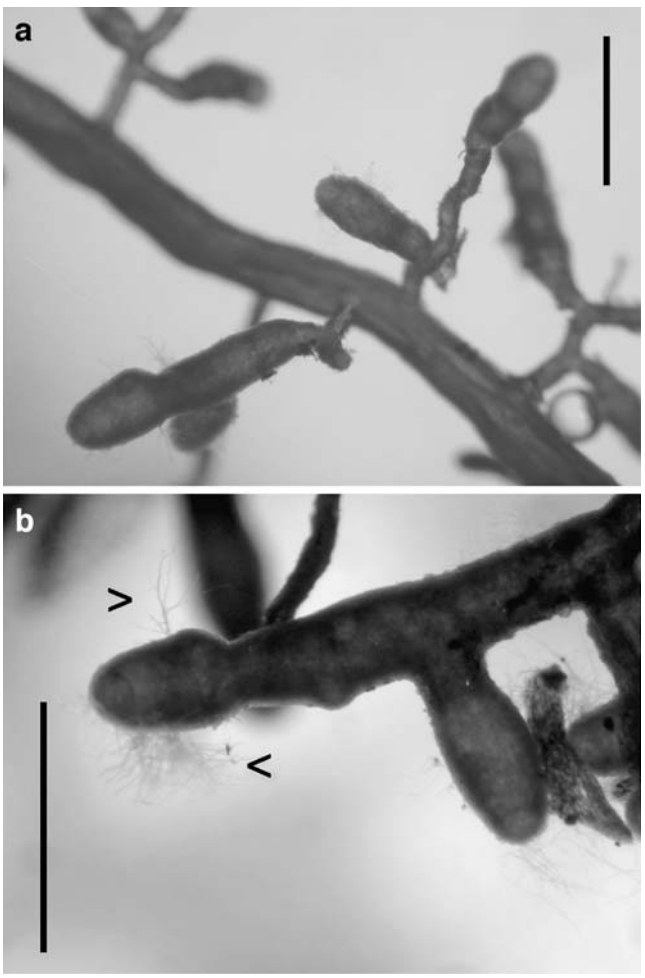

Fig. 2 Morphology of ectomycorrhizae formed by *Tuber melanosporum* and *Nothofagus obliqua*. **a** Small mycorrhizal systems. **b** Tip of larger mycorrhizal system with bundles of ramified cystidia (*arrowheads*); *bar*=1 mm

similar to *Quercus robur*, the second natural mycorrhizal host of black truffle used in this experiment, which has been identified as one of the most efficient trees in truffle culture (Hall et al. 2001; Reyna 2000): Both species showed a relatively high percentage of mycorrhization after six months in the greenhouse (almost 9%) and a high survival rate after inoculation and transplantation. Although much lower than *Q. ilex*, the proportion of active mycorrhizal roots in *N. obliqua* and *Q. robur* are not very dissimilar to infections found in natural, mixed mycorrhizal associations in the field which are usually between 15 and 20% (Palfner et al. 2005). Remarkable is the observation that although *Q. robur* formed almost twice as many fine root tips as *N. obliqua* in the same sample volume, the percentage of ectomycorrhizae was almost the same in both species. Our results show that *N. obliqua* forms good mycorrhizas with *T. melanosporum* despite being grown in a medium with a pH considerably higher than that where it is found growing naturally.

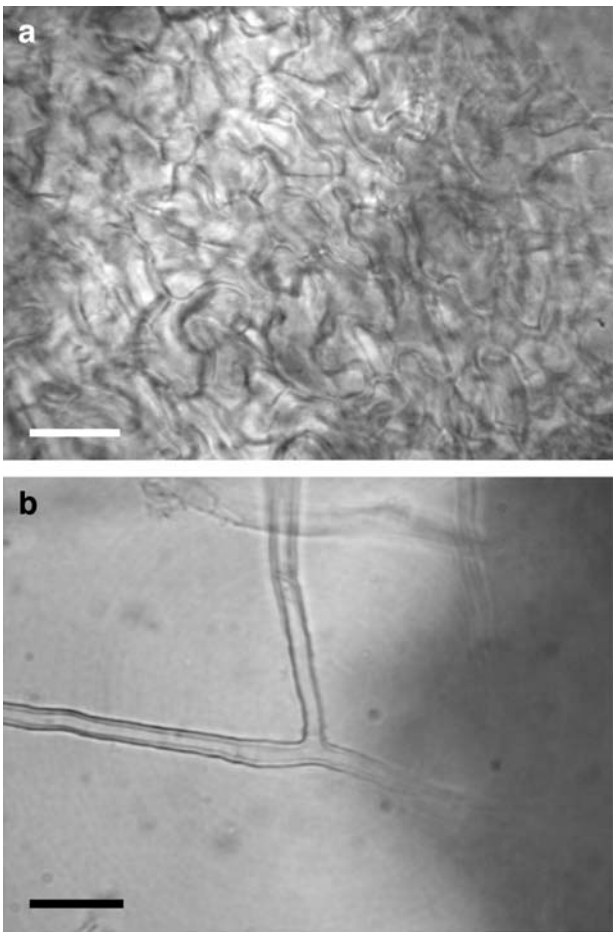

Fig. 3 Anatomy of ectomycorrhizae formed by *Tuber melanosporum* and *Nothofagus obliqua*. **a** Outermost mantle layer with pseudoparenchymatous, epidermoid cell pattern. **b** Section of cystidium with rectangular ramification; *bar*=10 μm

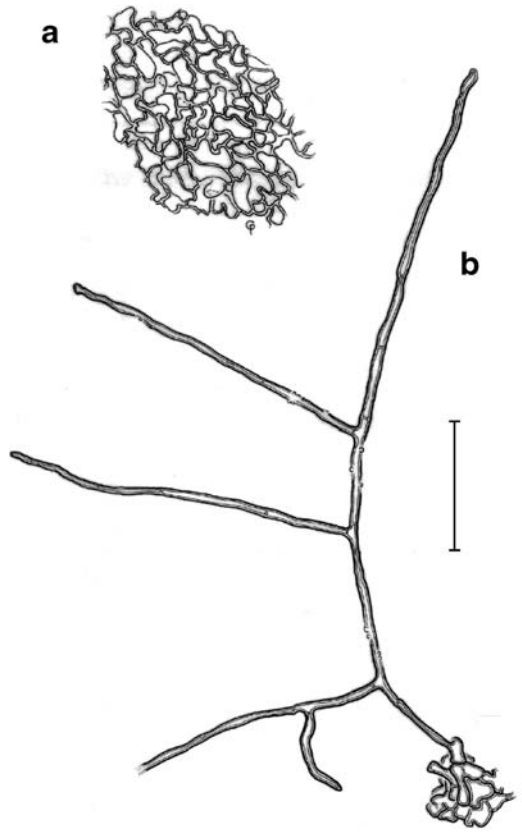

Fig. 4 Diagnostic anatomical details of ectomycorrhizae formed by *Tuber melanosporum* and *Nothofagus obliqua*. **a** Patch of outermost mantle layer close to mycorrhizal tip with pseudoparenchymatous, epidermoid cell pattern. **b** Single, branched cystidium, emerging from cell of outermost mantle layer; *bar*=50 μm

Ectomycorrhizae formed by *T. melanosporum* and both *Nothofagus* species matched all diagnostic key features like shape, colour, mantle pattern and cystidia, previously described for associations of this fungus with the natural hosts *Quercus* spp. and *C. avellana* (De Miguel and Sáez 1997; Etayo and De Miguel 1998; Rauscher et al. 1995; Sáez and De Miguel 1995; Zambonelli et al. 1993).

The high mortality and extremely low colonization rate by the Périgord black truffle of *N. glauca* seedlings was surprising considering that this species thrives under similar ecological conditions as *Q. ilex*, which performed extremely well in this study; the most probable reasons for the disappointing results could be a strong reaction to the abrupt change of soil pH, high susceptibility to transplantation stress during and after inoculation or, alternatively, low compatibility with *T. melanosporum*.

We conclude that *N. obliqua* is a promising symbiont for future truffle cultivation projects in Chile. However, our results reflect only the situation in a greenhouse over a period of 6 months. Work currently underway will examine the survival of *T. melanosporum* and the growth of *N. obliqua*, *Q. ilex* and *Q. robur* under field conditions. As there are no natural soils in the Chilean *Nothofagus* area

that offer an appropiate pH for *T. melanosporum*, cultivation of this truffle species will always require liming treatment (an estimated 20–25 tons of lime rock per hectare), which constitutes an additional cost for the land owner; however, granulated lime rock is readily available at a moderate price (approx. 120 USD/ton) and our experience during the ongoing monitoring of field plantations shows that after an initial treatment, renewal frequency decreases substantially as the required pH stabilizes after approximately 3 years.

Acknowledgements Funding for this study was provided by a grant from the Fundación para la Innovación Agraria (FIA), Chilean Ministry of Agriculture (grant no. BID-PI-C-2001-1-A-085). We would like to thank Dr. Santiago Reyna, CEAM, Valencia, Spain, for his valuable scientific and technical support, Mariela Flores, Talca, Chile, for her substantial participation in the project and Dr. Angelica Casanova, Universidad de Concepción, for critically reviewing the data. Special thanks to both anonymous reviewers who helped to improve the manuscript with their valuable comments.

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