Mycol Progress
DOI 10.1007/s11557-013-0923-5

ORIGINAL ARTICLE

The cultivation of oak seedlings inoculated with *Tuber aestivum* Vittad. in the boreal region of Finland

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Received: 19 December 2012 / Revised: 15 August 2013 / Accepted: 23 August 2013

Abstract Despite recent findings, truffles are rarely found in Finland. In 2006, we began to explore the cultivation potential of *Tuber aestivum/uncinatum* in Finland. In 2006–2008, roughly 1,200 *Quercus robur* seedlings and 200 *Q. pubescens* seedlings were planted in 20 orchards. We aimed to challenge the southern European (France) tree provenances of oak seedlings in a boreal climate. Additional winter coverings made up of fabric or plastic and twigs prevented the seedlings' mortality even when the air temperature was below −30 °C during the second winter. The results showed that the top soil temperature at 15 cm depth has to be above −5 °C to guarantee the survival of seedlings. *Q. pubescens* was more sensitive to low soil temperatures than *Q. robur*. Morphological and PCR analysis of root samples collected over 2007–2010 confirmed the presence of *T. aestivum* in all orchards despite unfavorable temperatures during the winter time. The first *T. aestivum* sporocarps were found under *Q. robur* in October 2012 in the orchards established in 2006 on old agricultural land, showing truffle cultivation to be successful in the boreal climate.

Keywords Burgundy truffle · Truffle cultivation · Soil temperature · Winter protection · *Q. robur* · *Q. pubescens*

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Introduction

Truffles are the most expensive edible fungi in the world. They belong to the Tuberaceae family, grow in symbiosis with several trees, such as oaks (*Quercus* spp.), hazel (*Corylus avellana*), beech (*Fagus sylvatica*), and even birch (*Betula* spp.), and produce hypogeous sporocarps (Riousset et al. 2001; Chevalier and Frochot 2002; Stobbe et al. 2012). Economically, the two most highly renowned truffle species are *Tuber melanosporum* Vitt., the Perigord black truffle, and *T. magnatum* Pico, the Italian white truffle. The summer truffle *T. aestivum* (syn. *T. uncinatum*; Wedén et al. 2005) also has significant commercial value (Mello et al. 2006; Hall et al. 2007; Streiblová et al. 2010).

The life cycle of a truffle involves an initial phase of growth as filamentous mycelium followed by a second phase of symbiotic association between fungal hyphae and host roots (ectomycorrhiza). If these first two stages are successful, the organization of hypogeous sporocarps may start (Peterson and Bonfante 1994). The last century has witnessed a continuously growing international market for truffles, while the world harvest of wild truffles has dropped dramatically from approximately 1,500 t to less than 100 t annually (Mello et al. 2006). This decline has led to the establishment of many truffle plantations worldwide (Mello et al. 2006; Hall et al. 2007) where truffle production is expected to begin in 5–7 years following orchard establishment (Chevalier and Frochot 2002; Sourzat 2000). Some findings have even suggested that the first production of these plants could begin in 3–4 years under optimal conditions (Lefevre et al. 2001; Streiblová et al. 2010). Currently, more than half the harvested truffles worldwide are produced in orchards (Hall et al. 2003). More than 80 % of French *T. melanosporum* production comes from truffle orchards (Mello et al. 2006).

The production of ectomycorrhizal plants in laboratory or greenhouse conditions was demystified and incorporated into

orchard preparation in the early 1970s in both France and Italy. The first successful harvest in France in the late 1970s led to a broad array of orchard set-ups (Lefevre et al. 2001). Following this period, much experience has accumulated on truffle cultivation and required growth conditions (Chevalier and Sourzat 2012). The most common, and the first species to be used in the commercial inoculation of plant seedlings, was *Tuber melanosporum* (Chevalier and Grente 1978). Other species, such as *T. magnatum* (Bencivenga and Granetti 1988) and *T. aestivum* (Chevalier and Frochot 2002), have gained cultivation interest over the last few decades, either due to the drop in natural production or increased demands on the market that have necessitated overcoming problems of cultivation and inoculation techniques (Hall et al. 1998).

T. aestivum is naturally widespread in Europe and North Africa from 37º to 57º N, and can be found as far north as Gotland in Sweden (Jeandroz et al. 2008; Wedén et al. 2004b; Stobbe et al. 2012). *T. aestivum* is cultivated quite commonly in Europe (Streiblová et al. 2010). The most northern orchard before our trials was established in 1999 in Gotland, in a mild, sub-boreal climate (Wedén et al. 2004b). With regard to site characteristics, *T. aestivum* is less demanding in terms of environmental conditions compared to other commercial truffle species (Chevalier and Frochot 2002). The species also exhibits a broader range of symbiotic tree partners (Gardin 2005), with many trees and shrubs successfully inoculated under laboratory conditions and transferred to orchards (Hall et al. 2007; Pruett et al. 2008). However, the presence of adequate exchangeable calcium in the soil is essential for *T. aestivum* growth (Chevalier 2012).

Finland is located between latitudes 60° and 70° N, and longitudes 20° and 32° E. As such, the country is not a traditional truffle-producing location, nor are truffles a part of the traditional Finnish kitchen. However, truffles have a long history in Finland, despite several truffle species (*T. borchii* Vittad, *T. maculatum*, *T. scruposum*, and *T. foetidum*, excluding *T. aestivum*) having only been recently identified there (Shamekh et al. 2009; Orczán et al. 2010). Over the last decade, we have established the first truffle orchards in Finland in order to study seedling survival, growth, and truffle ectomycorrhiza development in a boreal environment with long winters and low winter soil temperatures. To overcome these environmental restrictions, a combination of different orchard adaptations and management methods were studied.

Materials and methods

Establishment of truffle orchards

Twenty truffle orchards were established over 2006–2008, mainly in Southern Savo. Six out of 11 orchards planted in 2006 were included in the detailed analysis. Nine orchards were planted from 2007–2008 that were also used for examining the survival of seedlings. Altogether, approximately 1,200 *Quercus robur* seedlings (provenance north-east France) inoculated with *T. aestivum/uncinatum* were planted. Roughly 200 *Q. pubescens* seedlings (originating from Southern France) were also planted. All seedlings were inoculated under controlled conditions by Robin Pepinieres (Saint Laurent Du Cros, France) 1 year prior to planting. *Tuber aestivum* sporocarps originating from mild climate areas in northern France were used for spore inoculation.

Seedlings were planted in rows with 3–5 m between plants and 4–6 m between lines to ensure good future shading with canopies and access to harrowing machines (Chevalier and Frochot 2002; Sourzat 2000). Lime was added prior to planting to achieve a proper soil pH, and was continued during the following years. A total of 2.5 t ha^{-1} of lime was added to increase the pH value of the soil by 0.1 at a depth of 10 cm. The soil between the rows was ploughed, harrowed, or weeded mechanically. The top soil was managed and protected as summarized in Table 1. Other orchards not shown in Table 1 had similar protection.

The seedlings were cultivated with or without grazing protection tubes around the lower part of the trunk. When a protection tube was used, large side branches were removed. The orchards were irrigated by using a hose or bucket during the summer period, and especially during the first months after planting or when needed in years following. Fabric, plastic, twigs, or a sawdust layer (Table 1) was used for additional winter protection in a 1-m circular area around the seedlings from November until the snow melted in the spring. The selection of methods and soil protection was based on materials applied in plantation in Sweden (Wedén et al. 2004b, 2005) and locally available materials. Seedling survival was assessed after each winter. All orchards were maintained regularly by local land owners under the supervision of the Juva Truffle Center.

Environmental conditions

The soil properties were determined for all truffle orchards prior to the first liming and planting, as well as in the years following planting. The soil sample parameters were assessed according to Wedén et al. (2004b) by Savo Lab (Mikkeli, Finland). The soil temperature was measured by a datalogger (A-lab; Keuruu, Finland) for each orchard at two differently exposed points of 15 cm depth. A 15 cm depth was selected as the depth where truffle mycelium was most likely to develop (Suz et al. 2006). The temperature was measured every fourth hour throughout the year.

Analysis of ectomycorrhizae

To assess the survival of *T. aestivum* ectomycorrhiza on the roots of oak seedlings (*Q. robur*), root samples were taken in

Table 1 Summary of mulch, soil temperature, seedling survival and winter protection practices influencing soil temperature at 15 cm depth

Orchard number	Mulch (all year around)	Winter protection (October–Spring)	Lowest measured temperature at 15 cm depth: winter °C	Percentage of dead seedlings after winter 2007–2008 (*Q. robur*/*Q. pubescens*)
1	Fabric	Fabric and twigs	2006–2007, −1.5	0
			2007–2008, −1.5	0
2	Sawdust	Plastic and twigs	2006–2007, −0.6	0
			2007–2008, −0.25	0
3	No mulch	Twigs	2006–2007, −0.5	0
			2007–2008, −4.4	0
4	No mulch	No protection	2006–2007, −1.5	0
			2007–2008, −8.1	6/80
5	No mulch	Sawdust (thin layer)	2006–2007, −2.5	0
			2007–2008, −5.2	2/15
6	Fabric	No	2006–2007, −2.0	0
			2007–2008, −5.9	0/5

Each of these orchards had 50–100 *Q. robur* and 20 *Q. pubescens* seedlings

September of 2007 and 2010 from two orchards that had the highest mortality of seedlings in a given year (orchards 4 and 5; Table 1). Three seedlings per plantation were randomly selected for sampling among the vital plants. A part of the root system at about 10–15 cm depth (comprising approximately 1 L of soil volume around roots) was dug by a spatula. All parts of sampled roots were rinsed with water to remove any soil and soil particles attached to coarse and fine roots and kept in plastic tubes with 75 % ethanol until identification.

Prior to analysis, sampled parts of root systems were cut into 2-cm pieces and pooled for each year. Subsequently, pieces were randomly selected and analysed until the total count of fine roots reached 400 (Benucci et al. 2011). Three samples of vital ectomycorrhizae from each identified morphotype were removed at this point for DNA-based molecular identification.

For morphology-based identification, vital types of ectomycorrhiza, old mycorrhizal root tips and non-mycorrhizal root tips were differentiated, counted, and photographed under the Olympus SZX12 stereo-microscope (magnification ×3.5–45) and Olympus BX51 microscope (magnification ×100–2,000). Types of ectomycorrhiza were identified based on morphological and anatomical evaluation (Agerer 1987–2008; Agerer and Rambold 2004–2012).

For molecular identification, individual ectomycorrhizal root tips were used for DNA extraction with the Plant DNeasy Mini Kit (Promega). Extracted DNA was then resuspended in pre-warmed, sterile milli-Q water to the approximate final concentration of 100 ng/μl. General fungal primers ITS1 and ITS4 (White et al. 1990) were used for PCR amplification of the ITS region, including 5.8 S rDNA. Amplification reactions were performed according to Kraigher et al. (1995) in a PE 9700 DNA thermocycler with a lower annealing temperature. Negative controls lacking fungal DNA were run for each experiment to check for any contamination of the reagents. Amplified DNA was separated and analysed as described by Grebenc et al. (2000). Amplified fragments were first separated and purified from the agarose gel using Wizard SV Gel and the PCR Clean-Up System (Promega), then sequenced at a commercial sequencing service (Macrogen). Sequencher 4.8 (GeneCodes) was used to identify the consensus sequence from the two strands of each isolate. The sequences were submitted to an EMBL database and compared to the GenBank (BLAST tools) to confirm their identity.

Results

All the orchard locations had been used prior as agricultural land for the production of various agricultural products (e.g., crops, grass, vegetables, and fruit trees), which made a decrease in the number of potential competing ectomycorrhiza propagules abundant in the forest soil quite likely (Hall and Yun 2001; Unterseher et al. 2012). The liming of ploughed local soils resulted in a pH increase of 0.8–1.3 in the year following the first application. In the next 2–3 years, additional liming raised the pH from a suboptimal to an optimal pH of 7.0–7.5 at all analysed sites (Table 2). The liming was continued after 2009 when the soil pH was not in the proper range. The pH values were also comparable in the other orchards (data not shown).

Mulching is known to promote *T. aestivum/uncinatum* colonization and reduction of contaminating ectomycorrhizal fungi (Zambonelli et al. 2005). Mulches affect soil temperature and moisture (Zambonelli et al. 2005). Commonly used (plastic in strawberry farming) and readily available (fabric, twigs, sawdust) materials were used in our orchards as

t2.1 **Table 2** Soil pH in four selected orchards with *Q. robur* planted in 2006. Lime was added yearly

t2.2	Orchard	2006[a]	2007	2008	2009
t2.3	1	6.3	7.1	7.0	7.5
t2.4	2	5.1	6.0	6.4	7.0
t2.5	3	6.0	6.5	7.0	7.1
t2.6	4	5.8	6.5	7.0	7.4
t2.7	5	6.1	7.0	7.2	7.6
t2.8	6	5.5	6.6	7.0	7.3

[a] 2006 soil pH value is before liming

protection (Table 1). Truffle cultivation has not historically been a part of Finnish agriculture. The first Finnish truffle orchard was the one established for this very study in the summer 2006. Different mulching and winter protection methods were applied to find out which one was suitable for the boreal climate. The effect of the protection methods was noted in examining the soil temperature and survival of the seedlings. The soil temperature at a depth of 15 cm dropped below 0° C (−0.25 °C to −8.1 °C) at all measured sites during the winter of 2007–2008. The lowest temperatures (below −5 °C) were recorded on the sites where no additional protection was applied, or only a thin layer of sawdust was used during the winter (Table 1). Fabric or plastic together with twigs was most efficient for additional winter protection to prevent low soil temperatures throughout January 2008 (Table 1; Fig. 1).

Low soil temperatures coincided with the mortality of seedlings when the soil temperature at a depth of 15 cm dropped below −5 °C (Table 1). The mortality of Mediterranean provenances of *Q. pubescens* seedlings in two of the most affected plots was higher than with *Q. robur* (Table 1). While most *Q. pubescens* seedlings died in orchard 4 during the winter of 2007–2008, orchard 2, under 1 km distance from orchard 4, did not suffer any *Q. pubescens* deaths. Variation of the lowest soil temperature above −5 °C did not affect the survival of the seedlings. Protection was corrected for in orchard 4 (Table 1) in November 2007 by using sawdust and twigs. After this, no deaths of seedlings were detected in this orchard and other orchards save for 1–2 % of seedlings dying in some orchards over the following years due to attack by moles.

Microscopic investigation (Fig. 2) showed that *T. aestivum* ectomycorrhiza (sensu Agerer 1987–2008; Chevalier and Frochot 2002; Agerer and Rambold 2004–2012) was present at all studied sites during 2007–2011 with all diagnostic characters: the ramification of ectomycorrhizal roots absent or monopodial pinnate absent; shape of the unramified ends straight; surface irregularly hairy, cottony; mantle surface densely long-spiny under low magnification; colour of ectomycorrhizae ochre to yellow-brown and at the very tips the same colour or brighter, whereas older tips were darker brown; rhizomorphs were not observed; fungal cells of the outer and inner mantle layers formed pseudoparenchymatous mantle consisting of angular cells without septa. The mantle type of hypha was designated as "L" type after Agerer's (1987–2008) categorization (Fig. 2b). Emanating hyphae were present and abundant, growing from different parts of ectomycorrhizae, and several emanating hyphae exhibited a curly shape. The emanating hyphae were not ramified, septae on emanating hyphae were present, and no special structures or clamp connections were observed. Anastomoses of emanating hyphae and rhizomorphs were not observed at higher magnifications.

Molecular characterization of the ITS region yielded complete ITS1, 5.8S rDNA and ITS2 sequences. The representative sequence from three identical DNA sequences from the

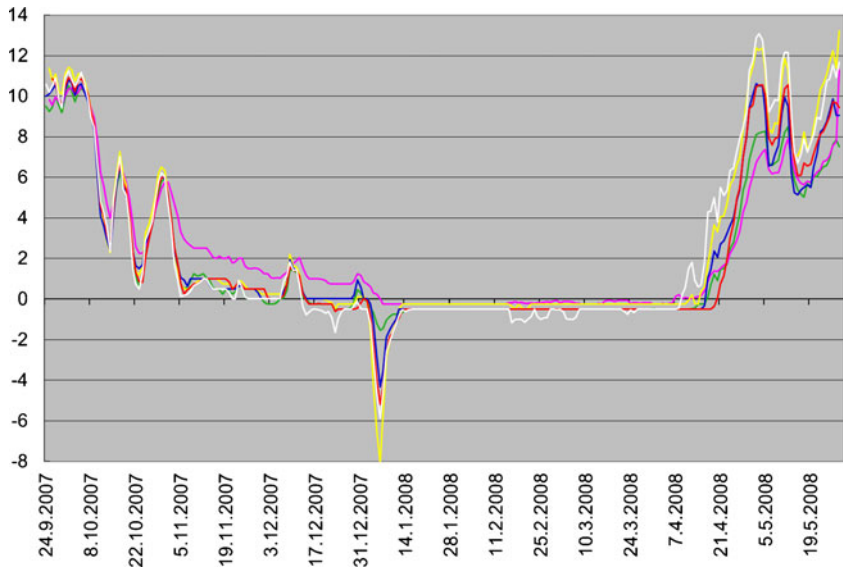

Fig. 1 Soil temperature profile at six orchards planted in 2006. The temperature was measured at 15 cm depth in intervals of 4 h during the extreme low-temperature winter of 2007–2008. The colors for different orchards are as follows: 1 *green*; 2 *pink*; 3 *blue*; 4 *yellow*; 5 *red*; 6 *white*

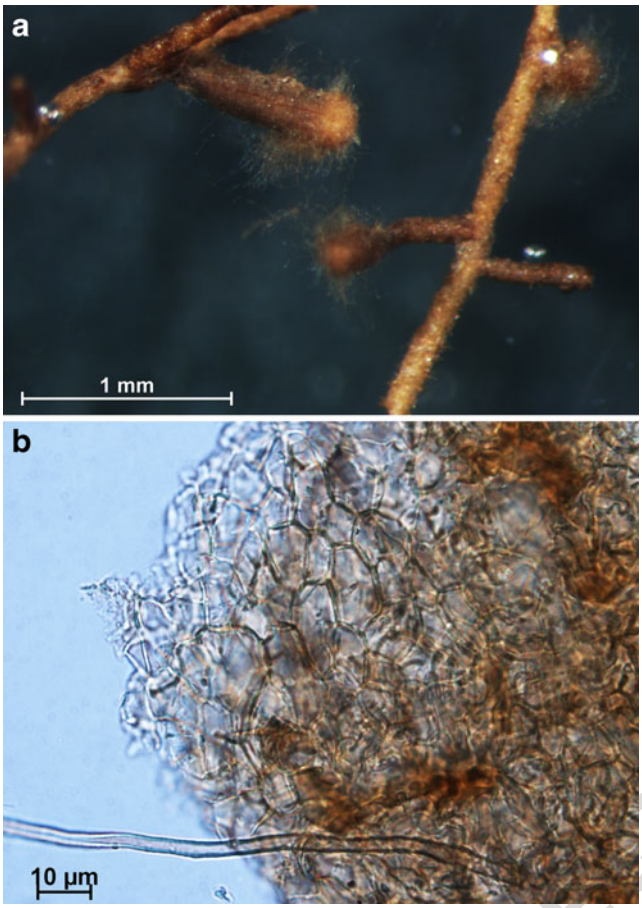

Fig. 2 **a** Morphology of anatomotype JUVA 01/2009 at ×45 magnification **b** Outer ectomycorrhiza mantle layers of anatomotype JUVA 01/2009, at ×1,000 magnification

analysed *T. aestivum* ectomycorrhiza was deposited at the EMBL database with accession number FN395017. The BLAST search gave multiple hits of query sequence with 99 % similarity to several *T. aestivum* ascocarp sequences published by Paolocci et al. (2004).

The ectomycorrhizae of *T. aestivum* were present at all analysed sites 4 years after planting. The fine root analysis of growing seedlings at the two plantations with the highest mortality (namely, orchards 4 and 5; Table 1) indicated a decrease in the percentage of vital *T. aestivum* ectomycorrhizae among the total mycorrhizal population, the appearance of two additional types of mycorrhiazae (including *Cenococcum geophilum*), and an increase of old ectomycorrhizal fine roots in the following years (Table 3).

In October 22, 2012, three truffle sporocarps from two orchards (established in 2006) in the city of Juva were found by the aid of a Lagotto truffle dog under *Q. robur*. Eleven orchards were screened. Two truffle sporocarps were harvested from orchard 2 and one from orchard 6. The protection system varied in these two orchards. The sporocarps were growing under roughly 2-m-tall oaks at about 10–20 cm distance from the trunks, a similar distance to the first

Table 3 The ectomycorrhizal community, old ectomycorrhizal fine roots and non-mycorrhizal fine roots on vital oak seedlings (*Q. robur*)

Fine root/ectomycorrhiza	Sampling in year 2007	Sampling in year 2010
Tuber aestivum	59.5 %	25.0 %
Unknown type 1	0.0 %	5.5 %
Cenococcum geophilum	0.0 %	1.0 %
Old ectomycorrhizal fine roots	39.5 %	68.5 %
Non-mycorrhizal fine roots	1.0 %	0.0 %

The root samples were collected in years 2007 and 2010. Each value represents the percentage from 400 analysed fine roots in the pooled sample

cultivated truffles in Gotland (Wedén et al. 2009). The findings were at 5–10 cm depth. Each of the sporocarps weighed approximately 30 g, and showed morphological characteristics (Fig. 3a, b) and had the fresh aroma of *T. aestivum*. Previous use of the land in orchard 2 included the production of grains and grass, and in orchard 6, grains and cabbage.

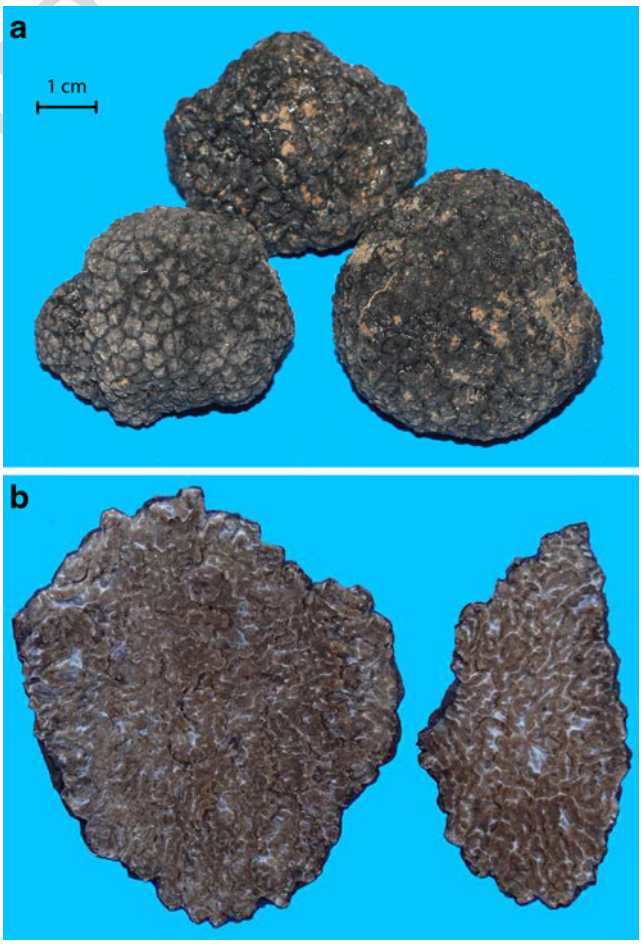

Fig. 3 The *T. aestivum* sporocarps found in October 2012 from the truffle orchards in Southern Savo. **a** Peridiums of all three sporocarps; the scale is in centimeters. **b** Cross-sections showing the spore tissue (gleba) of two sporocarps

Discussion

The conditions in Finland are challenging both for truffles and ectomycorrhiza host plants. *Quercus robur* grows in the hemiboreal zone in the southern coast of Finland (Koponen 2004), whereas *Q. pubescens* is a southern European species. We have established truffle orchards in the southern boreal forest region of Finland using both tree species. Plantations were established in the regions that are outside the natural oak range roughly 100 km to the north of the coastal oak-growing zone. In our orchards, *Q. robus* was growing well, but not *Q. pubescens*. The average air temperature in the area (data for nearby city of Mikkeli) in January 2006 was −7.1 °C, while peak air temperatures can occasionally drop below −30 °C, as happened in 2007, over a year after plantation. This explains the sudden very low soil temperature shown in Fig. 1. In general, the thick snow layer protects the soil from the temperature decrease to the same levels as the air temperature.

Truffle species generally require a relatively high pH level (between 7 and 8) (Bencivenga and Granetti 1988; Riousset et al. 2001; Mello et al. 2006). The optimal soil pH for the growth of *T. aestivum* is 6.8–8.0 (Chevalier and Frochot 2002; Wedén et al. 2004b). Thomas (2012) found the lowest optimal pH for commercial orchards to be 7.5. In our truffle orchards, pH elevated gradually during the first 3 years. Additional liming was used to obtain the optimal pH range as previously reported for many sites, including the unfavourable volcanic soils of New Zealand (Hall et al. 2007). The soil pH achieved the level of 7.0–7.5 in 1–3 years of liming depending on the site (Table 3). While the starting pH values in 2006 were in the range of 5.1–6.3, the pH values in the next year were in a range (6.5–7.1) adequate for *T. aestivum* growth (Chevalier and Frochot 2002; Thomas 2012).

The main challenge in truffle cultivation in Finland was the maintenance of soil temperature conditions that would ensure survival of *T. aestivum* ectomycorrhizae and the seedlings. Our results indicate that soil temperature at a depth where truffle mycelium is commonly present (Suz et al. 2006) should not drop below −5 °C, not even for a short time, as such temperatures increased the mortality of both host tree species and likely reduced the number of vital *T. aestivum* ectomycorrhiza on surviving seedlings.

The temperature in the boreal region can drop severely. To overcome this problem, we applied several permanent or seasonal soil protection approaches. Soil protection was applied similar to that of a previous truffle plantation in Gotland, Sweden (Wedén et al. 2004b, 2009), where it was primarily used not to protect the soil from low temperatures but to reduce competition from weeds and damage due to animal grazing. During the winter of 2006–2007 in the Finnish orchards, no additional winter protection appeared to be necessary. The extremely low temperature in January 2008 required additional winter protection to keep the soil temperature high enough. The combined use of plastic or fabric and twigs was most efficient in preventing the decrease of temperatures below −5 °C. Seedling survival was not generally affected by soil temperatures between −0.25 °C and −4.4 °C. Only when the soil temperature dropped below −5 °C was seedling death observed.

While *T. aestivum* ectomycorrhizae remained present 4 years after planting, the amount of competing mycorrhizae increased. The frost might be one reason for the change in the ectomycorrhizal community in the Finnish orchards, since it has been reported that the low soil temperatures can reduce the percentage of *T. aestivum* ectomycorrhizae and support the development of a stress-tolerant type of ectomycorrhizae formed by *Cenococcum geophilum* (Hasselquist et al. 2005). However, the relatively low number of other species in the plantation (compared to the plantation in Benucci et al. 2011) was likely due to the history of the site and relatively unfavourable climate conditions.

The sporocarps collected in 2012 were identified based on the morphological characters of sporocarps (Fig. 3), microscopy of spores (not shown), and the typical aroma of *T. aestivum*. The finding of the first sporocarps in October 2012 revealed that the Finnish truffle orchards started producing well-formed truffles 6 years after planting, much the same as the cultivated *T. aestivum* sporocarps obtained in Gotland (Wedén et al. 2009). The results of our work indicated that even a boreal area is suitable for the production of truffles, and it appears that *T. aestivum* is a promising truffle species for orchards in northern conditions. Geologically, Finland has Archaean bedrock, whereas surficial deposits of the country have developed during the ice age (Nenonen and Portaankorva 2009). Gotland, which holds the nearest southern *T. aestivum* findings, is formed of Silurian shallow-water marine sediment (Kershaw 1993). This comparison confirms the conclusion of Chevalier (2012) that the soil is not a limiting factor for the cultivation of *T. aestivum*. The summer temperatures in southern Finland and Gotland are similar, whereas the autumn temperatures are higher in Gotland. For example, in October the average temperatures in Southern Finland are 4–6 °C, and in Gotland 6–10 °C. Mean winter temperatures in Southern Finland are 2–4 °C lower (Tveito et al. 2000). Precipitation is slightly higher in southern Finland than in Gotland (Tveito et al. 2000). It appears that these climate differences did not affect the growth rate of the first cultivated *T. aestivum* sporocarps. In October 2012, when the fruit bodies were harvested, the average temperature in the Juva region was 4–5 °C. The production of *T. aestivum* fruit bodies occurs mainly when the temperature is between 3.5 and 15 °C (Wedén et al. 2004a; Bruhn et al. 2013).

In conclusion, the selection of proper tree species and provenances is needed to obtain positive results. These should be adapted to a shorter vegetation period and low winter air and soil temperatures. Our results show that southern European oak

seedlings and *T. aestivum* can adapt to the climate and ecological conditions of Finland. Our results also show that restrictions caused by the northern climate and low soil temperatures can be overcome with proper soil and winter protection management.

Acknowledgments We would like to thank Rajupusu Leader ry and the Regional Council of South Savo for financial support, as well as the orchard owners for their kind cooperation. Thanks also to Mrs Heli Valtonen for her assistance. The partner from the Slovenian Forestry Institute was co-financed by the Ministry of Higher Education, Science and Technology through Research Programme P4-0107 and the Targeted Research Program Project CRP V4-0492.

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