Black Truffles of Sweden

Systematics, Population Studies, Ecology and Cultivation of Tuber aestivum syn. T. uncinatum

BY

CHRISTINA WEDÉN
Dissertation presented at Uppsala University to be publicly examined in Friesalen, Evolutionsbiologiskt Centrum, Friday, November 26, 2004 at 13:00 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

Tuber aestival is an ectomycorrhizal ascomycete with underground fruit bodies. It is an economically important species, but has been regarded as endangered in Sweden. My inventory has increased the number of reported localities from 3 to 31.

It has long been debated whether T. aestival and T. uncinatum are conspecific or not, so a clarification would help conservation biology and cultivation. My study included 117 fruit bodies from 8 countries. The phylogenetic (ITS) and microscopic analyses showed that the two taxa were synonyms and that the spore reticulum height, used to separate the taxa, is not diagnostic. T. aestival was clearly different from T. mesentericum, which I reported new to Sweden.

The Gotland T. aestival population was genetically distinct (RAPD) from other European specimens. The genetic variation suggested sexual reproduction. The habitat of 18 T. aestival sites on Gotland were analysed and compared with data from France. No striking functional differences in soil chemistry were found, so a possible T. aestival ecotype on Gotland would rather be an adaptation to the colder and drier climate. Selecting local T. aestival inoculum for truffle orchards in Northern Europe could be important for successful truffle production.

In 1999, 10 experimental truffle orchards with a total of 240 oak and hazel seedlings were established on Gotland, and as a result of this project 3000 commercial oak seedlings were planted in 2000-2001. In 2004, T. aestival mycorrhiza was still present in all of the 22 orchards studied on Gotland, some in soils different from natural habitats. In addition, the project has also generated a truffle cultivation association, a truffle company, truffle dog breeding and export of T. aestival to France.

Keywords: Tuber aestival, Tuber uncinatum, Tuber mesentericum, ITS, spore reticulum, truffle cultivation, island ecology, populations, morphology, ecotype, Querus robur

Christina Wedén, Department of Evolution, Genomics and Systematics, Department of Systematic Botany, Norbyvägen 18D, Uppsala University, SE-752 36 Uppsala, Sweden

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ISSN 1104-232X
ISBN 91-554-6099-2
urn:nbn:se:uu:diva-4675 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-4675)
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I  Christina Wedén, Eric Danell and Leif Tibell. Species recognition in the truffle genus Tuber – the synonyms T. aestivum and T. uncinatum. (Submitted to Environmental Microbiology).


IV  Christina Wedén, Lina Pettersson and Eric Danell. Cultivation of the edible truffle Tuber aestivum syn. T. uncinatum in Sweden. (Manuscript)

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All papers included in this thesis are written by the first author, with comments and suggestions given by the co-authors. The studies were planned in co-operation with the co-authors. The first author is responsible for all field work, laboratory work and analyses, except for Fig. 1 and Fig. 2 in Paper II, by A.B. and F.J.C. respectively. Inoculation in Paper IV was carried out by L.P.
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Introduction

The history of *Tuber aestivum* research in Sweden

*Tuber aestivum* Vitt., the Burgundy truffle or summer truffle, is a hypogeous ascomycete which lives in ectomycorrhizal symbiosis with trees and shrubs such as *Quercus robur* L. (oak) and *Corylus avellana* L. (hazel) (Chevalier & Frochot 1997, Figure 1). In 1997, when *T. aestivum* was only reported three times from Sweden, I did my undergraduate masters project suspecting that *T. aestivum* was possibly an overlooked species in Sweden, due to its hypogeous fruit bodies (truffles) and the lack of a tradition of searching for and eating truffles in Sweden (Wedén & Danell 1998).

The earlier finds were all from the island of Gotland on the Swedish East coast (Sunhede 1978, Bohus-Jensen 1988, Kers 1992), which is why I concentrated my field work there. The discovery of a new site and large (225 g) fruit bodies with mature spores indicated that the species seemed well adapted to the local climate and soil conditions (Wedén & Danell 1998). These findings enabled further financing, making it possible to set up a PhD project in 1999 to study *T. aestivum* in Sweden. Such a project would bring new knowledge about a threatened species in Sweden. *Tuber aestivum* is also a species of potential economic importance for rural societies. This is important because the traditional management of the landscape on Gotland, resulting in a high biodiversity, needs a rural population.

Truffle cultivation presents an incentive for planting oak seedlings of importance for conservation biology. The oak (*Q. robur* and *Q. petraea*) is the Swedish tree to which the largest number of other species are connected and some 800-1000 mosses, lichens, fungi and invertebrates are restricted to oak (Gärdenfors 1994). Since the 17th century there has been a 50% decline in the volume and area of Swedish oaklands (Lars Kardell, SLU, Uppsala, pers. comm.). On Gotland, the reduction of forest meadows between 1700 and 2000 was 97% (Croneborg 2001, Länsstyrelsen i Gotlands län 2002). According to Stål (1994) the reduction of oaklands during the 20th century, was due to systematic removal of broadleaved trees to promote conifers.
Mycorrhiza and hydnology

The mutualistic symbiosis between fungus and plant was first postulated by Frank (1885), who discovered the association between _T. aestivum_ mycelia and lateral roots when trying to cultivate truffles. He named this mutualistic relationship mycorrhiza (Gr. *mucor*=fungus and *rhizos*=root).

The study of fungi with hypogeous fruit bodies, truffles, is called hydnology (Gr. *hydnon*=truffle). Truffles do not constitute a group of related taxa, but they share a hypogeous life form that has developed multiple times in a wide range of fungal orders (Trappe et al. 2001). This might have been an adaptation to better withstand e.g. drought than their epigeous ancestors (Percudani et al. 1999). About 65 hypogeous fungi have been reported from Sweden (Danell 1996, Wedén & Danell 1998). Truffles belonging to the ascomycetes are referred to as true truffles. In a more strict definition, true truffles include only the genus _Tuber_, where we find the Périgord truffle or ‘black diamond’, _T. melanosporum_ Vitt., the winter truffle _T. brumale_ Vitt., and _T. aestivum_, which all are characterised by a blackish peridium of 4-6-cornered, flat, closely adjacent pyramidal warts (Figure 1). _Tuber_ also in-
cludes species with a whitish smooth peridium, e.g. the white Piedmont or Alba truffle, *T. magnatum* Pico, and the bianchetto, *T. borchii* Vitt. These *Tuber* species are some of the most highly valued truffles, both gastronomically and economically (Brillat-Savarin 1825, Hall & Yun 2002, Olivier *et al.* 1996).

**Distribution of *Tuber aestivum***

*Tuber aestivum* is distributed from Northern Africa to Sweden, and from the United Kingdom to Russia (Pegler *et al.* 1993, Chevalier & Frochot 1997, Wedén & Danell 1998). Five *T. aestivum* sites have been recorded from Denmark (Rosenvinge 1906, Buchwald 1954, Lange 1994), but it is neither known from Norway (Gro Gulden and Finn-Egil Eckblad, pers. comm. 1997), Finland (Henry Väre and Seppo Huhtinen, pers. comm. 1997) nor from any of the Baltic states (Edgars Vimba, pers. comm. 1997), despite the stated Baltic occurrence by Chevalier and Frochot (1997). In the United Kingdom, it may be found even in beech woods on lime-deficient soils (Pegler *et al.* 1993).

**Food for gods, kings and pigs**

At spore maturity, truffles emit volatile substances attracting animals, which unearth and eat them, and thereafter spread the spores (Trappe & Castellano 1991, Figure 2). This scent is the reason for some species having high culinary values (Brillat-Savarin 1825). Wild boars are natural vectors of truffle spores and truffles have historically been searched for with the help of pigs, which is why truffles have been called ‘food for gods, kings and pigs’. Truffles are reported to have been a delicacy in Egypt of the Pharaohs (Giovannetti *et al.* 1994). They were mentioned in writing already in the 17th century B.C. (Delmas 1978) and belong to the few fungi that were important during the Middle Ages (Lobelius 1581). Paracelsus claimed plant and fungal tubers, e.g. truffles, to be potent aphrodisiacs (Brillat-Savarin 1825, Danell 1996). Due to their intense aroma, truffles are used as a spice in cooking and are best experienced fresh and unprocessed, since their volatile aroma quickly evaporates when dried or boiled.

**Truffle cultivation**

Truffle cultivation may have positive socioeconomic effects on rural communities (Samils *et al.* 2003). Truffles can be cultivated in two ways. The first method is called Talon’s method and is a passive way of truffle cultivation. In 1810 Talon planted acorns in areas where truffles occur naturally, to improve truffle production by introducing more host trees (Chevalier & Grente 1979). This method is
still in use today, but a new active method was developed in the late 1960’s and early 1970’s, following a pronounced decline in natural truffle production in Europe (Olivier et al. 1996). By using greenhouse tree seedlings grown in soil inoculated with truffle spores, seedlings with the desired mycorrhizal symbionts such as truffles, can be established on the root system. This also allowed growers to establish truffle orchards in regions, and even continents, where these truffles did not occur naturally, e.g. in New Zealand and North America (Hall & Yun 2002).
Objectives

The objectives of this thesis were to investigate which species of black *Tuber* truffles exist in Sweden (Paper I), to investigate if the Gotland *T. aestivum* was a genetically distinct population (Paper II), to characterise the Swedish biotopes and compare them with published reports in order to investigate any specific adaptations to the Swedish biotopes (Paper III) and to establish truffle cultivation in Sweden (Paper IV).

The hypotheses were:

1. *Tuber aestivum* Vitt. and *T. uncinatum* Chat. are synonyms.
2. There is a genetically distinct population on Gotland.
3. This population is adapted to local climate and/or soil conditions.
4. It is possible to use standard nursery plants and nursery equipment to inoculate seedlings and to create truffle orchards on Gotland.
Results and discussion

The recognition of species (Paper I)

Black truffles on Gotland

Previous to my study, *T. aestivum* had only been reported three times from Sweden, all from the island of Gotland (Sunhede 1978, Bohus Jensen 1988, Kers 1992). In 1997 a new site was confirmed (Wedén & Danell 1998) and in 1999 and 2000, I lead a truffle inventory on Gotland with the help of trained truffle dogs from France (Wedén et al. 2001) (Figure 9). Thereafter I trained my own dog to find additional truffle sites. Through these inventories I have increased the number of reported sites for *T. aestivum* on Gotland from three to 31 (classified data reported to the Endangered Species Unit, Uppsala, Sweden). The truffles collected by the research project will be deposited at the Uppsala herbarium (UPS) as classified material. All finds have been reported to landowners. The geographical site names of the deposited material will be available for researchers and authorities, but not available for publishing. Publishing site names could lead to reckless prospecting for truffles, which could harm the surrounding flora and affect landowners negatively. To protect surrounding flora and maintain good relations with landowners, as well as to stimulate new truffle reports, I have therefore taken this action (Danell et al. 2003). My inventories have also resulted in *T. mesentericum*, another edible black truffle species, being reported from Sweden for the first time (Wedén et al. 2001). The reason for this success is due to my inventory method of searching with trained dogs in areas selected by studies of vegetation and soil type maps. In 2003, Kers reported *T. aestivum* from Öland, an island close to the Swedish mainland and also reported an additional 12 *T. aestivum* sites from Gotland. I have also searched for truffles in Skåne on the mainland, but so far without finding *T. aestivum*.

The new black truffle species for Sweden, called the Bagnoli truffle, *T. mesentericum*, was found at ten sites, co-existing with *T. aestivum* in five of these sites (Wedén et al. 2001). In one site where both species exist, *T. mesentericum* has showed a tendency to both start and stop maturing a few weeks earlier than *T. aestivum*, but the maturity periods overlap. Immature fruit bodies of *T. mesentericum* and *T. aestivum* can easily be confused, but at full spore maturity *T. mesentericum* emits a strong and very distinct, naphthalene like scent. The gleba is similar to that of *T. aestivum*, but has a more
lilac shade of brown at full spore maturity. Phylogenetically, *T. mesentericum* forms a sister group to *T. aestivum* (Paper I). Additional *T. aestivum* and *T. mesentericum* sites are being discovered continuously by the land owners themselves, as a result of the growing interest for training truffle dogs on Gotland, initiated by this truffle research project. Some dogs have also found other hypogeous fruit bodies, such as *Genea, Elaphomyces* and *Melanogaster* spp. Consequently the future use of trained dogs during inventories may widen our knowledge of the distribution of overlooked hypogeous species.

![Figure 2. Spores of *Tuber aestivum*. x1000. The spores of *T. aestivum* have a reticulate-alveolate spore ornamentation with irregular, polygonal meshes numbering 3-4 (5) along the bigger spore dimension.](image)

*Tuber aestivum* and its synonym *T. uncinatum*

The truffles *Tuber aestivum* and *T. uncinatum* are morphologically very similar. The names have been used to market two different product qualities of truffles and has been kept as separate taxon names, despite some molecular data suggesting otherwise (Guillemaud et al. 1996, Gandebouef et al. 1997). After Mello et al. (2002) claimed to be able to separate *T. aestivum* and *T. uncinatum* using molecular techniques, it became necessary to inves-
tigate which species exist in Sweden, a matter of importance, both for conservation biology efforts and cultivation.

*Tuber uncinatum* has been regarded by different authors, as either a distinct species, variety, subspecies, or synonym of *T. aestivum* (Chevalier & Frochot 1997). There are also many other synonyms (Ceruti *et al.* 2003). *Tuber aestivum* was described by Vittadini in 1831 and in 1887 Chatin described a new species, *T. uncinatum*, and thereby also emended the circumscription of *T. aestivum* (Vittadini 1831, Chatin 1887). The new species was named *T. uncinatum* (lat. *uncinatus*="hook"), due to the presence of hooks in the spore reticulum, a key character for the species. But the hooks seen by Chatin were in fact only the flexible walls of the spore reticulum (Figure 2) bending slightly at the top (Chatin 1887, Fischer 1897, Chevalier *et al.* 1979, Chevalier and Frochot 1997). The gleba of *T. uncinatum* was, when compared with *T. aestivum*, claimed to possess a deeper colour; the peridium having smaller, not transversally striated facets, and it was further claimed to having a more pleasant scent and to mature later during the year. Some authors have suggested that fruit bodies with a spore reticulum height around 2 \(\mu m\) should be classified as *T. aestivum* and fruit bodies with a spore reticulum height around 4 \(\mu m\) as *T. uncinatum* (Chevalier and Frochot 1997, Riousset *et al.* 2001).

I wanted both to test the reliability of the height of the spore reticulum, the only quantitative morphological character used to distinguish the two taxa, and to compare sequences of the ribosomal DNA internal transcribed spacer (ITS) region. The ITS sequence has repeatedly been used to separate even closely related sister species in fungi (Johannesson *et al.* 1999, Roux *et al.* 1999, Ryman *et al.* 2003). I analysed 117 fruit bodies of *T. aestivum* and *T. uncinatum* originating from eight European countries. All fruit bodies, except for the Swedish specimens, had previously been determined to be either *T. aestivum* or *T. uncinatum* by experts (Paper I). The ITS region was sequenced in 81 fruit bodies and 100 were analysed with regard to their spore reticulum height.

The results of the spore reticulum height measurements from 4-spored asci, in 100 fruit bodies, showed that this character is not diagnostic (Figure 3). The spore reticulum height may vary within the same fruit body, within the same ascus and even within the same spore (Paper I, Figure 2).

The maximum parsimony analyses of the ITS sequences from the 81 fruit bodies and an additional 32 sequences from GenBank, showed that *T. aestivum* and *T. uncinatum* were intermingled in one highly supported (100% bootstrap) monophyletic clade, separate from *T. mesentericum*, a taxon which has even been regarded as a subspecies of *T. aestivum* (Trappe 1979), and the outgroup *T. magnatum* (Figure 4). Specimens determined as *T. aestivum* and *T. uncinatum* even had identical sequences (Paper I and Figure 4). The mean difference in the sequences among the *T. aestivum/*T. uncinatum clade was 0.8% (0-3.5%), a variation in accordance with what could be ex-
pected within a species (Johannesson et al. 1999, Bergius & Danell 2000, Ryman et al. 2003). The variation within the Swedish *T. aestivum* specimens was only 0-0.3%. No conclusions could be drawn as to the geographical origin of the Swedish population based on ITS analysis. The identical sequences of very different geographical origin, e.g. Spain and Sweden, could be a result of parallel evolution, but the ITS variation may also be a remnant from before Gotland was colonised by *T. aestivum* (Pamilo & Nei 1988).

![Box plot of spore reticulum heights [μm] illustrating medians, first and third quartiles, range and extreme values (o). 45 fruit bodies of *Tuber aestivum* syn. *T. uncinatum* from Sweden: ‘Swedish taxon’ (450 measurements). 30 fruit bodies of ‘*T. aestivum*’ (300 measurements) and 25 fruit bodies of ‘*T. uncinatum*’ (250 measurements) sensu Gérard Chevalier (INRA Clermont-Ferrand, France) and Sergio Arcioni (Istituto di Genetica Vegetale Sez. Perugia, CNR, Italy). The box plot clearly illustrates that the spore reticulum height is not a diagnostic character for separating ‘*T. aestivum*’ and ‘*T. uncinatum*’.

*Figure 3.* Box plot of spore reticulum heights [μm] illustrating medians, first and third quartiles, range and extreme values (o). 45 fruit bodies of *Tuber aestivum* syn. *T. uncinatum* from Sweden: ‘Swedish taxon’ (450 measurements). 30 fruit bodies of ‘*T. aestivum*’ (300 measurements) and 25 fruit bodies of ‘*T. uncinatum*’ (250 measurements) sensu Gérard Chevalier (INRA Clermont-Ferrand, France) and Sergio Arcioni (Istituto di Genetica Vegetale Sez. Perugia, CNR, Italy). The box plot clearly illustrates that the spore reticulum height is not a diagnostic character for separating ‘*T. aestivum*’ and ‘*T. uncinatum*’. 

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Figure 4. One of the most parsimonious trees (317 steps; CI=0.9338; RI=0.9707) found in the analysis of the ITS sequences representing *Tuber aestivum* (Tae), *T. uncinatum* (Tun) and their sister species *T. mesentericum* (Tms). *Tuber magnatum* (Tmg) is included as an outgroup. The German (D), Spanish (E), French (F), Hungarian (H), Italian (I), Danish (K) and unknown (X) specimens were determined previous to my study as either ‘*T. aestivum*’ or ‘*T. uncinatum*’. The Swedish (S) and
the English (U) specimens are all named *T. aestivum*, since I found it impossible to distinguish between *T. aestivum* and *T. uncinatum* based on my spore reticulum measurements (Paper I). The capital letter in the middle of the specimen number indicate the origin of the sequence: W=sequences obtained in this study (Paper I); A, B and V=GenBank sequences (Table 2 in Paper I). Identical sequences are excluded and listed in Table 3 in Paper I. *Tuber aestivum* and *T. uncinatum*, sensu Gérard Chevalier (INRA Clermont-Ferrand, France), Sergio Arcioni (Istituto di Genetica Vegetale, Sez. Perugia, CNR, Italy), Mello *et al.* (2002) and Paolocci *et al.* (2004), have identical sequences and the same identical sequence may also originate from several different countries. Numbers in brackets indicate the number of identical sequences (Table 3 in Paper I). Bootstrap percentages >50 are indicated above branches. Branches present in the strict consensus tree are indicated with bold lines. Branch lengths are proportional to number of changes. *Tuber aestivum* and *T. uncinatum* are mixed in a well supported monophyletic clade (bootstrap=100).

No material is mentioned in the protolog of *T. aestivum* (Vittadini 1831) and no neotypification has been undertaken. Thus no type is available for neither *T. aestivum*, nor *T. uncinatum*. According to Ceruti *et al.* (2003), a *T. aestivum* specimen seen by Vittadini is present in the Mattiroliano herbarium in Turin, Italy. This specimen may possibly be used for typification. Consequently, types have not been included in studies trying to resolve this question (Chevalier *et al.* 1994, Mello *et al.* 2002, Paolocci *et al.* 2004; Alfredo Vizzini, Università di Turin, pers. comm. 2004). A thorough nomenclatural study of this case, including typification, is needed.

Paolocci *et al.* (2004) kept *T. uncinatum* as a morphotype of *T. aestivum*, despite their inability to separate the two taxa based on DNA sequencing. My morphological analysis clearly illustrated that the spore reticulum height is not diagnostic. It displays a continuum, neither suited for the discrimination between species, nor for varieties or morphotypes (Figure 3).

Where a distinction is maintained between ‘*T. aestivum*’ and ‘*T. uncinatum*’, immature fruit bodies are being sold as a separate species devoid of scent. Accepting synonymy between these names would make such marketing more difficult, and make it less profitable to dig up immature fruit bodies. It is possible that organoleptic properties are a constant character and there exists a taxon with mature spores devoid of scent (Chevalier *et al.* 1994). If that taxon would be genetically distinct from *T. aestivum* it would deserve recognition, but it is not congruent with Chatin’s description of *T. uncinatum*.

The small variation in the ITS sequence among ‘*T. aestivum*’ and ‘*T. uncinatum*’ specimens neither corresponded to spore reticulum height, nor to any other morphological, chemical or ecological character studied. Although there could be problems connected with using ITS for species recognition (Álvarez & Wendel 2003), the absence of support for a separation between the two names, does not implicate any such problems in this case. I conclude that as traditionally used, *T. aestivum* and *T. uncinatum* are synonyms and
the species should be named _T. aestivum_, since the oldest name has priority according to the International Code of Botanical Nomenclature (Greuter 2000). Since both names are commonly used, a conservation of _T. uncinatum_ versus _T. aestivum_ is unwarranted. With a strict application of the Code, the name _T. albidum_ Fries should have priority over _T. aestivum_, but since _T. albidum_ has not been used for 100 years, its conservation is not likely (Trappe 2001). As this concept of _T. aestivum_ could initially confuse the truffle market, the double name ‘_T. aestivum_ syn. _T. uncinatum_’ could be used for mature fruit bodies, until conformity has been reached.

**_Tuber aestivum_ populations (Paper II)**

A distinct island population

I wanted to investigate if _T. aestivum_ on Gotland constituted a distinct population, separate from other European specimens. If a distinct population existed, this could be the result of an adaptation to local soil and climate, and of great importance for successful local cultivation efforts. The variation in such a limited geographical area would also help to illuminate the dispersal abilities of _T. aestivum_.

Genetic structure and homogeneity of the population was studied using principal component analysis (PCA) and maximum parsimony (MP) analysis of randomly amplified polymorphic DNA (RAPD) data.

The results of my RAPD analyses of 26 _T. aestivum_ fruit bodies from 21 sites on the island of Gotland, and four fruit bodies from France, England and Denmark, showed that _T. aestivum_ on Gotland forms a distinct population of closely related subpopulations (Figure 5 and 6).
Figure 5. The phylogenetic tree (Maximum Parsimony) based on randomly amplified polymorphic DNA (RAPD) data of *Tuber aestivum* samples from Gotland (Sweden), Denmark, Britain and France. The 27 Gotland samples (T1-T13, T15-T22, T28-T33) form a clade, while the two Danish samples (T23, T24), the British sample (T25) and the French sample (T26) are at least as different from each other as they are to the Gotland clade.
Figure 6. The Principal Component Analysis (PCA) graph based on the randomly amplified polymorphic DNA (RAPD) analysis of *Tuber aestivum* samples from Gotland (Sweden), Denmark, Britain and France (computer edited version). The Eigen values were 10.884 and 6.994 for axis one and two respectively. The percentage of variance of axis one and two were 17.276 and 11.101 respectively. The 27 Gotland samples (T1-T13, T15-T22, T28-T33: triangles) cluster together, while the two Danish samples (T23, T24), the British sample (T25) and the French sample (T26) (filled circles) are at least as different from each other as they are from the Gotland cluster.

**Truffles and sex**

The life cycle of *Tuber aestivum* is complex and to a large extent still unknown. Due to the impossibility to mate species of the *Tuber* genus under controlled conditions, the mating systems within this genus are still unknown. Sexual ascospores are formed in the *Tuber* fruit bodies, the asccarps, commonly called truffles. The haploid ascospores contain five chromosomes and nuclei of the diploid vegetative mycelia of the fruit body have ten chromosomes (Poma *et al.* 1998). Sexual reproduction could be either homothallic or heterothallic, or a mix of the two (secondary homothallism) (Webster 1980). In heterothallic ascomycetes, two haploid mycelia from two spores of opposite mating type fuse to form a heterokaryotic mycelium, followed by nuclear fusion, meiotic division and ascospore formation. In homothallic fungi, a mycelium originating from a single spore can form a dikaryotic mycelium by selfing. Fusion of the dikaryon into a homokaryotic diploid is followed by meiosis and ascospore formation. Secondary homothallism is when a heterothallic fungus appears to be homothallic, by forming binucleate ascospores which can germinate into a fertile mycelium, because they contain different mating types (Webster 1980).
The genetic variation observed in my molecular study of the *T. aestivum* population of Gotland (Paper II) indicates a heterothallic sexual reproduction, since pairwise comparisons of my RAPD data matrix obtained from 26 *T. aestivum* fruit bodies from 21 sites of Gotland resulted in a normal distribution of genotypes (Figure 7). The normal distribution indicates a randomly mating population, *i.e.* heterothallism. Homothallic reproduction would give rise to subpopulations with very little intragenetic variation, but with larger intergenetic variation, theoretically forming separate individual peaks, rather than a normal distribution. A similar assumption was made by Redecker *et al.* (2001) in their amplified fragment length polymorphism (AFLP) study on basidiomycetes. Paolocci *et al.* (2004) argue that *T. aestivum* is homothallic based on the perceived homozgyosity of ITS alleles. ITS is however known to undergo concerted evolution, *i.e.* a nonindependant evolution of duplicated genes towards conformity (Teshima & Innan 2004), and consequently homothallism cannot be assumed on the basis of ITS homozgyosity.

![Figure 7](image.png)

**Figure 7.** Pair-wise comparisons of random amplified polymorphic DNA (RAPD) data from 26 *Tuber aestivum* fruit bodies from 21 sites on Gotland, Sweden. The normal distribution indicates sexual reproduction within Gotland.

**How truffles attract vectors**

At full spore maturity, truffles emit many volatile aldehyde and sulphuric compounds that attract vectors for spore dispersal (Bellina-Agostinone *et al.* 1987, Pacioni *et al.* 1990, Pacioni *et al.* 1991a, Bellesia *et al.* 1998, Diaz *et al.* 2003). The truffle aroma consists of many components and descriptions diverge. Only in this project, the scent has been accredited by different people to smell like ‘dark chocolate and black olives’, ‘hazelnut’, ‘green mould
cheese’, ‘beestings’ (the first milk drawn from a cow after calving), ‘chocolate and strawberries’ and ‘newborn calf’. Sunhede (1978) described the scent as similar to boiled maize. Pigs, as natural vectors, have long been used in searching for truffles. It was thought that the ability of pigs to detect truffles depended on the presence of a steroid in the aroma, similar to a sexual hormone in boars (Giovanetti et al. 1994), but there are strong indications that the substance recognised by both mammals and insects in fact is thiobismethane (Talou et al. 1990).

An electric truffle detector capable of detecting thiobismethane has been developed, but trained dogs are still superior (Giovanetti et al. 1994) and most truffle hunters today, including myself, use trained truffle dogs (Rocchia 1992, Chevalier & Frochot 1997), which has proved extremely successful since dogs generally have no interest in eating the truffles, as opposed to pigs.

Vectors and populations
The question of how hypogeous fungi without active spore dispersal can colonize islands is intriguing. The hypogeous life form of truffles could have evolved to better withstand e.g. destruction by weather changes and predation prior to full spore maturity. On the other hand there is no active spore dispersal and the spread of spores depends entirely on vectors finding the fruit bodies, unearthing them, eating them and spreading them through their faeces (Trappe & Castellano 1991, Trappe et al. 2001). Since Gotland is an island, 90 km off the Swedish and 130 km off the Estonian shore, the surrounding Baltic Sea acts as a natural barrier for spore dispersal. Gotland has not been connected to the mainland since it rose from the sea after the last ice age, 11 600 years ago (Fredén 1998). Icing over of the Baltic Sea is rare, but could occur during extremely cold winters, when any remaining truffles would be deep frozen in the ground and animals would be reluctant to move long distances due to the energy loss. In the case of Tuber-truffles, mammals such as mice, squirrels and wild boars are the known vectors (Redshaw 1982, Miller 1985, Trappe & Castellano 1991). Mice and squirrels could explain the dispersal of T. aestivum within Gotland, but the mode by which T. aestivum and other hypogeous fungi are introduced to Gotland is still unknown.

Hydnophagous insects are specialised on truffles. The only invertebrate with a truffle associating Swedish trivial name is the lamellicorn beetle, Odontes armiger (tryffeldyvel; Coleoptera), suspected to eat e.g. Scleroderma sp. (Forshage 2000), but it has not been found in association to T. aestivum. The mycelium beetle Leiodes cinnamomea attacks fruit bodies of both T. melanosporum and T. aestivum, while the fly Suilla gigantea, the truffle fly, lays its eggs close to mature fruit bodies, which supply a food source for the larvae. In the field, these insects may be regarded as indicators
of the presence of mature truffles (Delmas 1978, Pacioni et al. 1991b, Rocchia 1992, Chevalier & Frochot 1997). *Leiodes cinnamomea* has been found in Denmark but not in Sweden (Mats Jonsell, SLU, Uppsala, pers. comm.). *Suillia gigantea* has not been found either, but other flies of the genus *Suillia* (Heleomyzidae), and also *Pegomya* (Antheromyiidae), have been used to spot mature truffles on Gotland.

I supervised a pilot study where we set up insect traps with a piece of mature *T. aestivum* fruit body as bait. A beetle of the Corylophidae family was captured and showed to have *T. aestivum* spores within its gastrointestinal system. Flying insects such as this beetle, may not be able to cover the long distance from the mainland to Gotland by themselves, but may sometimes be transported longer distances by wind drift and could hence be responsible for the dispersal of truffle spores over large water barriers.

There has not been any intentional dispersal of *T. aestivum* by humans on the island, since there has not existed a tradition of collecting, cooking or cultivating *T. aestivum* in Sweden, but unintentional introduction through soil, roots and tools is still possible. There are no records of *T. aestivum* being found on Gotland before 1977 (Sunhede 1978), but the abundance and distribution of *T. aestivum* on Gotland indicate a long presence on the island (Paper III). It is possible that the present population was established from one introduction, which might be the result of the pioneer’s ability to survive in this habitat rather than due to rare colonising events. The distinct *T. aestivum* population on Gotland might be an ecotype adapted to the island’s climate and/or soil conditions.

**The habitat of *T. aestivum* (Paper III)**

**Soil and climate of *T. aestivum* sites on Gotland**

To test the hypothesis that the genetically distinct *Tuber aestivum* population on the island of Gotland, Sweden (Paper II), is adapted to habitats different from French *T. aestivum* populations, I investigated the soil structure, soil chemistry, bedrock, climate, vegetation and host tree continuity of 18 *T. aestivum* sites on Gotland.

Table 1. *Overview of the soil composition at 18 studied Tuber aestivum sites on Gotland, Sweden.*
| Site identification | A-2 | B-T5 | C-T10 | D-T9 | E-T8 | F-T11 | G-T1 | H-T6 | I-T7 | J-T20 | K-T19 | L-T16 | M-T17 | N-T18 | O-T13 | P-T15 | Q-T21 | R-T22 |
|---------------------|-----|------|-------|------|------|-------|------|------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Soil particle size fractions (%) | < 2 μm (clay) | 21.4 | 15.0 | 22.4 | no data | 10.4 | 32.6 | 23.1 | 14.2 | 16.4 | 18.3 | 15.8 | 22.0 | 22.9 | 18.1 | 18.1 | 21.4 | 16.8 | 18.5 |
|                      | 2.20 μm (fine silt) | 14.7 | 16.2 | 49.0 | no data | 6.9 | 23.1 | 18.4 | 7.1 | 12.5 | 13.1 | 16.6 | 20.4 | 18.5 | 19.5 | 16.9 | 16.6 | 15.3 | 13.2 |
|                      | 20-50 μm (coarse silt) | 5.4 | 10.8 | 15.7 | no data | 2.9 | 9.5 | 8.8 | 4.6 | 6.4 | 7.2 | 8.2 | 10.4 | 7.2 | 5.4 | 11.4 | 4.3 | 6.8 | 4.2 |
|                      | 50-200 μm (fine sand) | 11.6 | 29.6 | 6.2 | no data | 9.3 | 16.9 | 22.8 | 20.6 | 42.1 | 17.7 | 28.9 | 26.7 | 19.4 | 13.3 | 25.0 | 10.9 | 20.4 | 8.1 |
|                      | 200-2000 μm (coarse sand) | 46.9 | 28.4 | 6.7 | no data | 70.5 | 17.9 | 26.9 | 53.5 | 21.6 | 43.7 | 30.5 | 20.5 | 32.0 | 43.7 | 28.6 | 46.8 | 40.7 | 56.0 |
| Water pH | 7.0 | 6.8 | 7.1 | 7.4 | 7.6 | 7.1 | 7.2 | 7.6 | 7.9 | 7.8 | 7.6 | 7.9 | 7.7 | 7.6 | 7.4 | 7.6 | 7.5 | 7.7 |
| Total lime stone (CaCO₃) [%] | 0.2 | 0.1 | 0.2 | 0.2 | 0.2 | 1.7 | 0.6 | 0.6 | 0.9 | 8.2 | 8.0 | 1.7 | 2.0 | 10.5 | 6.3 | 0.3 | 4.1 | 0.6 | 7.5 |
| Assimilable phosphorus (P₂O₅) [%] | 0.0161 | 0.0228 | 0.0100 | 0.0044 | 0.0064 | 0.0028 | 0.0039 | 0.0053 | 0.0068 | 0.0062 | 0.0048 | 0.0324 | 0.1174 | 0.0142 | 0.0038 | 0.0844 | 0.0016 | 0.0054 |
| Exchangeable calcium (Ca) [%] | 0.356 | 0.382 | 0.443 | 0.422 | 0.563 | 0.604 | 0.588 | 0.641 | 0.991 | 0.848 | 0.832 | 0.612 | 0.818 | 0.927 | 0.519 | 0.829 | 0.664 | 1.071 |
| Exchangeable magnesium (Mg) [%] | 0.0286 | 0.0233 | 0.0166 | 0.0091 | 0.0142 | 0.0186 | 0.0330 | 0.0130 | 0.0233 | 0.0206 | 0.0140 | 0.0129 | 0.0142 | 0.0137 | 0.0180 | 0.0451 | 0.0100 | 0.0178 |
| Exchangeable potassium (K) [%] | 0.0627 | 0.0276 | 0.0127 | 0.0112 | 0.0127 | 0.0331 | 0.0109 | 0.0102 | 0.0348 | 0.0086 | 0.0076 | 0.0309 | 0.0565 | 0.0145 | 0.0077 | 0.0187 | 0.0081 | 0.0160 |
| Ca/Mg | 12.4 | 16.4 | 26.7 | 46.4 | 39.6 | 32.5 | 17.8 | 49.3 | 42.5 | 41.2 | 59.4 | 47.4 | 57.6 | 67.7 | 28.8 | 18.4 | 66.4 | 60.2 |
| K/Mg | 2.2 | 1.2 | 0.8 | 1.2 | 0.9 | 1.8 | 0.3 | 0.8 | 1.5 | 0.4 | 0.5 | 2.4 | 4.0 | 1.1 | 0.4 | 0.4 | 0.8 | 0.9 |
|                      | Organic nitrogen [%] | 0.483 | 0.422 | 0.371 | 0.264 | 0.551 | 0.488 | 0.549 | 0.756 | 0.789 | 0.497 | 0.766 | 0.270 | 0.434 | 1.060 | 0.357 | 0.500 | 0.405 | 0.800 |
Soil data were compared with a similar investigation of 25 *T. aestivum* sites in four French regions (Chevalier & Frochot 1997) (Table 2). Even though the levels of different elements differed between Swedish and French soils, I could show that there were no striking functional differences due to similar ratios between these elements (Table 2).

The French and Swedish truffle populations both occupy land with high pH (7-8) soils and low phosphorus concentrations (0.002-0.080%). The only exception was the Swedish site B-T5 where the pH was 6.8 and M-T17 where there was a high phosphorus concentration of 0.12% (Table 1). The texture of the soil samples from the Swedish *T. aestivum* sites was silty to sandy, while French soils were more clayey (Figure 8). Due to the broad range in soil particle size in both Swedish and French sites, I conclude that *T. aestivum* has a wide soil texture tolerance and the differences in the sites simply reflected regional geology.

Table 2. A soil composition comparison between Swedish (Table 1) and French *T. aestivum* syn. *T. uncinatum* sites, presenting the mean values, standard deviations and ranges. French data originate from Chevalier and Frochot 1997.

<table>
<thead>
<tr>
<th>Measured parameter</th>
<th>Mean value ± standard deviation</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sweden</td>
<td>France</td>
</tr>
<tr>
<td>Clay &lt; 2 μm %</td>
<td>19.3 ± 4.9</td>
<td>34.1 ± 11.1</td>
</tr>
<tr>
<td>Silt 2-50 μm %</td>
<td>25.1 ± 11.9</td>
<td>48.3 ± 11.6</td>
</tr>
<tr>
<td>Sand 50-2000 μm %</td>
<td>55.6 ± 14.9</td>
<td>17.5 ± 16.1</td>
</tr>
<tr>
<td>Water pH</td>
<td>7.5 ± 0.3</td>
<td>7.6 ± 0.2</td>
</tr>
<tr>
<td>CaCO₃ (total) %</td>
<td>3.0 ± 3.5</td>
<td>16.7 ± 17.2</td>
</tr>
<tr>
<td>Exchangeable calcium* (Ca) %</td>
<td>0.67 ± 0.21</td>
<td>0.51 ± 0.14</td>
</tr>
<tr>
<td>Assimilable phosphorus (P₂O₅) %</td>
<td>0.020 ± 0.032</td>
<td>0.009 ± 0.017</td>
</tr>
<tr>
<td>Exchangeable magnesium* (Mg) %</td>
<td>0.019 ± 0.009</td>
<td>0.017 ± 0.011</td>
</tr>
<tr>
<td>Exchangeable potassium* (K) %</td>
<td>0.023 ± 0.017</td>
<td>0.059 ± 0.022</td>
</tr>
<tr>
<td>Ca/Mg ratio</td>
<td>40.6 ± 17.3</td>
<td>58.5 ± 31.6</td>
</tr>
<tr>
<td>K/Mg ratio</td>
<td>12.2 ± 0.9</td>
<td>4.5 ± 2.4</td>
</tr>
<tr>
<td>Organic matter %</td>
<td>11.9 ± 4.5</td>
<td>9.7 ± 4.2</td>
</tr>
<tr>
<td>Organic carbon %</td>
<td>6.9 ± 2.6</td>
<td>5.6 ± 2.4</td>
</tr>
<tr>
<td>Organic nitrogen %</td>
<td>0.54 ± 0.21</td>
<td>0.46 ± 0.14</td>
</tr>
<tr>
<td>C/N ratio</td>
<td>13.0 ± 2.2</td>
<td>11.9 ± 2.6</td>
</tr>
</tbody>
</table>

Unlike French *T. aestivum* soils, which always respond to the hydrochloric acid (HCl) test indicating CaCO₃ (Chevalier & Frochot 1997), some *T. aestivum* soils from Gotland failed to react indicating a lack of CaCO₃, a finding supported by our analyses (Table 1). However, the fraction of assimilable calcium was often much higher in the Swedish than in the French soils (Table 2). The Ca/Mg ratios were therefore of interest, since calcium
and magnesium compete for the same positions on soil colloids. This means that sites with low calcium levels in the soil may still be good sites for *T. aestivum*, providing that the magnesium and potassium levels are low and the pH is sufficiently high (Table 1), although both the Swedish and French *T. aestivum* soil showed great variation in this regard (Table 2).

*Figure 8.* Diagram of soil texture of 25 *T. aestivum* sites in Eastern France (numbers 1-25) (Chevalier & Frochot 1997) and of 17 *Tuber aestivum* sites on Gotland, Sweden (numbers 26–42 in italics). C = clayey, Si = silty, Sa = sandy, f = fine, vf = very fine. Illustration by Créations Philippe Toumire, France.
The comparatively high potassium levels in the French soils are unlikely to indicate different habitat preferences, since it is rather the K/Mg ratio, which is interesting biologically, i.e. a K/Mg ratio over 2 affects the plant uptake of magnesium negatively (Eriksson et al. 1997). According to both our Swedish data and the French data, *T. aestivum* can be found in soils with a K/Mg ratio well over 2, with a rather high variation both in Sweden and France (Table 2).

The Swedish and French C/N ratios are also similar. For agricultural land, a C/N ratio below 10 is known to be an indication that the soil has been N-fertilised (Eriksson et al. 1997). Only one Swedish soil sample (Table 1, site H-T6, C/N = 9.73) and two out of the 25 French soil samples (Chevalier & Frochot 1997) had a C/N ratio below 10, indicating that *T. aestivum* prefers soils which are poor in readily degradable nitrogen. This is well in accordance with the findings of Wallander (1992), that high nitrogen contents in the soil can act harmfully on mycorrhizas.

I therefore conclude that the French and Swedish soils are functionally similar, but may differ diagnostically for individual parameters, a finding that is essential when prospecting for new natural truffle sites or locations suited to the establishment of truffle orchards.

### Table 3. Monthly mean precipitation and temperature, and the duration of the *Tuber aestivum* fruit body season, on Gotland, Sweden and in Burgundy, France.

<table>
<thead>
<tr>
<th>Month</th>
<th>Gotland [mm]</th>
<th>Burgundy</th>
<th>Gotland [°C]</th>
<th>Burgundy</th>
<th>Gotland</th>
<th>Burgundy</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>43.3</td>
<td>73.6</td>
<td>-1.1</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>February</td>
<td>32.6</td>
<td>69.3</td>
<td>-1.8</td>
<td>3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>March</td>
<td>34.6</td>
<td>69.3</td>
<td>0.1</td>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>April</td>
<td>29.3</td>
<td>60.4</td>
<td>4.0</td>
<td>9.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>May</td>
<td>28.0</td>
<td>81.9</td>
<td>9.6</td>
<td>13.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>June</td>
<td>39.5</td>
<td>75.9</td>
<td>14.3</td>
<td>16.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>July</td>
<td>51.6</td>
<td>65.4</td>
<td>16.3</td>
<td>18.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>August</td>
<td>48.5</td>
<td>66.4</td>
<td>15.9</td>
<td>18.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>September</td>
<td>58.6</td>
<td>72.0</td>
<td>12.1</td>
<td>15.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>October</td>
<td>50.5</td>
<td>80.0</td>
<td>8.1</td>
<td>10.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>November</td>
<td>57.5</td>
<td>83.2</td>
<td>3.9</td>
<td>5.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>December</td>
<td>55.5</td>
<td>87.1</td>
<td>0.7</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
If the *T. aestivum* population on Gotland constitutes an ecotype it is rather an adaptation to the colder and drier climate on Gotland (Table 3). Selecting local *T. aestivum* inoculum for truffle orchards in Northern Europe could be important for successful truffle production (Trappe 1977, Bencivenga & Granetti 1988).

Vegetation and characterisation of *T. aestivum* sites

*Tuber aestivum* forms ectomycorrhiza foremost with deciduous hardwoods and hazel (Chevalier 1979, Zambonelli & Branzanti 1984, Chevalier & Frochot 1997). No *T. aestivum* indicator plants other than the host trees were found, but the host tree continuity on the *T. aestivum* sites on Gotland was more than 300 years. All *T. aestivum* sites on Gotland had a mixture of both oak (*Q. robur*) and hazel (*C. avellana*) (Figure 9), but the proportion between the two varied. In Denmark and England it has also been found together with lime (*Tilia* sp.) and beech (*Fagus sylvatica*) (Pegler et al. 1993, Lange 1994).

In a typical *T. aestivum* site on Gotland (Figure 9), there are both oak (*Q. robur*) and hazel (*C. avellana*) with an interspersion of hawthorn bushes (*Crataegus* spp.). The pH in the soil of a typical *T. aestivum* site is 7.5, with a Ca/Mg ratio of 25-55, a K/Mg ratio of 0.3-2.1, a level of organic matter around 12%, a C/N ratio of 13 and with a silty-sandy soil structure. The ground is only very sparsely vegetated with vascular plants and there is substantial shading of the ground by the trees, growing a few meters apart. This shaded, but still open character of the sites, is due to either canopy closure of e.g. old ashes (*Fraxinus excelsior*), or grazing in addition to some traditional meadow management. Mature fruit bodies of *T. aestivum* are at these sites mainly found during October to November.

Most *T. aestivum* sites on Gotland have been covered by semi-open deciduous forest meadows for more than 300 years (Paper III), which is why *T. aestivum* could be favoured by a stable environmental conditions and a long continuity of host plants. But it is also possible that it is indifferent to host tree age and continuity and is found there simply because these are the only available oak/hazel habitats. Oak trees were historically not favoured by farmers, as the foliage was unsuitable as cattle fodder. From 1647 the farmers were restricted by Swedish law from cutting down oak trees or removing oak seedlings, as oaks constituted potential ship construction wood for the state. This law was lifted in 1830, after which many oaks were cut down without being replaced. In a comparison of data from 1700 and 2000, there has been a 97% decline in deciduous forest meadows on Gotland (Croneborg 2001, Länsstyrelsen i Gotlands län 2002).
Many of the volatile substances that have been identified from the fruit bodies are also emitted by the vegetative mycelium (Pacioni 1991). A function appears to be to inhibit competitive forms of life in the surrounding soil volume, the hydnosphere (Fasolo Bonfante et al. 1971, Pacioni 1991). Plattner and Hall (1995) have reported that the mycelium of *T. melanosporum* can act as a parasite on weeds, causing necrosis of the root cortices. This inhibitory effect of *T. melanosporum* causes the area around the tree with *T. melanosporum* mycorrhiza to look burnt, hence the term truffle burn or brulé. This effect is less pronounced for *T. aestivum* mycorrhiza (Pacioni 1991). One reason why *T. aestivum* is mostly found at sites with very sparse ground vegetation (Paper III), could be the weed inhibiting effect of the shading conditions (Chevalier & Frochot 1997).

Even though *T. aestivum* might have been favoured by the increased shading from trees in deciduous forest meadows when the old management of cutting the twigs ceased, it is clearly disfavoured when the ground is densely overgrown with weeds and brushwood (Paper III). *Tuber aestivum* can act as a cultural and economic incentive for continuing old management practices of old deciduous meadows, thereby conserving high biological and cultural values. According to the Gotland County Administration, truffles, being under ground, are the landowner’s property, as opposed to epigeous fungi in Sweden. This means that a traditional management of meadows, allowing shaded parts, may also bring some economic return in the form of truffles. However, governmental support to open up meadows by cutting...
down trees and thinning hazel stands, only benefits vascular plant flora at the cost of some fungi, including threatened species like \textit{T. aestivum}. For the plant and fungal diversity as a whole, it is therefore better with a mosaic landscape, taking both fungal and plant communities into account.

**Truffle cultivation (Paper IV)**

**Truffle orchards in Sweden**

In June 1999, 10 \textit{T. aestivum} orchards of a total of 240 seedings, bought from the two French nurseries ROBIN pépinières and AGRI-TRUFFE, were initiated on Gotland. Each orchard consisted of 12 oak (\textit{Q. robur}) and 12 hazel (\textit{C. avellana}) seedlings. The seedlings and the \textit{T. aestivum} (syn. \textit{T. uncinatum}) inoculum used to produce the mycorrhiza, originated from Northern France and were chosen so as to endure the colder climate in Sweden as best as possible (Paper III). Seedlings were planted in 2-3 rows with 3-4 m between the seedlings within the rows, to enable fast colonisation of the soil by the truffle and root system, and enable future shade from the canopies (Olivier \textit{et al.} 1996, Chevalier & Frochot 1997, Figure 10).

![Figure 10. One of the experimental truffle orchards (2A) of hazel (Corylus avellana) and oak (Quercus robur) established in 1999. The ground around the trees is covered by a plastic weave to suppress weeds. The trees are protected against animals by sturdy net cages. The ground between the tree rows has been harrowed annually. Photograph taken in May 2004.](image-url)
Rows were planted 4-6 m apart, depending on the size of the machinery to be used for harrowing the soil. Weed control was executed at least 1 m around each seedling, either by covering the soil with straw, chipped bark or white plastic weave, or by manual weeding (Table 4, Figure 10). This strategy was tested since I wanted to avoid the use of herbicides, due to the large number of organic farmers on Gotland (15%). In between the rows, the soil was ploughed, harrowed or weeded manually, or being covered by regularly mowed grass.

In all 10 orchards, bucket or hose irrigation was possible and recommended in periods of drought during summer, especially the first months after plantation. The establishment of these experimental truffle orchards was done in order to study mycorrhizal development during a maximum number of seasons within the five-year project.

Table 4. Truffle orchards established in June 1999 on Gotland, Sweden, with 120 1.5 years old Quercus robur (ROBIN pépinières, France) and 120 1.5 years old Corylus avellana (AGRI-TRUFFE, France) seedlings with T. aestivum (syn. T. uncinatum) mycorrhiza. Seedling and truffle inoculum originated from Northern France. Each of the 10 truffle orchards consisted 12 Q. robur and 12 C. avellana seedlings. *Percentage surviving seedlings if the orchards 1A and 10A, where weed control was not executed, are excluded. aIn the orchard 5A and 6A, the plastic was removed the first year. bChipped bark was applied in <0.2 m thick layer, ineffective against weeds within two years, as was also the case with the straw. Some manual weeding and mowing the grass has been undertaken since.

<table>
<thead>
<tr>
<th>ID No</th>
<th>N surviving seedlings 1st yr</th>
<th>N surviving seedlings 2004</th>
<th>Seedling protection</th>
<th>Weed control 1 m around seedling</th>
<th>Main cause of seedling death</th>
<th>Height of majority of seedlings 2004</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>5 (21%)</td>
<td>0</td>
<td>None.</td>
<td>None</td>
<td>Competition from weeds</td>
<td>-</td>
</tr>
<tr>
<td>2A</td>
<td>24 (100%)</td>
<td>23 (96%)</td>
<td>Net cages</td>
<td>1/3 plastic, 1/3 bark &amp; 1/3 straw</td>
<td>Unknown</td>
<td>&gt;1.5 m</td>
</tr>
<tr>
<td>3A</td>
<td>23 (96%)</td>
<td>22 (92%)</td>
<td>Net cages</td>
<td>Manual weeding</td>
<td>Unknown</td>
<td>&gt;1.5 m</td>
</tr>
<tr>
<td>4A</td>
<td>21 (88%)</td>
<td>19 (79%)</td>
<td>None</td>
<td>1/3 plastic, 1/3 bark &amp; 1/3 straw</td>
<td>Unknown</td>
<td>&gt;1.5 m</td>
</tr>
<tr>
<td>5A</td>
<td>22 (92%)</td>
<td>20 (83%)</td>
<td>None</td>
<td>1/3 plastic, 1/3 bark &amp; 1/3 straw</td>
<td>Broken by plastic/wind</td>
<td>&gt;1.5 m</td>
</tr>
<tr>
<td>6A</td>
<td>21 (88%)</td>
<td>20 (83%)</td>
<td>Net cages</td>
<td>1/3 plastic, 1/3 bark &amp; 1/3 straw</td>
<td>Competition from weeds</td>
<td>&lt;0.5 m</td>
</tr>
<tr>
<td>7A</td>
<td>23 (96%)</td>
<td>21 (88%)</td>
<td>None</td>
<td>Manual weeding/mowed grass</td>
<td>Eaten by hares/rabbits</td>
<td>0.5-1.5 m</td>
</tr>
<tr>
<td>8A</td>
<td>24 (100%)</td>
<td>23 (96%)</td>
<td>None</td>
<td>1/3 plastic, 1/3 bark &amp; 1/3 straw</td>
<td>Competition from weeds</td>
<td>&gt;1.5 m</td>
</tr>
<tr>
<td>9A</td>
<td>24 (100%)</td>
<td>22 (92%)</td>
<td>Net cages</td>
<td>1/3 plastic, 1/3 bark &amp; 1/3 straw</td>
<td>None</td>
<td>1/5 m</td>
</tr>
<tr>
<td>10A</td>
<td>5 (21%)</td>
<td>0</td>
<td>None</td>
<td>None</td>
<td>Competition from weeds</td>
<td>-</td>
</tr>
</tbody>
</table>

192 (80%) (95%)* 170 (71%) (89%)*
I selected as varying sites as possible, covering different geographical locations on the island of Gotland, to enable a preliminary assessment of finding suitable soil and location types through the results of these experimental orchards. All soils were well drained (Chevalier & Frochot 1997) and had not been populated by trees for at least 20 years, to lower competition with other mycorrhizal fungi (Hall et al. 1994, Chevalier & Frochot 1997, Smith & Read 1997). All plants were financed by the truffle project, but owned and managed by the landowners. All orchards thus had the same batch of truffle seedlings and the same management manual, but different soils (local climate not studied) and different managers.

As a consequence of the truffle inventories (Wedén & Danell 1998, Wedén et al. 2001, Paper I, Paper II), interest for commercial production arose and in 2000 the company Cantharellus AB offered commercial truffle seedlings. Therefore another 3000 oak seedlings (Q. robur) were planted on Gotland by individual landowners in 2000 and 2001 (Table 5). These seedlings were produced by ROBIN pépinières, France, using T. aestivum inoculum from Gotland and acorns of Danish origin. Originally I tried to find Swedish acorns and contacted the largest seed supplier in Sweden (Sveaskog Frö & Plant AB). They failed to deliver indigenous seeds and the best compromise was to use Danish seeds instead. Fourteen truffle orchards were followed in my study (Table 5). One orchard was established after removing stems and roots of the existing trees, of mainly aspen (Populus sp.) and some birch (Betula sp.) and pine (Pinus sylvestris), a month prior to plantation. The remaining 13 orchards were planted on old agricultural or meadowland, without recent tree growth. Weeds around the seedlings were removed manually, by mulching with straw, by attaching cardboard discs (<0.4 m wide) around the bottom of the stems, or not at all (Table 5). Seedlings were protected against rabbits, hares or grazing animals, by net cages around each seedling, by fencing in the whole orchard, or not at all (Table 5). The area between the seedling rows, *i.e.* 1 m from the seedlings, was ploughed, harrowed, cultivated, grazed or covered with grass (free growing or mowed).

Root samples were taken yearly in all orchards. This was done in order to follow the development of the truffle orchard and to be able to take measures in the case of detecting a negative trend, *i.e.* little or no detection of mycorrhiza.
Table 5. Investigation of 14 commercial truffle orchards established during 2000-2001 on Gotland, Sweden, of in total 2149 Quercus robur (Danish acorn origin) inoculated with Tuber aestivum (origin Gotland) by ROBIN pépinières, France. Only truffle orchards where more than 10 seedlings had survived the first year were followed in this study.

<table>
<thead>
<tr>
<th>ID</th>
<th>N° seedlings planted</th>
<th>Plantation date</th>
<th>Seedling protection</th>
<th>Weed control 1 m around seedling</th>
<th>Height of majority of seedlings 2004</th>
</tr>
</thead>
<tbody>
<tr>
<td>11B</td>
<td>250</td>
<td>Oct 2000</td>
<td>Net cages</td>
<td>Some manual weeding. Cardboard discs 2002.</td>
<td>0.5-1.5 m</td>
</tr>
<tr>
<td>12B</td>
<td>200</td>
<td>Oct 2000</td>
<td>Net cages</td>
<td>Some manual weeding. Cardboard discs 2002.</td>
<td>0.5-1.5 m</td>
</tr>
<tr>
<td>16B</td>
<td>150</td>
<td>Oct 2000</td>
<td>Net cages</td>
<td>Manual weeding. Cardboard discs 2002.</td>
<td>0.5-1.5 m</td>
</tr>
<tr>
<td>17B</td>
<td>560</td>
<td>Dec 2001</td>
<td>Net fencing</td>
<td>None. Cardboard discs 2002.</td>
<td>&lt;0.5 m</td>
</tr>
<tr>
<td>18B</td>
<td>125</td>
<td>June 2001</td>
<td>Net fencing</td>
<td>Straw 0.3 m packed.</td>
<td>0.5-1.5 m</td>
</tr>
<tr>
<td>19B</td>
<td>154</td>
<td>May 2001</td>
<td>None</td>
<td>None.</td>
<td>&lt;0.5 m</td>
</tr>
<tr>
<td>20B</td>
<td>130</td>
<td>Oct 2000</td>
<td>Net fencing</td>
<td>None. Cardboard discs 2002.</td>
<td>&lt;0.5 m</td>
</tr>
<tr>
<td>21B</td>
<td>30</td>
<td>Oct 2000</td>
<td>Net cages</td>
<td>None.</td>
<td>&lt;0.5 m</td>
</tr>
<tr>
<td>22B</td>
<td>18</td>
<td>Oct 2000</td>
<td>Net fencing</td>
<td>None. Cardboard discs 2002.</td>
<td>&lt;0.5 m</td>
</tr>
<tr>
<td>23B</td>
<td>228</td>
<td>Oct 2000</td>
<td>Net cages</td>
<td>None/mowed grass. Cardboard discs 2002.</td>
<td>0.5-1.5 m</td>
</tr>
<tr>
<td>24B</td>
<td>72</td>
<td>Oct 2000</td>
<td>Net cages</td>
<td>None. Cardboard discs 2002.</td>
<td>0.5-1.5 m</td>
</tr>
</tbody>
</table>

All 22 orchards harboured T. aestivum mycorrhiza in 2004 (Table 6 and Table 7), and one experimental orchard showed so called truffle burns or brûlés in 2004 (Paper IV), a phenomenon caused by the T. aestivum mycelia. Tuber aestivum mycorrhiza survived even in orchards where soil parameters lie without the range of natural sites (Table 1 and Table 8), e.g. with high amounts of sand (95%), low pH (6.4) and low amounts of exchangeable Ca (0.1%).

Soil chemistry and texture range found in natural T. aestivum sites corresponded well with the perceived trends of mycorrhizal development in the T. aestivum orchards on Gotland, i.e. that the T. aestivum mycorrhiza seemed to disfavour soils with a sand content of > 85%. Only in one sandy orchard (9A), T. aestivum mycorrhiza was easily found during probing (Table 6). In this orchard the ground 2 m in diameter around the seedlings was covered by
plastic weave, chipped bark or straw at plantation, and irrigation has been practiced regularly during dry summer months. By more thorough investigation of the root system at one sandy site (15B), after failing to find mycorrhiza with my soil core probing method, a lot of *T. aestivum* mycorrhiza was found (Paper IV).

Interestingly this shows that the *T. aestivum* mycorrhiza can grow in soils, which differ in soil texture and pH from that found in natural sites, but conclusions of cultivation possibilities cannot be drawn until fruit bodies are found at these sites.

Table 6. Classification of mycorrhiza vitality judged by soil core probing in experimental truffle orchards on Gotland, established in 1999 with oak (*Quercus robur*) and hazel (*Corylus avellana*) inoculated with *Tuber aestivum* (origin Northern France). Classification is done in the following classes and are based on five arbitrarily taken root samples from each orchard 2004: + = presence of one or few *T. aestivum* mycorrhizae in 1-3/5 samples, or indicating a receding trend since establishment; ++ = presence of numerous *T. aestivum* mycorrhizae in 2-5/5 samples; +++ = presence of clusters of *T. aestivum* mycorrhizae with vigorous cystidia in 2-5/5 samples. C = clayey, Si = silty, Sa = sandy. *Table 8.

<table>
<thead>
<tr>
<th>ID No</th>
<th><em>T. aestivum</em> Mycorrhiza classification</th>
<th>pH*</th>
<th>Soil Texture Type*</th>
<th>Showing extreme differences from natural sites*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>+++</td>
<td>8.01</td>
<td>Si</td>
<td>No</td>
</tr>
<tr>
<td>3A</td>
<td>++</td>
<td>7.98</td>
<td>SiSa</td>
<td>No</td>
</tr>
<tr>
<td>4A</td>
<td>++</td>
<td>7.85</td>
<td>SiSa</td>
<td>No</td>
</tr>
<tr>
<td>5A</td>
<td>+++</td>
<td>8.18</td>
<td>SiC-Sa</td>
<td>No</td>
</tr>
<tr>
<td>6A</td>
<td>+++</td>
<td>7.94</td>
<td>SiSa</td>
<td>No</td>
</tr>
<tr>
<td>7A</td>
<td>++</td>
<td>7.83</td>
<td>SiSa</td>
<td>No</td>
</tr>
<tr>
<td>8A</td>
<td>++</td>
<td>7.30</td>
<td>SiSa</td>
<td>No</td>
</tr>
<tr>
<td>9A</td>
<td>++</td>
<td>7.30</td>
<td>SaSi</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 7. Classification of mycorrhiza vitality judged by soil core probing in commercial truffle orchards on Gotland, established in 2000-2001 with oak (Quercus robur of Danish origin) and Tuber aestivum inoculum from Gotland. Classification is done in the following classes and are based on five arbitrarily taken root samples from each orchard 2004: + = presence of one or few T. aestivum mycorrhizae in 1-3/5 samples, or indicating a receding trend since establishment; ++ = presence of numerous T. aestivum mycorrhizae in 2-5/5 samples; +++ = presence of clusters of T. aestivum mycorrhizae with vigorous cystidia in 2-5/5 samples. C = clayey, Si = silty, Sa = sandy. *Table 8.

<table>
<thead>
<tr>
<th>ID N°</th>
<th>T. aestivum mycorrhizal classification</th>
<th>PH*</th>
<th>Soil Texture Type*</th>
<th>Showing extreme differences from natural sites*</th>
</tr>
</thead>
<tbody>
<tr>
<td>11B</td>
<td>+</td>
<td>7.11</td>
<td>Sa</td>
<td>Yes</td>
</tr>
<tr>
<td>12B</td>
<td>+++</td>
<td>8.20</td>
<td>Si</td>
<td>No</td>
</tr>
<tr>
<td>13B</td>
<td>++</td>
<td>6.36</td>
<td>Sa</td>
<td>Yes</td>
</tr>
<tr>
<td>14B</td>
<td>++</td>
<td>7.20</td>
<td>Sa</td>
<td>Yes</td>
</tr>
<tr>
<td>15B</td>
<td>+</td>
<td>6.50</td>
<td>Sa</td>
<td>Yes</td>
</tr>
<tr>
<td>16B</td>
<td>++</td>
<td>8.00</td>
<td>SiSa</td>
<td>No</td>
</tr>
<tr>
<td>17B</td>
<td>+</td>
<td>6.41</td>
<td>Sa</td>
<td>Yes</td>
</tr>
<tr>
<td>18B</td>
<td>+++</td>
<td>7.80</td>
<td>SiC</td>
<td>No</td>
</tr>
<tr>
<td>19B</td>
<td>+++</td>
<td>7.59</td>
<td>SiSa</td>
<td>No</td>
</tr>
<tr>
<td>20B</td>
<td>+++</td>
<td>7.40</td>
<td>SiC-Sa</td>
<td>No</td>
</tr>
<tr>
<td>21B</td>
<td>++</td>
<td>8.00</td>
<td>SiSa</td>
<td>No</td>
</tr>
<tr>
<td>22B</td>
<td>+++</td>
<td>8.00</td>
<td>SiSa</td>
<td>No</td>
</tr>
<tr>
<td>23B</td>
<td>++</td>
<td>8.00</td>
<td>SiSa</td>
<td>No</td>
</tr>
<tr>
<td>24B</td>
<td>+</td>
<td>7.49</td>
<td>SaSi</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 8. Overview of the soil composition at eight experimental (2A-9A, established in 1999) and 14 commercial (11B-24B, established in 2000-2001) Tuber aestivum plantations on Gotland, Sweden. *Total nitrogen content was measured in these soil samples, instead of organic nitrogen content. C = clayey, Si = silty, Sa = sandy. Bold numbers indicate values falling below, and bold, italic numbers indicate values above the natural T. aestivum range on Gotland (Table 1): Soil texture values falling below or exceeding the range with >5% of the mean value in natural T. aestivum sites on Gotland; pH >0.2 out of natural range; all other parameters <half of lowest value or >two times the highest value of natural range. *Sum of fine and coarse silt. **Sum of coarse and fine sand.
<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil particle size fractions (%)</td>
<td>22.4</td>
<td>12.2</td>
<td>17.8</td>
<td>24.9</td>
<td>17.5</td>
<td>17.5</td>
<td>12.6</td>
<td>7.9</td>
<td>3.4</td>
<td>20.2</td>
<td>5.3</td>
<td>6.0</td>
<td>4.4</td>
<td>13.9</td>
<td>3.3</td>
<td>30.5</td>
<td>15.5</td>
<td>21.0</td>
<td>17.0</td>
<td>18.8</td>
<td>13.5</td>
<td>7.2</td>
<td>10.4-32.6</td>
</tr>
<tr>
<td>&lt; 2 µm (clay)</td>
<td>2.20 µm (fine silt)</td>
<td>25.0</td>
<td>11.5</td>
<td>12.0</td>
<td>17.4</td>
<td>12.7</td>
<td>15.0</td>
<td>9.7</td>
<td>4.9</td>
<td>0.6</td>
<td>25.0</td>
<td>2.3</td>
<td>2.9</td>
<td>2.2</td>
<td>11.9</td>
<td>0.8</td>
<td>21.3</td>
<td>15.6</td>
<td>16.6</td>
<td>13.3</td>
<td>15.4</td>
<td>8.6</td>
<td>3.7</td>
</tr>
<tr>
<td>20-50 µm (coarse silt)</td>
<td>15.0</td>
<td>3.5</td>
<td>8.2</td>
<td>8.6</td>
<td>7.4</td>
<td>11.2</td>
<td>5.3</td>
<td>2.6</td>
<td>1.2</td>
<td>13.2</td>
<td>1.5</td>
<td>1.9</td>
<td>2.0</td>
<td>10.5</td>
<td>1.7</td>
<td>5.5</td>
<td>8.8</td>
<td>8.2</td>
<td>6.4</td>
<td>8.3</td>
<td>2.3</td>
<td>1.9</td>
<td>9.8-64.7</td>
</tr>
<tr>
<td>50-200 µm (fine sand)</td>
<td>2.5</td>
<td>15.3</td>
<td>47.6</td>
<td>18.4</td>
<td>24.4</td>
<td>19.7</td>
<td>24.5</td>
<td>19.1</td>
<td>80.1</td>
<td>21.0</td>
<td>79.1</td>
<td>74.2</td>
<td>77.2</td>
<td>32.6</td>
<td>40.7</td>
<td>29.0</td>
<td>23.3</td>
<td>20.1</td>
<td>13.3</td>
<td>19.8</td>
<td>18.5</td>
<td>52.4</td>
<td></td>
</tr>
<tr>
<td>200-2000 µm (coarse sand)</td>
<td>35.1</td>
<td>57.5</td>
<td>14.4</td>
<td>30.7</td>
<td>38.0</td>
<td>36.5</td>
<td>47.9</td>
<td>65.5</td>
<td>14.5</td>
<td>19.6</td>
<td>11.8</td>
<td>15.0</td>
<td>14.2</td>
<td>31.1</td>
<td>53.5</td>
<td>13.5</td>
<td>32.8</td>
<td>34.1</td>
<td>50.1</td>
<td>37.7</td>
<td>57.1</td>
<td>34.8</td>
<td>12.9-79.8</td>
</tr>
<tr>
<td>Soil texture type</td>
<td>S1</td>
<td>S1S1a</td>
<td>S1S5a</td>
<td>S1C</td>
<td>S1S5a</td>
<td>S1S5a</td>
<td>S1S5a</td>
<td>Sa</td>
<td>Sa</td>
<td>S1</td>
<td>S5a</td>
<td>S1</td>
<td>S1S5a</td>
<td>S1C</td>
<td>S1S5a</td>
<td>S1S5a</td>
<td>S1S5a</td>
<td>S1S5a</td>
<td>S1S5a</td>
<td>Sa</td>
<td>S1S5a</td>
<td>S1S1a</td>
<td>S1S5a</td>
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<tr>
<td>Water pH</td>
<td>8.01</td>
<td>7.98</td>
<td>7.85</td>
<td>8.18</td>
<td>7.94</td>
<td>7.83</td>
<td>7.3</td>
<td>7.3</td>
<td>7.11</td>
<td>8.2</td>
<td>6.36</td>
<td>7.2</td>
<td>6.50</td>
<td>8.00</td>
<td>6.41</td>
<td>7.8</td>
<td>7.59</td>
<td>7.40</td>
<td>8.00</td>
<td>8.00</td>
<td>7.49</td>
<td>6.8-7.9</td>
<td></td>
</tr>
<tr>
<td>Total lime (CaCO₃) [%]</td>
<td>22.3</td>
<td>2.380</td>
<td>0.691</td>
<td>4.170</td>
<td>5.49</td>
<td>5.68</td>
<td>0.400</td>
<td>0.200</td>
<td>0.122</td>
<td>27.50</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>2.060</td>
<td>0.100</td>
<td>1.900</td>
<td>0.327</td>
<td>0.600</td>
<td>12.90</td>
<td>16.6</td>
<td>5.500</td>
<td>0.143</td>
<td>0.1-10.5</td>
</tr>
<tr>
<td>Available phosphorus (P₂O₅) [%]</td>
<td>0.016</td>
<td>0.018</td>
<td>0.012</td>
<td>0.075</td>
<td>0.025</td>
<td>0.045</td>
<td>0.032</td>
<td>0.024</td>
<td>0.012</td>
<td>0.013</td>
<td>0.009</td>
<td>0.006</td>
<td>0.002</td>
<td>0.014</td>
<td>0.007</td>
<td>0.009</td>
<td>0.036</td>
<td>0.003</td>
<td>0.008</td>
<td>0.014</td>
<td>0.004</td>
<td>0.002</td>
<td>0.002-0.120</td>
</tr>
<tr>
<td>Exchangeable calcium (Ca) [%]</td>
<td>0.689</td>
<td>0.581</td>
<td>0.409</td>
<td>0.944</td>
<td>0.623</td>
<td>0.684</td>
<td>0.245</td>
<td>0.198</td>
<td>0.155</td>
<td>0.693</td>
<td>0.255</td>
<td>0.320</td>
<td>0.113</td>
<td>0.438</td>
<td>0.186</td>
<td>0.724</td>
<td>0.279</td>
<td>0.642</td>
<td>0.941</td>
<td>0.818</td>
<td>0.724</td>
<td>0.266</td>
<td>0.36-1.07</td>
</tr>
<tr>
<td>Exchangeable magnesium (Mg) [%]</td>
<td>0.026</td>
<td>0.005</td>
<td>0.006</td>
<td>0.015</td>
<td>0.006</td>
<td>0.010</td>
<td>0.005</td>
<td>0.004</td>
<td>0.004</td>
<td>0.013</td>
<td>0.014</td>
<td>0.024</td>
<td>0.004</td>
<td>0.004</td>
<td>0.005</td>
<td>0.019</td>
<td>0.008</td>
<td>0.008</td>
<td>0.013</td>
<td>0.006</td>
<td>0.004</td>
<td>0.009</td>
<td>0.0045</td>
</tr>
<tr>
<td>Exchangeable potassium (K) (K₂O) [%]</td>
<td>0.032</td>
<td>0.008</td>
<td>0.007</td>
<td>0.029</td>
<td>0.019</td>
<td>0.023</td>
<td>0.013</td>
<td>0.012</td>
<td>0.006</td>
<td>0.003</td>
<td>0.009</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.007</td>
<td>0.012</td>
<td>0.004</td>
<td>0.003</td>
<td>0.008-0.063</td>
</tr>
<tr>
<td>C/Na</td>
<td>0.85</td>
<td>1.60</td>
<td>1.23</td>
<td>1.91</td>
<td>3.05</td>
<td>2.16</td>
<td>2.68</td>
<td>2.79</td>
<td>1.59</td>
<td>0.46</td>
<td>0.24</td>
<td>0.38</td>
<td>0.92</td>
<td>1.66</td>
<td>0.53</td>
<td>1.22</td>
<td>1.79</td>
<td>0.96</td>
<td>0.89</td>
<td>0.32</td>
<td>0.60</td>
<td>0.3-4.0</td>
<td></td>
</tr>
<tr>
<td>Carbon and nitrogen</td>
<td>3.46</td>
<td>3.13</td>
<td>1.96</td>
<td>3.38</td>
<td>2.74</td>
<td>4.9</td>
<td>1.449</td>
<td>2.032</td>
<td>1.92</td>
<td>4.937</td>
<td>4.27</td>
<td>5.07</td>
<td>1.57</td>
<td>2.1</td>
<td>2.96</td>
<td>6.274</td>
<td>2.29</td>
<td>4.824</td>
<td>5.952</td>
<td>5.065</td>
<td>3.603</td>
<td>2.6</td>
<td>3.5-12.3</td>
</tr>
<tr>
<td>Organic matter [%]</td>
<td>0.361</td>
<td>0.230</td>
<td>0.170</td>
<td>0.298</td>
<td>0.239</td>
<td>0.461</td>
<td>0.141</td>
<td>0.180</td>
<td>0.142</td>
<td>0.532</td>
<td>0.29</td>
<td>0.335</td>
<td>0.145</td>
<td>0.168</td>
<td>0.225</td>
<td>0.646</td>
<td>0.171</td>
<td>0.345</td>
<td>0.496</td>
<td>0.526</td>
<td>0.335</td>
<td>0.213</td>
<td>0.3-1.1</td>
</tr>
</tbody>
</table>
The main causes of seedling death were competition from weeds and destruction by animals. In two of the experimental orchards (1A and 10A) the majority of seedlings died during the first year because of competition for light and water from weeds (Table 4). Two commercial orchards (not included in my study) were eliminated by hares within weeks of plantation. Keeping the area >1 m around the trees weed free was of great importance for seedling growth (Table 4 and table 5). In all experimental orchards where ground coverage (straw, chipped bark or plastic weave) or manual weeding was executed, the majority of the seedlings had grown to be >1.5 m high in 2004 (Table 4). In the remaining two orchards, weeds were removed directly around the seedlings, and the grass was mowed, which had helped seedling survival but lead to slow growth. In the commercial orchards, the highest seedling growth was observed in the manually weeded orchards (Table 5). The cardboard discs used by many of the commercial growers were too small and hence ineffective for suppressing weeds (Paper IV). Manually removing weeds or covering the ground >1 m around the seedlings is thus recommended, as well as choosing sites with low weed pressure (Paper IV).

The downy mildew (*Microsphaera alphitoides*), which may stunt the growth of younger seedlings (Chevalier & Frochot 1997), has been observed on oaks in all orchards. Careful application of a sulphur fungicide (Kumulus DF) to the leaves has been practiced in one orchard (3A) and has not resulted in noticeable damage on the truffle mycorrhiza according to our probing procedure (Table 6).

In October 2004, *T. rufum* (red truffle) was found in four (3A, 5A, 7A and 9A) of the eight experimental orchards, which means that the seedlings are now large enough to support fruit body production (Paper IV). This small species is native to Gotland.

The implication of the survival of *T. aestivum* in all plantations is not only that *T. aestivum* production on Gotland in planted orchards is probable in the future, but also that *T. aestivum* does not necessarily need the long host tree continuity observed in natural sites on Gotland. That *T. aestivum* has been found in many places with old trees and long host tree continuity is a promising result for future long term production in truffle orchards on Gotland.

Survival of the first inoculated seedlings in Sweden

Formation of mycorrhiza in green house experiments has been reported for several *Tuber* species, but is not uncomplicated (Hall *et al.* 2003). Large truffle seedling producers, like ROBIN pépinières (France) and Crop & Food Research (New Zealand) guard their successful production methods well. On hand experience seemed to be the only way to attain knowledge of the importance of details in the procedure.

In 2000, a pilot study was set up to test if it was possible to produce truffle seedlings in a Swedish nursery. Potted, 1.5 years old seedlings of Swed-
ish oak (*Q. robur*), hazel (*C. avellana*) and hornbeam (*Carpinus betulus*) were inoculated with spores of *T. aestivum* from Gotland in 2000. The seedlings were dormant when the experiment was set up and their roots had grown very little into the inoculated substrate after the first 4.5 months. As a consequence, little *T. aestivum* mycorrhiza had formed after 4.5 months (Table 9). After 11 months, *T. aestivum* mycorrhiza had formed on 34% of the seedlings (Table 9, Figure 11).

The seedlings with *T. aestivum* mycorrhiza were planted on Gotland in 2001. In 2004, *T. aestivum* mycorrhiza was still present on all three tree species and they can thus be recommended, in pure or mixed stands, for future truffle orchard establishment in Sweden. Local tree seed and local *T. aestivum* inoculum is recommended because of their adaption to local habitats (Paper III).

**Figure 11.** An ectomycorrhiza of *Tuber aestivum*. The short root is embedded in truffle mycelia, giving it an inflated appearance, and the mycelia also grow in between the outer root cells, where the nutrient exchange takes place between the fungus and the tree. Some of the for *T. aestivum* characteristic curly hyphae (cystidia) can be seen radiating from the mycorrhizal tip. Photo: Lina Pettersson.

Based on our results (Table 9), I recommend using sterilised natural soil of similar character to that found at natural *T. aestivum* sites (Table 1) and inoculating with 1 g frozen fruit body (5x10^6 mature spores) per seedling. Seedlings should germinate in sterilised soil, to avoid competition from other mycorrhizal fungi. Seedlings should be repotted in inoculated substrate in early spring and be checked for *T. aestivum* mycorrhiza after growing in the inoculated substrate for at least eight months. I did not study at which seedling age it was suitable to inoculate, but for economical reasons seedlings
should be 2-3 months of age. At that stage healthy seedlings can be transferred to inoculated soil, not wasting valuable truffle inoculum on bad seedlings. Raising seedlings for a longer period of time would also be costly, if that would lead to having the seedlings in the nursery for two seasons instead of one. Defining a suitable artificial substrate and further investigating the lowest amount of truffle inoculum needed per seedling, as well as the minimum amount of time needed for good mycorrhizal development, is important to facilitate future truffle seedling production. With the results from this inoculation experiment (Paper IV), a local co-operation with greenhouse production of seedlings inoculated with *T. aestivum* spores has been initiated using seeds and spores of local origin and heat sterilized natural soil of pH>7.

Table 9. *Number of seedlings which had formed Tuber aestivum mycorrhiza after 4.5 months and after 11 months from inoculation by potting in substrates containing 5x10^5, 5x10^6 or 5x10^7 T. aestivum spores/seedling. Time=time from inoculation. MTOS=Modified truffle orchard soil, i.e. unsterilised soil taken from a field adjacent to site E-T8 in Table 1. Numbers in brackets=number of seedlings with *T. aestivum* mycorrhiza out of total number of checked seedlings. Quercus robur: Broken asci=asci broken to liberate the ascospores prior to inoculation. Carpinus betulus: Dipped roots=Seedling root system dipped in a slurry of *T. aestivum* spores and water prior to potting in inoculated MTOS. Corylus avellana. "Remaining nine seedlings in batch dead. " Remaining one seedling in batch dead. " Remaining two seedlings in batch dead."

<table>
<thead>
<tr>
<th>Quercus robur</th>
<th>Nº of spores/pot; Time</th>
<th>5x10^5; 4.5 months</th>
<th>5x10^5; 11 months</th>
<th>5x10^6; 4.5 months</th>
<th>5x10^6; 11 months</th>
<th>5x10^7; 11 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peat:perlite</td>
<td>0% (0 of 5)</td>
<td>0% (0 of 6)</td>
<td>0% (0 of 5)</td>
<td>33% (5 of 15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTOS</td>
<td>0% (0 of 5)</td>
<td>40% (6 of 15)</td>
<td>0% (0 of 5)</td>
<td>67% (10 of 15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTOS + broken asci</td>
<td>20% (2 of 10)</td>
<td>53% (8 of 15)</td>
<td>20% (2 of 10)</td>
<td>53% (8 of 15)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carpinus betulus</th>
<th>Nº of spores/pot; Time</th>
<th>5x10^5; 11 months</th>
<th>5x10^5; 11 months</th>
<th>5x10^6; 11 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peat:perlite</td>
<td>0% (0 of 10)</td>
<td>20% (2 of 10)</td>
<td>50% (5 of 10)</td>
<td></td>
</tr>
<tr>
<td>MTOS</td>
<td>20% (2 of 10)</td>
<td>10% (1 of 10)</td>
<td>78% (7 of 9)</td>
<td></td>
</tr>
<tr>
<td>MTOS + dipped roots</td>
<td>50% (5 of 10)</td>
<td>20% (2 of 10)</td>
<td>50% (4 of 8)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Corylus avellana</th>
<th>Nº of spores/pot; Time</th>
<th>5x10^5; 11 months</th>
<th>5x10^5; 11 months</th>
<th>5x10^6; 11 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peat:perlite</td>
<td>0% (0 of 9)</td>
<td>0% (0 of 10)</td>
<td>11% (1 of 9)</td>
<td></td>
</tr>
<tr>
<td>MTOS</td>
<td>20% (2 of 10)</td>
<td>38% (3 of 8)</td>
<td>44% (4 of 9)</td>
<td></td>
</tr>
</tbody>
</table>

Planting seedlings in orchards is recommended in autumn. A lot of irrigation during the first months after plantation in June was needed to assure seedling survival in the experimental orchards established in 1999. Commercial seedlings planted 2000-2001, as well as the seedlings from our inoculation experiment, were mostly planted in the end of October-November, which was successful (Paper IV).
A crucial factor for future truffle production from inoculated seedlings is also that adequate mycorrhizal checking of the seedlings is executed, to ensure that the seedlings used to establish truffle orchards are well colonised by *T. aestivum* and devoid of mycorrhiza from other fungi (Fischer & Colinas 1996).

**Testing of ecotypes**

Development and survival ability of inoculated seedlings of French seed and spore origin is studied in the eight experimental truffle orchards on Gotland, established in 1999 (Paper IV). Five years (2004) after plantation mycorrhizal development was good (Paper IV), but it is possible that both the French seedlings and *T. aestivum* mycelia would be disfavoured by e.g. an extremely cold winter in Sweden. To test if *T. aestivum* from Gotland is an ecotype better adapted to the colder and drier climate on Gotland (Paper III), it will be interesting to compare the development of the French strains in the experimental orchards, with Swedish strains in commercial orchards. Seedlings inoculated with *T. aestivum* from Gotland have also been planted in USA (Missouri) (Pruett et al. 2004), New Zealand (North Canterbury, South Island) and France (Champagne). The seedlings planted in Champagne in 2001 grew poorly, but had excellent *T. aestivum* mycorrhiza when checked in 2004 (Gérard Chevalier, INRA, France, pers. comm. 2004).

**Impact on society**

The impact of my research on society should be documented to facilitate future socioeconomic studies (Samils et al. 2003). At present (2004) there are 25 truffle orchards on Gotland. A truffle cultivation association (Gotlands tryffelodlarförening) has been formed and one truffle company has been established (Gotlandstryffel). The first Swedish truffle dog, Alice (Golden Retriever), was trained in 1999. Today there are ten Swedish truffle dogs and truffle dog training classes have been given. In 2001, a blind test of Swedish and French truffles was made in Périgueux, France, conducted by the head of L’écomusée de la truffe in Sorges, Dominique Delange. The blind test was organized together with the society Måltidsakademin Vänner. Five Swedish and five French *T. aestivum* (syn. *T. uncinatum*) truffles, picked on the same date and of approximately the same size, were compared. Four truffles were classified as top quality, two were Swedish and two were French. Swedish truffles have been exported to France (2002), New Zealand, USA, England and Denmark. The initial market price suggested by the research project in 1999, was 2000 SEK/kg. For some years it was hard to get that price, but in 2004 the demand had increased. The price to pickers for wild growing *T. aestivum* was about 3500 SEK, and e.g. NK and Hötorgs-
hallen in Stockholm sold Gotland truffles for 7900 and 8400 SEK. Processing of truffles is now also being studied at LivsTek, Gotland (Centre for practical food technology).
Concluding remarks

The objectives of this thesis were to investigate which species of black *Tuber* truffles exist in Sweden, if the Gotland *T. aestivum* was a genetically distinct population, to characterise the Swedish biotopes to investigate possible adaptations and to establish cultivation of the Burgundy truffle, *T. aestivum*, in Sweden.

I have shown that there does not exist any support for separating *Tuber aestivum* Vitt. and *T. uncinatum* Chat., as traditionally used, into two taxa. They are to be regarded as synonyms. The name *T. aestivum* should be used, since it is the oldest name and thus has priority according to the Botanical Code. Not to confuse the market, the name *T. aestivum* syn. *T. uncinatum* may be used until conformity is reached.

I have shown that *T. aestivum* is widely distributed in suitable habitats on Gotland. The number of reported *T. aestivum* sites has been increased from three to 31. I reported a new species for Sweden, the edible black truffle *T. mesentericum*. *Tuber mesentericum* was found at ten sites. Many other truffle species in Sweden might have been overlooked. By the use of trained truffle dogs, the knowledge of the distribution of hypogeous fungi can be increased significantly.

The Gotland population of *T. aestivum* is genetically distinct as compared to other European specimens and may constitute an ecotype. Although different in individual parameters, soils in natural *T. aestivum* sites on Gotland are functionally similar to those of natural *T. aestivum* sites in France. The population of *T. aestivum* on Gotland is likely to be an adaptation to the colder and drier climate on Gotland.

There are at present 25 truffle orchards on Gotland established in 1999-2003, with a total of about 2500 trees. In 2004, *T. aestivum* mycorrhiza was still present in all of the 22 truffle orchards followed in this project. *Tuber aestivum* mycorrhiza has survived even in orchards where soil parameters outside the range of natural sites. In a pilot study using standard nursery plants and nursery equipment, *T. aestivum* mycorrhiza was successfully produced on 1-2 years old seedlings of Swedish oak (*Q. robur*), hazel (*C. avellana*) and hornbeam (*C. betulus*) by inoculation with spores of Swedish *T. aestivum*.

It is probable that fruit bodies, *i.e.* truffles, of *T. aestivum*, will occur in the truffle orchards on Gotland. Through this research project, I hope to have contributed to the local economy, to have strengthened a formerly endan-
gered species by restoration and new knowledge, and created an economical incentive for oak plantation.

The success of the truffle research project may lead to more research on edible and pharmaceutical macrofungi in Sweden, which today is extremely marginalized. Future challenges lie in further investigating ecology and cultivation of *T. mesentericum*, to study the role of insects for spore dispersal of truffles, to further study the genetic structure of *T. aestivum* populations on Gotland and other islands in order to investigate possible adaptations to local conditions, dispersal and sexual reproduction. A new challenge would also be to develop a cultivation technique for the aromatic hypogeous fungus *Choiromyces venosus*, which occur naturally in Southern Sweden, and to infer its taxonomic relationship with the truffles of the *Tuber* genus. The nomenclature of many truffles is still largely uninvestigated and many names are in need of conservation. In the future it would be interesting to launch a socioeconomic study of the impact on truffles on the Gotland community.

*Figure 12.* I trained my own truffle dog Biscuit (Welsh Springer Spaniel), here with her Gotland born truffle dog colleagues Alice (Golden Retriever) and Lizzy (Lagotto Romagnolo). Gotland, November 2003.
Svensk sammanfattning

Vad är en tryffel?
Bourgognetryffel (Fig. 1), även kallad sommartryffel, är en svamp som bildar underjordiska fruktbroprar, s.k. tryfflar. Tryfflar som tillhör sporsäcksvampsläktet *Tuber*, brukar kallas ädeltryfflar. Flera av dessa ädeltryfflar är p.g.a. sin arom eftertraktade delikatesser och är därför ekonomiskt viktiga. Bland dessa finns de svarta tryfflarna bourgognetryffel (*T. aestivum*), périgordtryffel (*T. melanosporum*), vintertryffel (*T. brumale*), samt de vita tryfflarna albatryffel/piemontesertryffel (*T. magnatum*) och ’bianchetto’ (*T. borchii*).

Bourgognetryffeln i Sverige

Varför forska om tryffel i Sverige?
Syftet med forskningsprojektet var att öka kunskapen om en hotart, skapa ett nytt incitament för att plantera ekar och bevara ekbiotoper med hög biodiversitet, och bidra till att utveckla en ny närings i en glesbygd. Forskningsprojektet finansierades av Gotlands kommun, Länsstyrelsen i Gotlands län och EG:s strukturfonder Mål 2-Öarna och Mål 5B, med målsättningen att kunskap om tryffel och tryffelodling skulle etableras på Gotland.
Att finna tryffel

Tryffeln saknar förmåga att aktivt sprida sina sporer (Fig. 2) och avger istället en stark doft vid spormognad. Ofta attraherar djur som bökar upp tryffeln, åter och sprider dess sporer med sin spillning. För att hitta tryffeln använde man sig förr av grisar, som är naturliga spridare av tryffel. Då grisar åter tryffel har man övergått till betydligt mer lätthanterliga hundar, som tränas att söka tryffel. Höstarna 1999 och 2000 inventerade jag tryffel på Gotland med hjälp av två franska tryffelhundar och deras förare (Fig. 9). Genom denna för Sverige nya metod har antalet rapporterade tryffelställen på Gotland ökat från tre till 31. Tryffelställen rapporteras till markägaren och till Artdatabanken, men publiceras ej, av hänsyn till markägare och omgivande flora som kan ta skada av ovarsam insamling.

Bagnolitryffel – en ny art för Sverige


Svart tryffel på Gotland (Artikel I)

hund inte kan användas då den omgna tryffeln är doftlös. En omgen tryffel avslutar sin mognadsprocess i och med att den tas ur marken.

En Gotlandsanpassad bourgognetryffel (Artiklarna II & III)
I ytterligare en DNA-analys har jag visat att de gotländska tryfflarna sins-
emellan är nära släkt, tydligt skilda från andra europeiska tryfflar av samma
art. Jag ville därför undersöka om det fanns fog för hypotesen att den got-
ländska tryffeln kunde vara speciellt anpassad till lokala klimat-
och/eller jordförhållanden. Genom analyser av jordprover från 18 naturliga tryffelstäl-
len på Gotland, kunde jag dra slutsatsen att jorden på Gotland var funktio-
nellt sett lik den i Frankrike, även om det fanns vissa skillnader (Tab. 2). 
Gotland utgör den nordligaste kända utposten för bourgognetryffel, med ett
både kallare och torrare klimat än i Mellaneuropa. Anpassningen har därför
förmodligen skett för att klara det kallare och torrare klimatet (Tab. 3). Med
jämna mellanrum infaller mycket kalla vintrar, då vissa individer slås ut.
Över tiden sker därmed ett naturligt urval. Man talar om ekotyper, d.v.s. en
getisk anpassning till en viss miljö.

Tryffelodling (Artikel IV)
Bourgognetryffel växer på rötter av bl.a. ek och hassel. För att odla tryffel
idag, producerar man trädplantor, vars rotsystem i växthus koloniserats av
tryffelns svamptrådar och bildat s.k. mykorrhiza (Gr. mucor=svamp och
rhizom=rot), eller svamprot. I juni 1999 etablerade jag 10 försöksodlingar
med totalt 240 ek- och hasselplantor med tryffelmykorrhiza inköpta från
Frankrike (Fig. 10). Anledning till att använda utländska plantor, och inte
vänta tills svenska planter kunnat produceras, var att under en så lång period
som möjligt under femårsprojektet kunna följa utvecklingen i odlingarna,
ona utarbeta lämpliga skötselinstruktioner. P.g.a. intresset som tryffel-
forskningsprojektet skapade, planterades 2000-2001 ytterligare 3000 ekplan-
tor av privatpersoner på Gotland. De var producerade i Frankrike, från dans-
ka ekollen (svenska var ej tillgängliga) och gotländsk tryffel.
Genom att årligen ta rotprov i experimentplanteringarna från 1999 och i
14 av de kommersiella planteringar, har jag kunnat bekräfta att tryffelmy-
korrhiza finns kvar i alla planteringar. Detta är lovande resultat, och innebär
att vi kanske kan sköra frioffset i planteringarna inom fem år. Det viktigaste
skötselkravet var att hålla ogräs borta.
En metod för att ta fram tryffelplantor i Sverige, med ek, hassel och aven-
bok är under utveckling och testas just nu på Gotland.
Positiva samhällseffekter


Tryffeln innebär en sporre att bevara gamla ängar och att nyplantera ek. Att ge bidrag till kraftiga gallringar av hässlen i ängen, kan visserligen främja kärlväxtfloran, men skada tryffeln och andra svampar, varför ett mosaiklandskap kanske ger störst biodiversitet. Jag hoppas att tryffelodlingar skall kunna bidra till att markägare kan bo kvar, och på så sätt bidra till att kulturlandskapets rika biodiversitet bevaras. Tryffelforskningsprojektets framgång kan leda till mer forskning på ätliga och medicinskt viktiga storsvampar. Denna forskning är idag mycket marginaliserad i Sverige, trots en världsmarknad på fem miljoner ton. Framtida utmaningar består bl.a. i att studera och utveckla odling av bl.a. bagnolitryffel och den på fastlandet växande stjärnhovstryffeln.
Acknowledgements

I would like to thank the head of the Department of Systematic Botany, Bengt Oxelman, for letting me complete my study here. It has been a very rewarding experience.

My supervisor, Eric Danell, first introduced me to truffles in 1997. Your brilliant original idea about truffle cultivation on Gotland and your ability to launch this project, have given me the opportunity to work with and learn from you. Your scientific enthusiasm is very catchy and I thank you for sharing your vast mycological knowledge, and for believing in me.

I thank my supervisor, Leif Tibell, for sharing his systematic knowledge and for welcoming me into the lichen research group. I have very much enjoyed the opportunity to work with and learn from you.

My Gotland supervisor, Bertil Widbom, has contributed importantly by handling the project finances and for valuable discussions and great company during field trips.

I have had the good fortune of having one of the leading truffle researchers, Gérard Chevalier, as my supervisor. I would like to thank you very much for contributing with valuable expertise and your extensive contact net of truffle researchers and truffle growers. I have enjoyed working with you very much, and you and Chantal Dupré have made me feel very welcome during my visits in France.

Many people have made very valuable contributions to my project, and the space here will not be enough to thank you all. I hope you know this and that I get an opportunity to thank you in person.

My truffle research on Gotland was given a great start with the very appreciated help of Olle Persson, Elsa Bohus Jensen and Eva Pettersson. I am very grateful for all your help and I have really enjoyed sharing truffle experiences with you over the years.

Rector Gunhild Beckman made this project possible by her swift and resolute action, enabling a co-operation with Gotland University College. I am very grateful to you, and I have also enjoyed very much being an employee at Gotland University College. I would like to thank all my colleagues at Gotland, for always having provided such a welcoming environment during my visits.

I would like to thank all my colleagues and friends at Forest Mycology and Pathology (SLU), for help, advise and great company during my first years as a PhD student. I would also like to thank my new colleagues at Sys-
tematic Botany and Systematic Zoology, for taking me in and making me feel so welcome. You have also helped me greatly in my systematic studies and have generally just been so much fun to be around!

Michel Jalade, Marcelle and Pierre Poinsot, and their truffle dogs Everest and Olympe, helped me to make important truffle inventories and greatly widen our knowledge of the *T. aestivum* distribution on Gotland. Thank you for all the happy memories of truffle exploring on Gotland and in Burgundy. Ingvar Jakobsson helped me find suitable sites for the experimental truffle orchards. I thank you for sharing your field trial experience and for the many fruitful discussions!

I am very grateful to Hjalmar Croneborg at the Swedish Threatened Species Unit, for sharing his knowledge on forest meadows on Gotland and for giving me a lot of help with historical maps. Stellan Hedgren, Karin Wägström and Magnus Martinsson at the Gotland County Administration, have facilitated my work and always been very helpful. I would also like to acknowledge Tommy Knutsson and Sigvard Svensson for helping me with truffle inventories on Öland and in Skåne.

I thank Francisco Camacho and Anders Backlund for contributing to my work and Ian Hall, Françoise Beaucamp and the Danish truffle group with Christian Lange, Ole Pedersen and Iben Thomsen, for valuable discussions on truffle cultivation.

Local mushroom consultants, Kerstin Gahne and Åke Edwinsson, landowners and truffle orchard owners on Gotland, are thanked for contributing to the project. I would like to extend special thanks to the eight owners of my experimental orchards, for managing these pioneer truffle orchards on Gotland so well.

Lina Pettersson, Jonas Johansson, Nicklas Samils and Per Tjernby, it has been great working with you and you have made important contributions to the truffle project, through your studies of inoculation, powdery mildew, socioeconomy and insect vectors!

Lars Hedström is greatly acknowledged for determining the insects found in association with truffles.

I thank Carl-Jan Granqvist for arranging a great opportunity to test the Gotland truffle in an exhilarating blind test in Périgueux.

Mary Cole and Grechen Pruett are acknowledged for their very valuable comments on my thesis and manuscripts. Mary also introduced me to fungi, for which I am very glad. Ola Lundström kindly processed the graphs and tables for this thesis.

My project was financed by the Municipality of Gotland, the European Agriculture Guidance and Guarantee Fund under the Objective 5b Gotland Programme, the European Regional Development Fund under the Objective 2 Islands Programme, the Gotland County Administration, and further supported by Konsul Faxes Fund, the KK Foundation (Kunskaps- och kompe-
tensutvecklingsstiftelsen), Carl the XVI Gustafs 50th Anniversary Fund and Carl Tryggers Foundation. I am very grateful for your support.

I would like to thank my friends and my family. I am very lucky to have you. My parents, Elisabeth Rudbeck Wedén and Anders Wedén, have always offered me great support and advice. My grandmother Anna-Brita Rudbeck has generously let me, and many truffle researchers from all over the world, use her cottage on Gotland. And most of all I would like to thank my husband, Göran Ulväng, for all your support.


Lobelius, M. (1581) *Plantarum Seu Stirpium Icones. I.*


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