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Ecology of *Armillaria cepistipes*: population structure, niches, pathogenicity and interactions with *Armillaria ostoyae*

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SIMONE PROSPERO

Dipl. Forst. Ing. ETH
Swiss Federal Institute of Technology Zurich
born 05.10.1972
citizen of Malvaglia, TI

accepted on the recommendation of

Prof. Dr. Ottmar Holdenrieder, examiner
Prof. Dr. Wolfgang Osswald, co-examiner
Dr. Daniel Rigling, co-examiner

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to my parents

Table of contents

List of tables

List of figures

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Abstract

Root and butt rot fungi are important agents of disturbance in forest ecosystems worldwide and they can also cause significant economic losses. The basidiomycete genus *Armillaria* is a major component of the wood-decaying mycoflora. *Armillaria* species differ in geographical distribution as well as in ecological behaviour. In coniferous forests in central Europe, the preferentially saprotrophic *A. cepistipes*, which is probably the most common *Armillaria* species, frequently co-exists sympatrically with the pathogenic *A. ostoyae*. Understanding the interaction between saprotrophic and pathogenic species of *Armillaria* will provide important information about the opportunities for using the former to control the latter. The aim of this dissertation was to investigate the ecology of *A. cepistipes* with special emphasis on its population structure, primary resource capture, virulence, and interaction with *A. ostoyae*.

The spatial population structures of both *Armillaria* species were investigated by intensively sampling the soil and the stumps in three 1 ha plots (Ludiano, Lurengo, and Dalpe) established in managed Norway spruce (*Picea abies*) stands in the Swiss Alps. Two to six genets of each species were identified in each plot. Genets of the same species rarely overlapped, indicating a mutual spatial exclusion. On parts of the investigated areas, interspecific overlaps were rare, which suggests a competitive interaction between the two species. However, on other parts, genets of *A. cepistipes* and *A. ostoyae* overlapped widely, indicating a more neutralistic co-existence.

The primary capture of stump resources by *A. cepistipes* and *A. ostoyae* was investigated both in the field and in pot experiments. For the field investigation, the incidence of stump colonisation by *Armillaria* species and genets was determined in Ludiano, Lurengo, and Dalpe. The study showed that *A. cepistipes* and *A. ostoyae* are efficient colonisers of fresh stumps, which represent therefore an important resource for the survival of the two species. In both *Armillaria* species, the newly created stumps are captured through the network of rhizomorphs present in the soil. Stumps located along interspecific boundaries were preferentially colonised by *A. ostoyae*, indicating a better competitive ability of this species in primary stump capture. This conclusion was confirmed by an experimental study in which the two *Armillaria* species were either simultaneously or sequentially (*A. cepistipes* prior to *A. ostoyae*) inoculated in pots containing a Norway spruce seedling. The subsequent rhizomorph production as well as the colonisation of the stump were then investigated. This experiment also confirmed that both *A. cepistipes* and *A. ostoyae* efficiently colonise stumps

when inoculated alone. The mutual ability in stump colonisation was, however, reduced by the presence of the other species, indicating that competition occurred for the stump resources. At the level of rhizomorphs in the soil, evidence for a more neutralistic association between the two species was found, with *A. cepistipes* exhibiting a better ability to produce rhizomorphs than *A. ostoyae*.

The virulence of 20 isolates of *A. cepistipes* and 16 isolates of *A. ostoyae* on two-year-old Norway spruce seedlings of four provenances was determined in an inoculation experiment. Within 30 months after inoculation, all *A. cepistipes* isolates showed a low virulence indicated by little host mortality or infection. In contrast, there was a considerable variation in virulence among the isolates of *A. ostoyae*, ranging from highly to weakly virulent. One seedling provenance showed a significantly lower susceptibility to *A. ostoyae* than the other three provenances, which could be due to specific genetic traits. Both *Armillaria* species were able to attach rhizomorphs to the root surface. However, *A. ostoyae* caused significantly more lesions than *A. cepistipes*, suggesting it is better able to penetrate non-wounded roots.

The study indicates that *A. cepistipes* and *A. ostoyae* are ecologically very similar in managed Norway spruce forests. The most relevant differences are the better ability of *A. cepistipes* to produce rhizomorphs and the higher virulence of *A. ostoyae*. The ecological similarity suggests that competition occurs between the two species. Considering all the results, the outcome of the competitive interaction will rather be a dynamic equilibrium of co-existence than the replacement of one species by the other.

Riassunto

I funghi che causano marciumi radicali sono agenti di disturbo di notevole importanza negli ecosistemi forestali di tutto il mondo. Essi possono provocare importanti perdite economiche nei boschi di produzione e possono compromettere la stabilità dei boschi di protezione. Il basidiomicete *Armillaria* è uno dei principali funghi responsabili della decomposizione del legno. Si conoscono diverse specie di *Armillaria*, che differiscono fra di loro sia quanto a distribuzione geografica che quanto a comportamento ecologico. *A. cepistipes*, a comportamento preferenzialmente saprofita, è probabilmente la specie più frequente nelle foreste di conifere dell'Europa centrale. Spesso, essa coesiste simpatricamente con *A. ostoyae*, una specie patogena primaria. Conoscenze sull'interazione fra specie di *Armillaria* saprofite e patogene possono fornire importanti indicazioni sul potenziale uso delle prime per il controllo biologico delle seconde. L'obiettivo della presente tesi è la comprensione delle caratteristiche ecologiche di *A. cepistipes*, ponendo una particolare attenzione alla struttura delle popolazioni, all'insediamento su ceppaie fresche (*primary resource capture*), alla virulenza e all'interazione con *A. ostoyae*.

La struttura spaziale di tre popolazioni delle due specie di *Armillaria* è stata studiata in tre aree di studio (Ludiano, Lurengo e Dalpe), di un ettaro di superficie ciascuna, situate in popolamenti di abete rosso (*Picea abies*) sottoposti a normale gestione selvicolturale nelle Alpi svizzere. Abbiamo campionato *Armillaria* sia nel suolo che sulle ceppaie. Da due a sei cloni di ogni specie sono stati identificati in ogni area di studio. Soltanto raramente l'area di distribuzione di diversi cloni della medesima specie si sovrapponeva, indicando quindi una reciproca esclusione spaziale. La rara sovrapposizione delle aree di distribuzione dei diversi cloni delle due specie in alcune zone delle aree di studio indica la presenza di fenomeni di competizione interspecifica. Tuttavia, in altre zone delle aree studiate, abbiamo trovato ampie zone di sovrapposizione nell'area di distribuzione dei diversi cloni di *A. cepistipes* e *A. ostoyae*, fatto che suggerisce una possibile coesistenza delle due specie con poca influenza reciproca.

L'insediamento di *A. cepistipes* e *A. ostoyae* su ceppaie fresche è stato investigato sia con studi *in situ* che con esperimenti di laboratorio in vaso. Per l'indagine *in situ*, nelle aree di Ludiano, Lurengo e Dalpe abbiamo determinato la frequenza di ceppaie colonizzate da cloni di *A. cepistipes* e *A. ostoyae*. Ambedue le specie di *Armillaria* colonizzano efficientemente le ceppaie fresche, le quali rappresentano di conseguenza un'importante risorsa per la

sopravvivenza di questi due funghi. *A. cepistipes* e *A. ostoyae* colonizzano le ceppaie per mezzo della rete di rizomorfe presente nel terreno. Le ceppaie situate lungo i limiti dell'area di distribuzione delle due specie vengono preferenzialmente colonizzate da *A. ostoyae* che sembra perciò avere una migliore abilità competitiva. Questa conclusione è stata confermata da un nostro esperimento in vaso, in cui piantine di abete rosso sono state inoculate con le due specie di *Armillaria*, simultaneamente o in due fasi successive (*A. cepistipes* prima e *A. ostoyae* dopo). Questo esperimento ha pure confermato che, se inoculate separatamente, sia *A. cepistipes* che *A. ostoyae* colonizzano le ceppaie. La capacità di colonizzare le ceppaie è tuttavia ridotta quando è presente anche l'altra specie, indicando l'esistenza di fenomeni di competizione interspecifica. Per quanto riguarda le rizomorfe nel terreno, sembra che le due specie coesistano in modo passivo. *A. cepistipes* è tuttavia caratterizzata da una migliore capacità di produrre rizomorfe rispetto a *A. ostoyae*.

Tramite un'inoculazione sperimentale è stata determinata la virulenza di 20 isolati di *A. cepistipes* e 16 isolati di *A. ostoyae* su piantine di abete rosso di due anni di età appartenenti a quattro diverse provenienze. Trenta mesi dopo l'inoculazione tutti gli isolati di *A. cepistipes* mostravano una bassa virulenza, indicata da una ridotta mortalità o infezione delle piantine ospiti. Al contrario, gli isolati di *A. ostoyae* presentavano una virulenza molto variabile. Alcuni di essi erano altamente virulenti mentre altri lo erano solo leggermente. Una provenienza di abete rosso mostrava una suscettibilità verso *A. ostoyae* significativamente inferiore rispetto alle altre tre provenienze. Ciò potrebbe essere dovuto a tratti genetici specifici. Ambedue le specie di *Armillaria* erano in grado di attaccare rizomorfe alla superficie delle radici. Ciononostante, *A. ostoyae* causava significativamente più lesioni che *A. cepistipes*, suggerendo una maggiore capacità di penetrazione in radici intatte.

La presente tesi indica che in foreste di abete rosso sottoposte a normale gestione selvicolturale *A. cepistipes* e *A. ostoyae* hanno un comportamento ecologico simile. Le differenze più rilevanti sono la miglior capacità di *A. cepistipes* di produrre rizomorfe e la maggior virulenza di *A. ostoyae*. L'affinità ecologica delle due specie suggerisce l'esistenza di fenomeni di competizione interspecifica. Considerando tutte le varie indicazioni scaturite dallo studio, molto probabilmente questi fenomeni di competizione fra le due specie sembrano condurre ad un equilibrio dinamico di coesistenza delle specie, piuttosto che alla sostituzione di una specie da parte dell'altra.

1

Main introduction, objectives, and main conclusions

1.1 Main introduction

1.1.1 The genus *Armillaria*

Taxonomy and nomenclature

In 1819, the Swedish mycologist Elias Fries established the taxon *Armillaria*, which included 12 species (Watling *et al.* 1991). The name *Armillaria* was based on the Latin word *armilla* (ring), referring to the annular veil present on the stipe (Pegler 2000). About forty years later, Staude raised *Armillaria* to generic rank (Burdsall & Volk 1993). After considerable debate, he is now generally accepted as the validating author of the genus (Watling *et al.* 1982).

At present, the basidiomycete genus *Armillaria*, whose taxonomic characteristics are described in Table 1.1, mainly comprises facultatively parasitic root and butt rot fungi, which produce rhizomorphs (Burdsall & Volk 1993). In current classifications, *Armillaria* is linked with the *Tricholomataceae*, a family of the *Agaricales*. However, there is no strong relationship between *Armillaria* and the other genera in the *Tricholomataceae* (Watling *et al.* 1991).

Table 1.1. Main morphological characteristics of fruiting bodies in the genus *Armillaria* (Watling *et al.* 1991).

Character	Description
Habit	Clitocyboid with slightly sinuate, adnexed, subdecurrent or decurrent gills; solitary, gregarious, or caespitose.
Pileus	Fleshy, thinning towards margin, expallant, hygrophanous; color variable yellow to brown; surface glabrous, scurfy, squamulose.
Stipe	Central, fibrous-fleshly, not characteristically cartilaginous.
Lamellae	Close to subdistant; moderately thick; sinuate; adnexed to deeply decurrent.
Flesh	Of pileus pale and of stipe white at first, becoming dark.
Spore-print	White to cream colour

Ecological behaviour

The genus *Armillaria* is an important natural component of the mycoflora of many forest ecosystems worldwide. All *Armillaria* species can survive saprotrophically on woody substrates, such as roots, debris, and stumps (Redfern & Filip 1991). They degrade all wood components, causing a white rot (Morrison *et al.* 1991a). Because of extensive and persistent substrate colonisation, *Armillaria* species contribute significantly to wood decomposition and mineral cycling (Kile *et al.* 1991). Except for some species that may be obligate saprotrophs, most *Armillaria* species also exhibit a parasitic capability and can be considered as facultative necrotrophs (Cooke & Whipps 1980, Termorshuizen 2000). Primary plant pathogenic species (e.g. *A. mellea*) are able to infect healthy and vigorous plants. Secondary pathogenic species (e.g. *A. gallica*), on the other hand, opportunistically infect plants which have already been weakened by abiotic factors (stress) or by other parasitic organisms.

On the basis of their ecological strategies, *Armillaria* species may be regarded as relatively K-selected organisms with long individual lifespans and low reproductive effort (Kile *et al.* 1991).

Life cycle

The specific structures involved in the life cycle of *Armillaria* are rhizomorphs, mycelium, pseudosclerotial tissue, fruiting bodies, and basidiospores.

Armillaria species typically produce highly differentiated, fully autonomous, and apically growing discrete filamentous aggregations named rhizomorphs (Garraway *et al.* 1991). Rhizomorphs consist of an external cortex, which contains melanin and protects the rhizomorph against colonisation by other fungi or bacteria, and a white inner layer (medulla), consisting of a lose network of hyphae, responsible for the water and nutrient transport.

Regarding form and function, subterranean (*Rhizomorpha subterranea*) and subcortical (*Rhizomorpha subcorticalis*) rhizomorphs can be distinguished. The root-like (*rhizo*-morph) subterranean rhizomorphs generally have a brown to black cortex and can be up to 5 mm in diameter. They grow in the upper soil layer (0-20 cm depth), where they sometimes form a large, monopodially or dichotomously branched network, which may persist over centuries (Smith *et al.* 1992). The vegetative extension of a thallus via rhizomorphs leads to the formation of clones with a definite spatial domain (Rizzo & Harrington 1993). Since these clones occupy discrete patches in the soil and are not repeatedly recovered from different

sites, they can be considered to represent genetic individuals or genets (Anderson & Kohn 1995). Subterranean rhizomorphs enable the absorption and translocation of nutrients and permit the extension of the fungus and an enhancement of the inoculum potential (Garraway *et al.* 1991, Fox 2000). Rhizomorphs, growing from a food base into a medium without nutrients, can advance over ten times faster than mycelial hyphae (Carlile 1995). When rhizomorphs come into contact with a root, they may attach themselves to the surface and cause an infection. At the point of attachment, hyphae (*Rhizomorpha fragilis*) emerge from the rhizomorph and penetrate the root-bark tissues (Solla *et al.* 2002).

After penetration of the root bark, the fungus spreads laterally and radially through the cambial region, forming the typical white mycelial fans. The cambium, as well as the xylem and the phloem of the roots, are destroyed and crown symptoms appear. These symptoms are, in approximate chronological order, reduction of shoot growth, changes in foliage characteristics, crown dieback, and finally death of the plant (Morrison *et al.* 1991). According to Morrison *et al.* (1991a), the host responds to an *Armillaria* infection by cork and callus formation (meristematic activity), by production of exudates (resin, gum), or by biochemical reactions. Sometimes these defences can restrict the infection to a single root. If heartwood is already present in such a root, the fungus may infect it and cause a white rot of the heartwood, which can extend above ground level (stem and butt rot). The presence of root lesions and heart rot reduces tree growth (Mallett & Volney 1999), wood quality and volume (Morrison *et al.* 1991a), and increases the risk of stem breakage (Kile *et al.* 1991). *Armillaria* infection can also occur following contact between a healthy root and an infected root (Fox 2000). This infection pathway is probably important for pathogenic species which produce less rhizomorphs, such as *A. mellea* (Guillaumin *et al.* 1989). After killing the host plant, *Armillaria*, as a necrotroph, can continue to use the colonised substrate as a food base. More or less flattened subcortical rhizomorphs develop from the margins of the mycelial fans, forming a dense network under the bark. The fungus penetrates into the wood and begins to degrade it. In infected wood, *Armillaria* typically forms pseudosclerotial plates, visible as dark zone-lines in cross-section (Fox 2000). They are crust-like aggregations of either highly branched and anastomosed or pseudoparenchymatous hyphae, surrounding the column of wood occupied by the fungus (Boddy 2000). The function of the pseudosclerotial plates is to maintain a favourable internal milieu and to resist resource capture by competing fungi (Rayner & Boddy 1988). The formation of pseudosclerotial plates is promoted by physical factors, the presence of antagonistic fungi (including different genets of the same species), and genetic factors (Garraway *et al.* 1991).

Armillaria fruiting bodies (basidiomes) often develop in groups, from the basis of infected trees, trunks, stumps, or on the ground near infected roots (Fox 2000). Fructification occurs irregularly in autumn, but not all species fructify during the same period (Marxmüller *et al.* 1990). There is no evidence, so far, that basidiospores are able to infect living roots (Redfern & Filip 1991). Similarly, the colonisation of the cut surface of fresh stumps seems to be an infrequent event (Rishbeth 1988). Nevertheless, the production of basidiospores provides a source of genetic diversity, facilitates the long-range spread of the fungus, and enables infection of forests established on former agricultural land (Redfern & Filip 1991).

Species identification

To date, about 40 species of *Armillaria* are known worldwide (Watling *et al.* 1991). Probably the number of species in the genus *Armillaria* is higher since detailed information about *Armillaria* in some areas (e.g. Africa and China) is still lacking. Some of the known species have a cosmopolitan character and occur in Europe, America, and Asia (e.g. *A. ostoyae* and *A. gallica*). Other species, on the other hand, are restricted to a specific geographical area, such as *A. gemina* in North America and *A. luteobubalina* in Australia.

Taxonomic species of organisms are traditionally identified using morphological characteristics. In *Armillaria* and other basidiomycetes, this method of species identification is difficult to apply because intraspecific variations in morphology can be considerable (Marxmüller & Holdenrieder 2000). Consequently, the concept of biological species indicating a group of individuals with a common gene pool is frequently applied in mycology. Biological species are identified with interfertility (mating) tests. In Europe, these tests have shown that the biological *Armillaria* species correspond to the morphological species. Beside morphology, the following two methods for identifying *Armillaria* species are used:

a) Interfertility tests

In basidiomycetes, a single basidiospore germinates into a homokaryotic mycelium (primary mycelium), which consists of haploid, monokaryotic cells. The primary mycelium may grow for an indefinite time in a substrate. In heterothallic species (self-sterile), sexually compatible haploid monokaryons anastomose with one another upon contact, resulting in a dikaryotic mycelium (secondary mycelium), which contains both parental nuclei separately in a single cell (Carlile 1995). In *Armillaria*, the two nuclei fuse (somatic nuclear fusion), forming a diploid mycelium with uninucleate cells (Guillaumin *et al.* 1991). Therefore, *Armillaria*, as

the only genus within the hymenomycetes, has a persistent and widespread diploid vegetative stage in the field.

In basidiomycetes, mating can also occur between a heterokaryotic and a homokaryotic mycelium (Buller phenomenon). In this case, the heterokaryon donates a nucleus to the homokaryon, which subsequently becomes heterokaryotic (Korhonen 1978). In *Armillaria*, an analogous process takes place. When an unknown diploid isolate (i.e. from rhizomorphs, mycelium, or fruiting bodies) is paired with a haploid isolate (i.e. from basidiospores) belonging to the same species, the latter isolate becomes diploid (diploidisation) and develops the macro-morphological characteristics of the former. Haploid *Armillaria* isolates generally produce a white and fluffy aerial mycelium. By contrast, diploid cultures are in most cases flat, crustose, and produce rhizomorphs or grow submerged in an agar medium (Guillaumin *et al.* 1991).

b) PCR-RFLP analysis of the ribosomal DNA

The nuclear rDNA sequences evolve slowly and are, therefore, particularly useful for studying distantly related organisms (Bruns *et al.* 1991). However, the evolutionary pressure for the conservation of the non-coding regions between the rDNA units is low. Therefore, sequences of these regions can exhibit high degrees of variability and are useful for studies at the level of species (Pérez Sierra *et al.* 2000).

Table 1.2. *Alu*I, *Nde*I, and *Bsm*I restriction fragments of the amplified IGS region of the rDNA of the five annulated European *Armillaria* species.

Species	Type[a]	PCR product	Fragment sizes (bp) after digestion with		
		(bp)	*Alu*I	*Nde*I	*Bsm*I
A. borealis	1	920	310, 200, 104	550, 370	920
	2	920	310, 200, 135	550, 370	920
A. cepistipes	1	920	399, 200, 183	920	920
	2	920	310, 200, 135	920	920
A. ostoyae		920	310, 200, 135	550, 370	620, 300
A. mellea	1	875	320, 155	875	600, 275
	2	875	320, 180, 155	875	600, 275
A. gallica		920	399, 240, 183	920	920

[a]According to Pérez Sierra *et al.* (1999).

For the identification of *Armillaria* species, a highly polymorphic portion of the intergenic spacer (IGS) region of the rDNA is used (Harrington & Wingfield 1995, Pérez Sierra *et al.* 1999). By means of the polymerase chain reaction (PCR), the target sequence is exponentially amplified. The PCR products are analysed for restriction fragment length polymorphism (RFLP) using restriction enzymes, which cleave the DNA molecule at the point where specific sequences are present. European species of *Armillaria* can be identified by using three different enzymes (*Alu*I, *Nde*I, and *Bsm*I). After separating the restriction DNA fragments by agarose gel electrophoresis, *Armillaria* species are identified by analysing the different restriction patterns and fragment sizes (Table 1.2).

General and economic impact on forestry

Armillaria species, as primary pathogens, can cause mortality and growth reduction in natural and planted forests, resulting in economic losses. Lethal primary disease has been reported in (i) boreal forests in North America and Scandinavia, (ii) mixed conifer forests in western North America, (iii) pine (*Pinus mugo* Turra var. *uncinata* Ram. and *Pinus sylvestris* L.) forests in the French Pyrénées, and (iv) dry sclerophyll eucalypt forests in Australia (Kile *et al.* 1991). Mortality due to *Armillaria* can affect seedlings as well as older trees both individually and in patches. For example, in Alberta (Canada), the annual average mortality in a lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm) regeneration was about 6% (Morrison & Mallett 1996). In a Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) plantation in British Columbia, Morrison & Pellow (1994) observed that 2% of the trees were killed annually by *A. ostoyae*. Primary disease is not necessarily lethal since trees affected by Armillaria root and butt rot often survive. In five plantations in Ontario, Whitney *et al.* (1989) found that an average of 58% of the apparently healthy coniferous trees were infected by *A. ostoyae*. The presence of butt rot reduces the growth and stability of the infected trees, as well as the timber quality and production (Morrison *et al.* 1991b, Mallett & Volney 1999). For example, in production forests in British Columbia, Armillaria root disease causes annual losses of 2-3 million m^3 (Morrison & Mallett 1996). In summary, in natural and planted forests, primary pathogenic *Armillaria* species significantly affect a forest economy by (i) reducing the merchantable volume of timber, (ii) reducing the wood quality, (iii) influencing the forest composition and structure, and thus (iv) influencing management activities (Hagle & Shaw 1991, Morrison & Mallett 1996).

1.1.2 *Armillaria* in Europe

History and species classification

Armillaria root disease has long been known as a serious problem of several coniferous and broadleaved tree species in Europe (Guillaumin *et al.* 1993). In 1874, Hartig was the first author to describe the disease, including the causal fungus *Agaricus* (*Armillaria*) *melleus*. However, the nomenclature and taxonomy of the genus *Armillaria* have been confused for over a century (Watling *et al.* 1991). Until the 1970's, all European annulated *Armillaria* species were commonly considered to be polymorphic members of the single species *Armillaria mellea* (*sensu latu*). In 1973, Hintikka showed that *Armillaria* is heterothallic and tetrapolar. Five years later, Korhonen (1978) demonstrated the existence of five sexually intersterile groups (A-E) within the *A. mellea* complex. Now, the five groups are considered as distinct species, namely: *Armillaria borealis* Marxmüller and Korhonen (group A), *Armillaria cepistipes* Velenovsky (group B), *Armillaria ostoyae* (Romagnesi) Herink (group C), *Armillaria mellea* (Vahl: Fr.) Kummer (group D), and *Armillaria gallica* Marxmüller and Romagnesi (group E). These species differ in their geographical and ecological distribution, host range, and pathogenicity (Table 1.3).

Additional to the five annulated *Armillaria* species, two exannulated species occur in Europe, namely *A. tabescens* (Scop.: Fr.) Emel and *A. ectypa* (Fr.) Lamoure. The thermophilic *A. tabescens* seems not to produce rhizomorphs *in natura* and mostly behaves saprotrophically on hardwood (Rishbeth 1982, Guillaumin *et al.* 1993). *A. ectypa* is a rare saprotrophic species, confined to *Sphagnum* peat bogs of high latitude or altitude (Zolciak *et al.* 1997).

Armillaria cepistipes

The northern limit of A. *cepistipes'* distribution is near the Arctic Circle, whereas the southern limit is in Greece and Calabria (Guillaumin *et al.* 1993, Grillo *et al.* 1996, Tsopelas 1999). The distribution of this species shows an inverse relationship between altitude and latitude: the more southern the latitude, the higher the altitude (Guillaumin *et al.* 1993).

Table 1.3. Geographical distribution and ecological behaviour of the European annulated *Armillaria* species (Guillaumin *et al.* 1993).

Current name	Old names	Geographical distribution[a]	Ecological behaviour
A. borealis Marxmüller & Korhonen		**N**: near 69th parallel.	Mainly saprotroph.
		S: probably central Europe.	Mixed forests and hardwoods.
A. cepistipes Velenovsky		**N**: near the Arctic Circle.	Saprotroph, weak parasite.
		S: Greece and southern Italy.	Hardwood and coniferous forests.
A. ostoyae (Romagnesi) Herink	*A. obscura* Schaeff.: Fr.	**N**: around 61 °N.	Primary parasite, saprotroph.
		S: Mediterranean zone.	Conifer, mixed, and hardwood forests.
A. mellea (Vahl: Fr.) Kummer		**N**: probably Denmark.	Occasionally aggressive primary parasite, saprotroph.
		S: Sicily, Greece, North Africa.	Hardwood and mixed forests, vineyards, parks, gardens, orchards.
A. gallica Marxmüller & Romagnesi	*A. bulbosa* (Barla) Kile & Watling	**N**: South Sweden, Georgia, Ukraine.	Saprotroph, weak parasite.
	A. lutea Gillet	**S**: Mediterranean regions (Sardinia, Sicily).	Hardwood and mixed forests.

[a]Northern (N) and southern (S) limits of the distribution.

A. cepistipes is rare in England but is probably the most common *Armillaria* species in central Europe (Guillaumin *et al.* 1993, Legrand *et al.* 1996, Rigling *et al.* 1998, Marxmüller & Holdenrieder 2000). A low interfertility exists between *A. cepistipes* and the North American biological species (NABS) V (*A. sinapina*) (Bérubé *et al.* 1996). Fruiting bodies of *A. cepistipes* show a low morphological variability. Typical characteristics are the stipe with yellow to white floccules, a fine and short-lived ring, and a hazel brown pileus with a few squamules at the disk (Marxmüller & Printz 1982, Marxmüller & Holdenrieder 2000).

Field observations indicate that *A. cepistipes* is a preferentially saprotrophic or weak pathogenic species, which occurs both in hardwood and coniferous forests (Guillaumin *et al.* 1993). In Scandinavia this species is also known as a causal agent of heart rot of conifers (Piri *et al.* 1990). *A. cepistipes* has a very good ability to produce thick, vigorous, and monopodially branched rhizomorphs, forming a dense network in forest soils (Guillaumin *et al.* 1989, Legrand *et al.* 1996, Rigling *et al.* 1998). By means of these rhizomorphs, the fungus can easily reach and colonise dead wood such as stumps, which means that *A. cepistipes* is an efficient decomposer of dead wood (Guillaumin *et al.* 1993). In its ecology, *A. cepistipes* is very similar to *A. gallica,* which, however, is more frequent at low altitudes (Guillaumin *et al.* 1989, Legrand & Guillaumin 1993).

Armillaria ostoyae

The northern limit of *A. ostoyae* in Scandinavia is around 61°N (Guillaumin *et al.* 1993). In continental Europe, *A. ostoyae* is quite common in coniferous forests, but in Mediterranean zones, it is only found at high altitudes (1000-1500 m a.s.l.) (Guillaumin *et al.* 1993, Grillo *et al.* 1996, Tsopelas 1999).

The fruiting bodies of *A. ostoyae* are characterised by a dark to ochraceous brown pileus with numerous, concentric, and dark brown squamules (Pegler 2000). The stipe is also decorated by persistent, white to brown floccules (Marxmüller & Printz 1982, Marmüller & Holdenrieder 2000).

A. ostoyae is mainly associated with conifers (Guillaumin *et al.* 1985, Marxmüller *et al.* 1990, Guillaumin *et al.* 1993). In European climax forests, *A. ostoyae* probably behaves mostly as a saprotroph and secondary parasite, killing suppressed or weakened trees (Guillaumin *et al.* 1989). As a lethal primary disease it is only locally of importance, for example in the forest ecosystem (*Pinus mugo* Turra var. *uncinata* Ram. and *Pinus sylvestris* L.) of Cerdagne, in the French eastern Pyrénées (Durrieu *et al.* 1985). In conifer plantations,

however, *A. ostoyae* can act as an aggressive primary pathogen, for example, in France on maritime pine (*Pinus pinaster* Ait) (Lung-Escarmant *et al.* 1998). Beside mortality, *A. ostoyae* causes butt and stem rot (Rishbeth 1982, Holdenrieder *et al.* 1994). In managed forests, *A. ostoyae* is an efficient coloniser of stumps created by silvicultural interventions (Legrand *et al.* 1996). However, it is not as good at developing rhizomorphs as *A. cepistipes* (Mohammed & Guillaumin 1989). Networks of *A. ostoyae* rhizomorphs in the soil are not very dense and are sometimes restricted to the zone around the stumps (Legrand *et al.* 1996). Fruiting bodies are formed on killed trees or on colonised stumps (Rishbeth 1982, Anselmi & Lanata 1989).

1.1.3 Interactions among *Armillaria* species

Species interaction in general

Two organisms interact when the presence of one in some way affects the performance of the other (Cooke & Rayner 1984). In natural communities, interactions occur frequently and knowledge of their nature is essential for understanding the functioning of the community. In contemporary ecology, species interactions are usually classified as indicated in Table 1.4 (Culver 1992).

Table 1.4. Possible interactions between two species.

		Effect of species A on species B[a]		
		0	-	+
Effect of species B on species A	0	Neutralism	Amensalism	Commensalism
	-	Amensalism	Competition	Predation, Parasitism
	+	Commensalism	Predation, Parasitism	Mutualism

[a]0, no effect; +, positive effect; and -, negative effect.

Amensalism occurs where two or more organisms of different species live in close proximity to one other when one member is unaffected by the relationship and the other is negatively affected by it. Neutralistic and mutualistic associations involve no detrimental effects on either participant, and benefits may be absent, unilateral or bilateral (Rayner & Boddy 1988). The complete absence of either benefit or harm and just passive co-existence is

probably rare among fungi (Cooke & Rayner 1984). In commensalism, one member benefits, whereas the other is unaffected by the relationship. In mutualism, benefits from the relationship are bilateral (Cooke & Rayner 1984). Examples of mutualistic associations are lichens (fungus-alga) and mycorrhiza (plant root-soil fungi). In predation and parasitism, one species experiences a negative effect as a result of the interaction while the other species benefits from the relationship (Culver 1992). In competition, both or all the organisms involved experience negative effects from the interactions. Concretely, competition occurs when two or more individuals of the same or different species simultaneously require a necessary common resource which is in limited or potentially limited supply (Cooke & Rayner 1984, Wicklow 1992).

Competition

Most ecologists divide competition into exploitation competition and interference competition. Exploitation involves co-utilisation of the limiting resources, whereas interference involves a denial of access to limiting resources (Culver 1992, Lockwood 1992). Interference competitors influence the access to the resource through behavioural or chemical interaction before the actual use of the resource (Wicklow 1992). Competition, which can occur at any stage of the life cycle, is an important driving force in the biology of ecologically similar fungal species (Shearer 1995). In wood-decaying fungi, two stages of competition are commonly distinguished: primary resource capture, i.e. the process of gaining initial access to an available resource, and secondary resource capture, i.e. the combat for resources after their capture (Cooke & Rayner 1984). The main requirement of fungal species colonising a vacant domain is to achieve maximal primary resource capture at least cost (time and energy resources) before the arrival of potential competitors (Rayner & Boddy 1988). The success in primary resource capture depends on several factors, such as a good spore dispersal and rapid germination, rapid mycelial extension, and the ability to use the organic compounds available in previously uncolonised resources (Boddy 2000). Some pathogenic fungal species, such as *Heterobasidion annosum* (Fr.) Bref, are efficient in primary resource capture (Holmer & Stenlid 1996). Saprotrophic species can exhibit both types of resource capture, but they sometimes have a stronger ability for secondary resource capture (Rayner & Todd 1979). Success in secondary resource capture depends on antagonistic mechanisms. According to Boddy (2000), combative interactions can be mediated at a distance through the release of diffusible or volatile factors or antibiotics, or following contact at the level of individual

hyphae or mycelia. Hyphal degeneration following contact or very close proximity is described as hyphal interference and is common among basidiomycetes (Rayner *et al.* 1995). The term mycoparasitism is used to indicate the parasitism of one hypha by another, following contact and recognition of the host (Boddy 2000). Contact at the mycelial level seems to be important among wood-decaying higher fungi (Boddy 2000). A wide range of interactions may follow mycelial contact, such as growth inhibition, redistribution of the mycelium, development of a dense and pigmented zone of mycelium in the contact region, changes in the pattern of the secondary metabolites, or stimulation of fruiting (Cooke & Rayner 1984, Sonnenbichler *et al.* 1997).

The consequences of competitive interactions are influenced by different factors, including the competitive ability of the combatants, the size and quality of the available resources, and the environmental factors (Shearer 1995, Boddy 2000). The outcomes of competition can be either exclusion or a stable equilibrium (Culver 1992). Competitive exclusion, which often occurs when competition is strong enough, involves the replacement of one fungus by the opponent (Boddy 2000). An equilibrium results when neither competitor gains headway (deadlock) or when the competitors may intermingle without damage to either (Cooke & Rayner 1984).

Biological control

Competitive interactions among antagonistic organisms provide opportunities for employing inoffensive organisms to control pathogens (biological control). By "biological control" I mean here reducing the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man (Cook & Baker 1983). According to Holdenrieder & Greig (1998), the main advantages of biological control are: (i) the biodegradability of the biocontrol agents and their metabolites, (ii) the potential sustainability of the effect, and (iii) a wide acceptance of this technique by the public. Antagonistic organisms can interfere with the pathogenic target organism at different stages of its life cycle. Facultatively pathogenic wood-rotting fungi, such as *Armillaria* spp., exhibit a saprotrophic life phase. During this phase, they are vulnerable to competition from aggressive lignicolous saprotrophs. In this context, the fresh stumps created by forest management represent a readily available resource (Holdenrieder & Greig 1998). Stump colonisation is important for the establishment, development, and survival of many root and butt rot fungi, including *Armillaria* (Cooke & Rayner 1984). By colonising the stumps they

can accumulate a considerable biomass and, consequently, stumps become an important source of inoculum (Holdenrieder & Greig 1998). Therefore, stumps are privileged targets for biocontrol measures.

To control *Armillaria*, an organism might function by (i) inhibiting or preventing rhizomorph and mycelial development, (ii) restricting the pathogen to substrate already occupied, (iii) actively pre-empting the substrate, or (iv) replacing *Armillaria* in an already occupied substrate (Hagle & Shaw 1991). Rishbeth (1976) pointed out that two *Armillaria* characteristics make its control by antagonistic organisms difficult. First, the positional advantage of *Armillaria*, which may already occupy a considerable portion of the substrate, over introduced fungi; and second, the rapid spread of *Armillaria* in the cambial zone of freshly killed trees. Thus, ideally an *Armillaria* antagonist should be efficient in both primary and secondary resource capture, and have a combative strategy towards other organisms (Cooke & Rayner 1984, Raziq 2000). The most studied antagonists of *Armillaria* are probably species of the hyphomycete genus *Trichoderma,* which are common and ubiquitous soil fungi (Hagle & Shaw 1991). Several *Trichoderma* spp. are antagonistic to other fungi, including many basidiomycetes (Holdenrieder & Greig 1998). Another group of potential agents for the control of *Armillaria* are the cord-forming wood-decay fungi, such as *Resinicium bicolor* (Alb. & Schw. ex Fr.) and *Hypholoma* spp. In these species, secondary resource capture is facilitated by the presence of mycelial cords, which can grow under the bark as well as in soil and litter (Hagle & Shaw 1991, Boddy 1993, Holmer & Stenlid 1996). Theoretically, preferentially saprotrophic *Armillaria* species are also potentially biocontrol agents for pathogenic *Armillaria* species and other root rot fungi such as *H. annosum*. Such an application would have three basic advantages. First, the frequent, natural co-existence of the pathogenic target organism and the antagonistic organism in the same forest ecosystem (Table 1.5). Second, the rapid stump colonisation following spread in the soil through rhizomorphs (Holdenrieder & Greig 1998). Third, the persistence of the *Armillaria* genets for hundreds of years, which could guarantee the sustainability of this method. But the use of a natural antagonist to control a target pathogen necessitates a thorough understanding of the biology and ecology of both organisms. For this, further study of the different *Armillaria* species is essential.

Table 1.5. Co-occurring preferentially saprotrophic and frequently pathogenic *Armillaria* species.

Region	*Armillaria* species		Forest type	Author(s)
	Preferentially saprotrophic	Primary pathogenic		
USA, California	*A. gallica*	*A. mellea*	Mixed hardwood forests	Baumgartner & Rizzo (2001)
USA, Pennsylvania	*A. gemina, A. calvescens*	*A. mellea*	Sugar maple stands	Marçais & Wargo (2000)
USA, New Hampshire	*A. gemina, A. calvescens*	*A. ostoyae*	Mixed coniferous stands	Rizzo & Harrington (1993)
USA, Michigan	*A. gallica*	*A. ostoyae*	Red pine plantation	Smith *et al.* (1994)
USA, Missouri	*A. gallica, A. tabescens*	*A. mellea*	Mixed Oak stands	Bruhn *et al.* (2000)
USA, New York	*A. calvescens, A. gemina*	*A. ostoyae*	Conifer-hardwood stand	Worrall (1994)
Canada, BC	*A. sinapina*	*A. ostoyae*	Spruce forest	Dettman & van der Kamp (2001)
Canada, BC	*A. sinapina*	*A. ostoyae*	Coniferous forests	Cruickshank *et al.* (1997)
Europe, Germany	*A. borealis, A. gallica*	*A. ostoyae*	Norway spruce forest	Siepmann (1985)
Europe, Germany	*A. cepistipes*	*A. ostoyae*	Silver fir forest	Holdenrieder (1986)
Europe, England	*A. gallica, A. tabascens*	*A. mellea*	Broad-leaved woodland	Rishbeth (1991)
Europe, Germany	*A. cepistipes*	*A. ostoyae*	Coniferous forests	Marxmüller & Holdenrieder (2000)
Europe, Italy	*A. cepistipes*	*A. ostoyae*	Coniferous forests	Anselmi & Lanata (1989)
Europe, France	*A. cepistipes*	*A. ostoyae*	Mixed forests	Legrand & Guillaumin (1993)
Europe, France	*A. gallica*	*A. mellea*	Hardwood forests	Legrand & Guillaumin (1993)
Europe, France	*A. cepistipes, A. gallica*	*A. ostoyae*	Beech stands	Legrand *et al.* (1996)

1.2 Objectives of the dissertation

The aim of this study was to investigate the ecological behaviour (population structure, ability in primary resource capture, and virulence) of the preferentially saprotrophic *A. cepistipes* and its interactions with the pathogenic *A. ostoyae*. The dissertation uses data from both field and experimental investigations (Fig. 1.1).

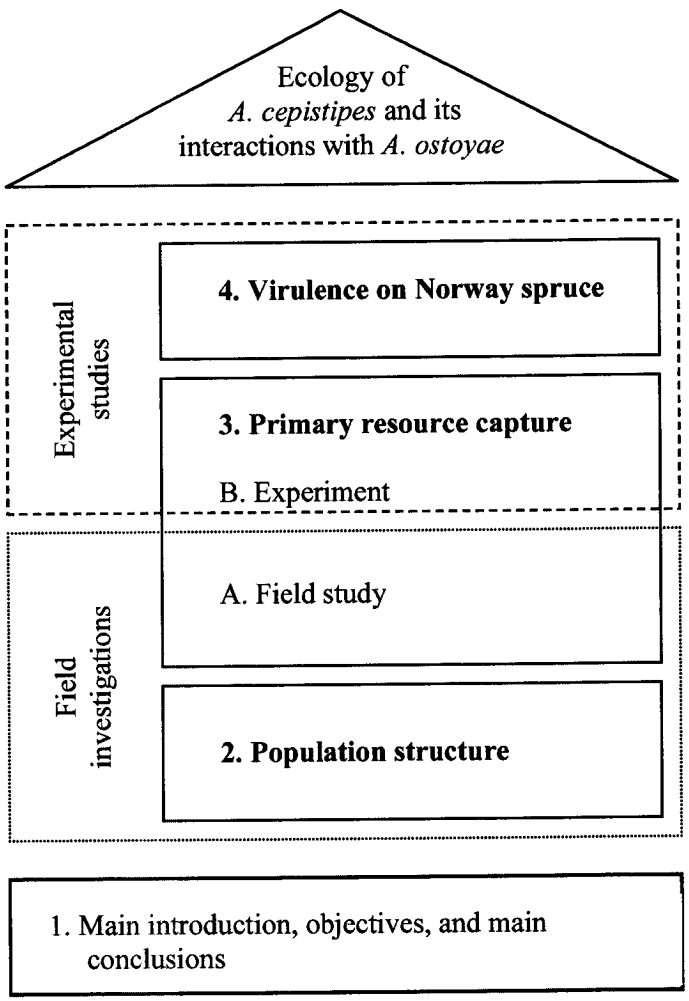


Figure 1.1. Structure of the dissertation.

Field investigations into the population structure of the two species and their abilities in primary resource capture were conducted in three managed spruce forests where both *Armillaria* species co-occur sympatrically. The virulence and the competitive ability of *A. cepistipes* and *A. ostoyae* in capturing fresh stumps were investigated experimentally. The

dissertation is structured in four different chapters (Fig. 1.1), with each of them addressing a number of specific questions.

Chapter 2: Population structure of *Armillaria* species in managed Norway spruce stands in the Alps

The majority of recent studies on the ecology of basidiomycetes are based on population structure analysis. Using the phenomenon of somatic incompatibility (Guillaumin *et al.* 1996, Worrall 1997, Malik & Vilgalys 1999), it is possible to identify genetically distinct secondary mycelia of the same species co-existing within a population. These mycelia are called genets and can be considered the units of a population. Knowledge of the size and spatial distribution of genets can provide important information on the processes of establishment and growth of a particular fungal population (Kirby *et al.* 1990, Worrall 1994).

The purpose of this chapter was to analyse the spatial population structure of *A. cepistipes* and *A. ostoyae* co-existing in three managed Norway spruce stands in the Alps.

Specific questions addressed were:

(i) *What is the spatial distribution of the genets of the two species?*

(ii) *Are there overlaps between genets of the same or of different species?*

(iii) *What are the spatial patterns of genets along interspecific boundaries?*

Chapter 3: Primary resource capture by *Armillaria cepistipes* and *Armillaria ostoyae*

All *Armillaria* species exhibit a saprotrophic life phase, during which they survive on woody substrates (Redfern & Filip 1991). In managed forests, stumps created by silvicultural interventions represent an intermediate stage between living trees and dead wood (Rayner & Boddy 1988, Holdenrieder & Greig 1998). If a facultatively parasitic species efficiently captures fresh stumps, it can accumulate a considerable biomass and, consequently, stumps become an important source of inoculum (Holdenrieder & Greig 1998).

The purpose of this chapter was (i) to determine the importance of fresh Norway spruce stumps as a resource for *A. cepistipes* and *A. ostoyae*, and (ii) to investigate the ability of these two species in primary stump colonisation. For this, a field investigation (part A) and an experimental study (part B) were conducted.

Specific questions addressed were:

(i) *To what extent are stumps colonised by* Armillaria *spp.?*

(ii) *What is the relationship between the incidence of* Armillaria *species and genets in the soil and on the stumps?*

(iii) *What is the mutual competitive ability of the two species to capture fresh stumps?*

(iv) *Does the presence of rhizomorphs of* A. cepistipes *in the soil prevent or reduce the stump colonisation by* A. ostoyae*?*

Chapter 4: Comparison of the virulence of *Armillaria cepistipes* and *Armillaria ostoyae* on four Norway spruce provenances

Circumstantial evidence suggests that *A. cepistipes* behaves preferentially as a saprotroph (Guillaumin *et al.* 1993). However, little experimental data are available on the pathogenicity of this species. By contrast, a number of inoculation trials involving isolates of *A. ostoyae* have been conducted (e.g. Shaw 1977, Morrison & Pellow 2002), but no extensive inoculation experiments have been performed with Norway spruce, which is the most common conifer in central and northern Europe.

The purpose of this chapter was to determine the virulence of a representative number of isolates of *A. cepistipes* and *A. ostoyae* on Norway spruce seedlings of four provenances.

Specific questions addressed were:

(i) *How does the virulence of* A. cepistipes *compare with that of* A. ostoyae*?*

(ii) *What is the variation in virulence among isolates of the same species?*

(iii) *Are there differences in the susceptibility of seedlings with different provenances?*

1.3 Main conclusions

The study presented in ***chapter 2*** shows that, in comparable Norway spruce (*Picea abies* (L.) Karst) stands where *A. cepistipes* and *A. ostoyae* co-exist, the incidence of each species as well as the population structure (i.e. number, spatial patterns, and sizes of genets) vary considerably. It can be assumed that site characteristics (i.e. exposition, soil, and topographical heterogeneity) and stand characteristics (history, management practice, and species composition) probably influence the population dynamics of *Armillaria*. In all three populations investigated, genets of the same species rarely overlap. This implies a strong intraspecific antagonism probably because of somatic incompatibility and the use of identical ecological strategies. A general type of interaction between *A. cepistipes* and *A. ostoyae* is difficult to deduce. On parts of the investigated area, both species seem to exclude each other, which would indicate they are in competition. On other parts, however, there are considerable interspecific overlaps, which would suggest a more neutralistic co-existence.

Conclusion 1: *Genets of the same species generally exclude each other, suggesting a strong intraspecific antagonism.*

Conclusion 2: *Between* A. cepistipes *and* A. ostoyae, *a varying degree of competition seems to occur, ranging from a rather neutralistic co-existence to a mutual exclusion.*

The stumps created by forest management represent a readily available resource which is exploited by many root and butt rot fungi (Cooke & Rayner 1984). The study presented in ***chapter 3A*** demonstrates that, in managed Norway spruce stands, *A. cepistipes* and *A. ostoyae* both efficiently capture fresh stumps. In both *Armillaria* species, internal stump capture (i.e. heartwood colonisation) occurs when the trees are still standing and alive. On the other hand, external stump capture, which is much more important for *A. cepistipes* and *A. ostoyae*, primarily occurs within one to four years following tree felling. The ability of both *Armillaria* species to produce a network of rhizomorphs enveloping living trees as potential food-bases is probably decisive for rapid stump capture. Stumps located along interspecific boundaries are preferentially colonised by *A. ostoyae*, suggesting that this species is slightly better at primary resource capture than *A. cepistipes*.

In the ***chapter 3B*** an experiment is described which aimed to investigate the competitive ability of *A. cepistipes* and *A. ostoyae* in primary stump capture further. The two *Armillaria* species were simultaneously or sequentially (*A. cepistipes* prior to *A. ostoyae*) inoculated in pots containing Norway spruce seedlings. The subsequent rhizomorph production and colonisation of newly created stumps were investigated. The study provides evidence that *A. cepistipes* is a better rhizomorph producer than *A. ostoyae*. When inoculated alone, both species efficiently colonise the stumps. Their ability in primary stump capture is reduced by the presence of the other *Armillaria* species, indicating that competition occurs for the stumps, as was hypothesised in chapter 3A. *A. ostoyae* is relatively more frequent on the stumps than in the soil, whereas for *A. cepistipes* the opposite applies. This indicates that *A. ostoyae* has a better competitive ability in primary resource capture than *A. cepistipes*, which confirms the results of the field investigation described in chapter 3A.

Conclusion 3: *In managed spruce forests, stumps are an important resource for the development of both* A. cepistipes *and* A. ostoyae.

Conclusion 4: A. ostoyae *is more competitive than* A. cepistipes *in primary stump capture.*

Conclusion 5: *The primary stump capture ability of both species is reduced by the presence of the other species, indicating competition for this resource.*

Conclusion 6: *The presence of* A. cepistipes *in the soil can inhibit stump colonisation by* A. ostoyae.

The inoculation experiment reported in the ***chapter 4*** demonstrates that the virulence of *A. cepistipes* isolates on Norway spruce seedlings is generally low. In contrast, the virulence of *A. ostoyae* varies considerably among isolates and is positively correlated with the ability to form rhizomorphs. Seedling provenances vary in how susceptible they are to *A. ostoyae*, possibly due to differing genetic traits. Both *Armillaria* species attach rhizomorphs to the surface of the seedling roots. However, *A. ostoyae* causes significantly more lesions, which implies it is better able than *A. cepistipes* to directly penetrate non-wounded root bark.

Conclusion 7 : A. cepistipes *is a weakly pathogenic species.*

Conclusion 8 : *In* A. ostoyae *there is a considerable variation in virulence among isolates, ranging from highly to weakly virulent.*

Conclusion 9 : *The susceptibility to* A. ostoyae *varies among host provenances.*

Conclusion 10: A. ostoyae *is better able than* A. cepistipes *to penetrate intact root bark and cause lesions.*

The study shows that the behaviour of *A. cepistipes* and *A. ostoyae* in managed Norway spruce stands in the Alps is ecologically similar. Thus both species: (i) form a network of rhizomorphs in the soil, (ii) can cause heart rot on standing trees, and (iii) intensively capture fresh stumps. The most relevant differences between the two *Armillaria* species concern their ability to form rhizomorphs and their pathogenicity. *A. cepistipes* is a better rhizomorph producer than *A. ostoyae*. Regarding pathogenicity, *A. cepistipes* can be considered as a species with non- or only low-virulent genotypes. In contrast, there is considerable variation in virulence among *A. ostoyae* genotypes, ranging from highly to weakly virulent.

Because of the two species' ecological similarity it could be assumed that, when co-occurring in the same stand, competition occurs between *A. cepistipes* and *A. ostoyae*. This competition takes place on the boundaries between genets of the two species and primarily concerns the exploitation of the limited resources (i.e. fresh stumps). By contrast, in the soil (i.e. as rhizomorphs) the two species seem to co-exist in a more neutralistic association. Since the present study gives just a snapshot of a dynamic system, it is difficult to make long-term predictions. The better competitive ability of *A. ostoyae* in primary stump capture could suggest that this species may have a certain advantage over *A. cepistipes*. Highly virulent genotypes of *A. ostoyae* are also able to produce new resources actively by killing living trees. *A. cepistipes*, however, is probably more competitive than *A. ostoyae* in colonising the soil because of its better ability to produce rhizomorphs. Considering that *A. cepistipes* too is an efficient stump coloniser where it has been established in the soil, the outcome of the competitive interactions will, therefore, be a dynamic equilibrium of co-existence rather than the replacement of *A. cepistipes* by *A. ostoyae*. This hypothesis is supported: (i) by the local dominance of *A. cepistipes* genets, and (ii) by the inhibition of *A. ostoyae* in primary resource capture in the presence of *A. cepistipes*. Obviously, various other factors, such as the size and

quality of the available resources and more general environmental factors, are likely to influence the outcome of the interactions between the two *Armillaria* species.

Competitive interactions between preferentially saprotrophic and facultatively pathogenic organisms may provide opportunities for using the former to control the latter (Holdenrieder & Greig 1998). Although this study gives some basic information on the ecological behaviour of *A. cepistipes* and *A. ostoyae*, as well as on their interspecific interactions in managed spruce forests, further investigations are necessary to evaluate the potential of *A. cepistipes* as a biocontrol agent for *A. ostoyae*. At present, it seems there is some potential, but several important questions need to be addressed before clear conclusions can be drawn:

- *What are the long-term dynamics of* Armillaria *populations in managed forest stands? Will one species replace the other, or will there be a long-term co-existence of the two species?*
- *How significant is* A. cepistipes *as a causal agent of heart rot in Norway spruce and other species of conifers?*
- *Is it possible to manipulate* Armillaria *populations in order to promote* A. cepistipes*? For example, is it possible to inoculate fresh stumps with* A. cepistipes *to prevent or reduce their colonisation by* A. ostoyae*?*
- *Can low-virulent* A. ostoyae *genotypes be used to control virulent genotypes by exploiting the strong intraspecific antagonism?*

2

Population structure of *Armillaria* species in managed Norway spruce stands in the Alps*

*Prospero S, Rigling D, Holdenrieder O. 2003. *New Phytologist* 158: 365-373

Abstract

The preferentially saprotrophic basidiomycete *Armillaria cepistipes* and the pathogenic *Armillaria ostoyae* occur sympatrically in many European forests.

The spatial population structure of both *Armillaria* species was investigated in three 1 ha plots established in managed Norway spruce (*Picea abies*) forests in the Swiss Alps (1400 m a.s.l.). A total of 740 *Armillaria* isolates were recovered, 296 from rhizomorphs in the soil and 444 from the stumps.

The incidence of *A. cepistipes* and *A. ostoyae* varied greatly among the plots. Two to six genets of each *Armillaria* species were identified and mapped in each plot. Genets of the same species overlapped rarely and only on the borders. Large spatial overlaps between *A. cepistipes* and *A. ostoyae* genets were observed in the plot with the highest incidence of fragmented genets. In five subplots (approx. 0.1 ha) established along interspecific boundaries, overlaps were found in all three plots.

Our study suggests a strong intraspecific competition in both *Armillaria* species. Evidence for competition between the two species as indicated by spatial mutual exclusion was only observed on parts of the investigated area.

Key words: *Armillaria cepistipes*, *Armillaria ostoyae*, *Picea abies* (Norway spruce), population structure, genets, spatial pattern, interaction, soil-borne fungi.

2.1 Introduction

The majority of recent studies on the ecology of basidiomycetes are based on population structure analysis. Using the phenomenon of somatic incompatibility (Guillaumin *et al.* 1996, Worrall 1997, Malik & Vilgalys 1999), it is possible to identify genetically distinct secondary mycelia of the same species co-existing within a population. These mycelia are called genets and can be considered the units of a population. Knowledge about the size and spatial distribution of genets can provide important information on the processes of establishment and growth of a particular fungal population (Kirby *et al.* 1990, Worrall 1994). The population structure of several basidiomycetes has been studied, including that of *Heterobasidion annosum* (e.g. Swedjemark & Stenlid 1993), *Phellinus weirii* (e.g. Hansen & Goheen 2000), *Fomitopsis pinicola* (Högberg *et al.* 1999), *Resinicium bicolor* (Kirby *et al.* 1990), *Marasmius androsaceus* (Holmer & Stenlid 1991), *Suillus bovinus* (e.g. Dahlberg & Stenlid 1994), and *Armillaria* spp. (e.g. Worrall 1994).

The genus *Armillaria* comprises several species which are important components of the mycoflora in many forest ecosystems worldwide (Shaw & Kile 1991). *Armillaria* spp. can behave as primary or secondary pathogens causing root and butt rot on numerous coniferous and broadleaved tree species in both naturally regenerated forests and plantations (Guillaumin *et al.* 1993, Morrison *et al.* 2000). As parasites, *Armillaria* spp. can cause significant economic losses (Morrison & Mallett 1996) and influence the tree species composition of forests (Kile *et al.* 1991). In addition, all *Armillaria* species can survive saprotrophically in woody substrates such as roots, stumps, and debris. To date, about 40 species of *Armillaria* are known, seven of which occur in Europe (Watling *et al.* 1991). The European species differ in geographical distribution, ecological behaviour, host range, and pathogenicity (Guillaumin *et al.* 1993).

Different species of *Armillaria* frequently coexist in the same forest stand (Rizzo & Harrington 1993, Legrand *et al.* 1996, Bruhn *et al.* 2000, Marxmüller & Holdenrieder 2000). In central Europe, the pathogenic *Armillaria ostoyae* (Romagnesi) Herink and the preferentially saprotrophic *Armillaria cepistipes* Velenovsky often occur sympatrically in mountainous forests (Legrand *et al.* 1996, Marxmüller & Holdenrieder 2000). *Armillaria* species are probably significant competitors of the root and butt rot pathogen *Heterobasidion annosum* (Holdenrieder & Greig 1998) and they also compete with each other for resources (Chapter 3). Therefore, potential agents for the biological control of *H. annosum* and parasitic *Armillaria* species could be primarily saprotrophic *Armillaria* species and their interaction

with these fungi deserves attention.

The purpose of the present study was to analyse the population structure of *A. cepistipes* and *A. ostoyae* coexisting in three managed Norway spruce (*Picea abies* (L.) Karst.) stands in the Alps. Specifically, we addressed the following questions: (i) What is the spatial distribution of the genets of the two species?; (ii) Are there overlaps between genets of the same or of different species?; and (iii) What are the spatial patterns of genets along interspecific boundaries? Considering these aspects, inferences are made about intra- and interspecific interactions.

2.2 Materials and Methods

2.2.1 Study sites

The three study sites, Ludiano (46° 25' 26'' N, 8° 56' 28'' E), Lurengo (46° 30' 5'' N, 8° 44' 55'' E), and Dalpe (46° 27' 50'' N, 8° 47' 25'' E) are located in naturally regenerated and managed Norway spruce stands in the southern Swiss Alps at about 1400 m a.s.l and 5 to 17 km apart. The stands are comparable in vegetation type (spruce-fir forest), tree age (140-160 years), and past management practice.

In each stand, a 100 m x 100 m (1 ha) plot was established in summer 1999 containing numerous one- to three-year old stumps. Tree species were recorded and all stumps over 12 cm in diameter were mapped.

2.2.2 Sampling

In each plot, a systematic sampling was conducted by taking a cube of soil (15 cm side) at each point in a 10 m x 10 m square grid. If no rhizomorphs were found in the systematic sample, additional soil samples (max. 4) were taken within 2 m from the grid points. The soil samples were sieved through a 9 mm square mesh to separate the roots and rhizomorphs from the soil. All rhizomorphs were collected and brought to the laboratory for isolation.

All stumps present in the plots were examined for *Armillaria* colonisation as follows: portions of bark were removed from the collar region of three main lateral roots using an axe and the presence of subcortical mycelial fans or rhizomorphs was recorded. For isolation, pieces of wood with mycelial fans or rhizomorphs on the surface were brought to the laboratory.

2.2.3 Subplots

After determining the distribution of *A. cepistipes* and *A. ostoyae* genets in the soil, five subplots (12 m x 12-14 m) were established within the three plots (one in Ludiano and two each in Lurengo and Dalpe) at the borders of two *Armillaria* species or where they overlapped in the soil (Figs. 1a-d). In each subplot, a systematic sampling was conducted by taking a cube of soil (15 cm side) at each point of a 1.4 m x 1.4 m square grid (61-72 samples per subplot).

2.2.4 Isolation of *Armillaria* spp.

The collected rhizomorphs were first dipped in 50% ethanol for 15-20 seconds. Then, from each rhizomorph three segments of 1 cm length were surface sterilised in 30% hydrogen peroxide (H_2O_2) for 25-40 seconds (depending on the thickness of the rhizomorphs) and placed on a Petri plate (diameter 8.5 cm) containing malt extract agar (12 g l^{-1} malt extract; 15 g l^{-1} Bacto Agar) amended with 2 mg l^{-1} benomyl and 100 mg l^{-1} streptomycin (Maloy 1974).

The pieces of wood with subcortical mycelial fans were washed under running tap water with a brush and blotted dry between paper towels. For each sample, six small pieces (2-5 mm x 2-5 mm) of mycelium were surface sterilised in sodium-hypochlorite (7% active chlorine; Chemische Fabrik Schweizerhalle, CH-4013 Basel) for 5-10 seconds and rinsed in sterile distilled water for 10-15 seconds (Prospero *et al.* 1998). The samples were dried between paper towels and placed on MATS plates (20 g l^{-1} malt extract; 15 g l^{-1} Bacto Agar; 230 mg l^{-1} thiabendazole in 1 ml concentrated lactic acid; 100 mg l^{-1} streptomycin) modified according to Legrand & Guillaumin (1993).

All isolation plates were incubated in the dark at 20-25°C. After one to three weeks, pure cultures were transferred to malt extract agar (15 g l^{-1} Bacto Agar; 20 g l^{-1} Diamalt, Hefefabriken AG, CH-3324 Hindelbank).

2.2.5 Identification of *Armillaria* genets and species

Genets were identified by pairing diploid isolates on Shaw & Roth's medium as described by Harrington *et al.* (1992). Mycelial plugs (3-4 mm side), cut from the margin of growing cultures, were placed approximately 5 mm apart onto the agar surface and incubated in the dark at 20-25°C. Three pairings were performed in each Petri plate (diameter 8.5 cm). After three weeks, somatic incompatibility reactions between the isolates were classified according

to Rizzo & Harrington (1993). Each pairing was repeated twice and each isolate was also self-paired. To reduce the number of pairings, we first paired 8-10 isolates of each plot in all combinations to identify some genets. The other isolates were then paired with the nearest isolate representing an identified genet. Finally, the isolates which could not be assigned to an identified genet were paired in all combinations. Subcultures representing each genet were transferred to malt extract agar and stored at 4°C in the dark. The genets were designated as follows: plot (Lud = Ludiano, Lur = Lurengo, and Da = Dalpe), species (B = *A. cepistipes*, C = *A. ostoyae*, and E = *Armillaria gallica* Marxmüller & Romagnesi), and genet number.

Species identification was performed by pairing three isolates (if available) of each genet with three haploid tester strains (Korhonen 1978) of the five European annulate *Armillaria* species as described by Harrington *et al.* (1992). The tester strains were kindly provided by J.-J. Guillaumin (INRA, Clermont-Ferrand, France). In addition, all genets were also identified to species with PCR-RFLP analysis of a portion of the intergenic spacer (IGS) region of the ribosomal DNA (Harrington & Wingfield 1995). The PCR-products were digested using the restriction enzymes *Alu* I, *Hinc* II, *Mva 1269* I (*Bsm* I), and *Nde* I (MBI Fermentas). Three isolates (if available) of each genet were analysed with the PCR-RFLP method.

2.2.6 Estimation of genet boundaries, sizes, and spatial overlaps

An isolate recovered from a point on the 10 m x 10 m sample grid was considered representative for a genet area of 100 m^2 (corresponding to a circular area with a radius of about 5.64 m). Each stump colonised by *Armillaria* was considered to represent a circle with a radius of about 2.82 m (approx. 25 m^2), corresponding approximately to the diameter of the large root system. Two isolates of the same genet, distant ≤ 20 m, were considered to represent a contiguous (non-fragmented) genet regardless of whether there was an isolate belonging to another genet in-between. This criterion was set in respect to the 10 m x 10 m sample grid and allowed one negative grid point between two positive points without affecting the contiguity of a genet. Within the subplots, each isolate of the 1.4 m x 1.4 m grid was considered representative for a genet area of 2 m^2 and only isolates of the same genet ≤ 3 m away were considered contiguous.

Genet boundaries were outlined by modifying the method of Worrall (1994). First, we drew circles around each positive soil sample point (r = 5.64 m) and around each colonised stump (r = 2.82 m) from which isolates of the same genet were recovered. Then, for each

genet the shortest outline enclosing all circles was drawn as a smoothed polygon. Genet sizes were calculated using the software ArcView, Version 3.2a (Environmental Systems Research Institute, Inc., Redlands, CA, USA). The maximum linear extent of a genet was determined as the maximum spatial distance between two isolates of the same genet, even if they belong to different ramets.

To determine the extent of spatial overlaps among genets of the same or different *Armillaria* species, polygons were superimposed in ArcView. The size of areas occupied by more than one genet was then calculated with ArcView. In addition, overlaps were also quantified as the percentage of the total territory covered by *Armillaria* spp.

2.2.7 Statistical analysis

Statistical analysis of data was performed with the software DataDesk, Version 6 (Data Description, Inc., Ithaca, NY, USA). The quantitative variables of genets (i.e. size and linear extent) were analysed using one-way analysis of variance (ANOVA).

2.3 Results

2.3.1 Population structure

The incidences of *Armillaria* species and genets in the soil and on the stumps in the three study plots are summarised in Table 2.1. From 100 soil samples collected in each plot, 54 to 85 per plot contained rhizomorphs. Stumps colonised by *Armillaria* were detected at proportions of 39% (77 out of 199) in Ludiano, 79% (160 out of 202) in Lurengo, and 86% (115 out of 133) in Dalpe. *Armillaria* isolates were successfully recovered from 184 soil samples and 269 stumps. From 77 soil samples and 134 stumps more than one isolate was obtained. In total, 740 *Armillaria* isolates were identified to species and genets, 296 from rhizomorphs in the soil and 444 from the stumps. *A. cepistipes* and *A. ostoyae* were found in all three plots (Table 2.1). Both species occurred in similar proportions in Dalpe while *A. ostoyae* dominated in Lurengo and *A. cepistipes* in Ludiano. In this last plot, *A. gallica* was isolated from seven soil samples and 12 stumps.

Most soil samples (62 out of 77) and stumps (112 out of 134) from which more than one isolate was recovered, yielded isolates of a single genet. Soil samples containing rhizomorphs

Table 2.1. Incidence of *Armillaria* species and genets in the soil and on the stumps in the three plots, Ludiano, Lurengo, and Dalpe.

	All samples					Samples from which more than one *Armillaria* isolate was recovered								
			N of samples with species of[b]				N of samples with genets of[c]							
Origin	N	Positive[a]	*A. cep*	*A. ost*	*A. gal*	N	*A. cep*	*A. ost*	*A. gal*	*A. cep* + *A. cep*	*A. ost* + *A. ost*	*A. gal* + *A. gal*	*A. cep* + *A. gal*	*A. cep* + *A. ost*
Soil														
Ludiano	100	54 (50)	38	8	7	23	15	1	2	1	0	1	3	0
Lurengo	100	60 (52)	2	50	0	21	2	19	0	0	0	0	0	0
Dalpe	100	85 (82)	54	35	0	33	15	8	0	2	1	0	0	7
Total	300	199 (184)	94	93	7	77	32	28	2	3	1	1	3	7
Stumps														
Ludiano	199	77 (63)	44	9	12	35	24	4	5	0	0	0	2	0
Lurengo	202	160 (111)	2	110	0	47	0	40	0	0	6	0	0	1
Dalpe	133	115 (95)	53	54	0	52	22	17	0	1	0	0	0	12
Total	534	352 (269)	99	173	12	134	46	61	5	1	6	0	2	13

[a]Number of soil samples containing rhizomorphs of *Armillaria* and stumps colonised by *Armillaria*. Numbers in brackets refer to positive samples from which *Armillaria* could be isolated.

[b]Number of soil samples containing rhizomorphs of *A. cepistipes (A. cep)*, *A. ostoyae (A. ost)*, and *A. gallica (A. gal)*. A sum exceeding the number of positive soil samples from which *Armillaria* could be isolated indicates the presence of samples with two species.

[c]Number of soil samples or stumps with one genet of a species (e.g. *A. cep*), two genets of a species (e.g. *A. cep*+*A. cep*), or two genets of different species (e.g. *A. cep*+*A. gal*).

of both *A. cepistipes* and *A. ostoyae* were only found in Dalpe (7 out of 33). Likewise, several stumps (12 out of 52) in Dalpe were colonised by both species. In all plots, stumps colonised by more than one *Armillaria* genet were located on the borders between genets or where they overlapped in the soil (Figs. 2.1a-d).

Using somatic incompatibility tests, two to six different genets of *A. cepistipes* and *A. ostoyae* were identified in each plot (Table 2.2). This corresponds to a mean density of eight *Armillaria* genets per hectare. More genets were detected in the soil than on the stumps. In Ludiano and Dalpe, a few small (100-200 m^2) genets present in the soil (Ludiano: Lud B3, Lud C2; Dalpe: Da C4) were not found on the stumps. Fragmented genets were detected in all plots, and were particularly frequent in Dalpe (Table 2.2). The estimated sizes of the genets ranged from 100 (i.e. found at only one sample point) to about 4100 m^2 and the maximal linear extent from 10 to 105 m. No significant ($P < 0.05$) differences in the genet sizes were

Table 2.2. Characteristics of the *Armillaria* populations in the three plots.

	Ludiano			Lurengo		Dalpe	
Characteristics	*A. gal*	*A. cep*	*A. ost*	*A. cep*	*A. ost*	*A. cep*	*A. ost*
Territory occupied (m^2)	800	4100	1270	250	7070	6500	5200
Genets (N)	2	3	4	2	6	3	4
Genets in the soil (N)	2	3	4	2	6	3	4
Genets on the stumps (N)	2	2	3	2	6	3	3
Fragmented genets[a] (N)	1	0	0	0	1	2	2
Maximal linear extent (m)	45	74	41	10	105	105	105
Mean area of a genet (m^2)	475	1425	340	125	1285	2310	1350
Intraspecific overlaps (m^2)	150	180	100	0	650	440	200
(%)[b]	18.8	4.4	7.9	0	9.2	6.8	3.8
Interspecific overlaps (m^2)	350		20	250		3200	
(%)[c]	7.7		0.4	3.5		37.6	

[a]A genet was considered fragmented if the distance between two isolates was more than 20 m.

[b]Percentage refers to the total area occupied by the *Armillaria* species indicated.

[c]Percentage refers to the total area occupied by the two *Armillaria* species indicated.

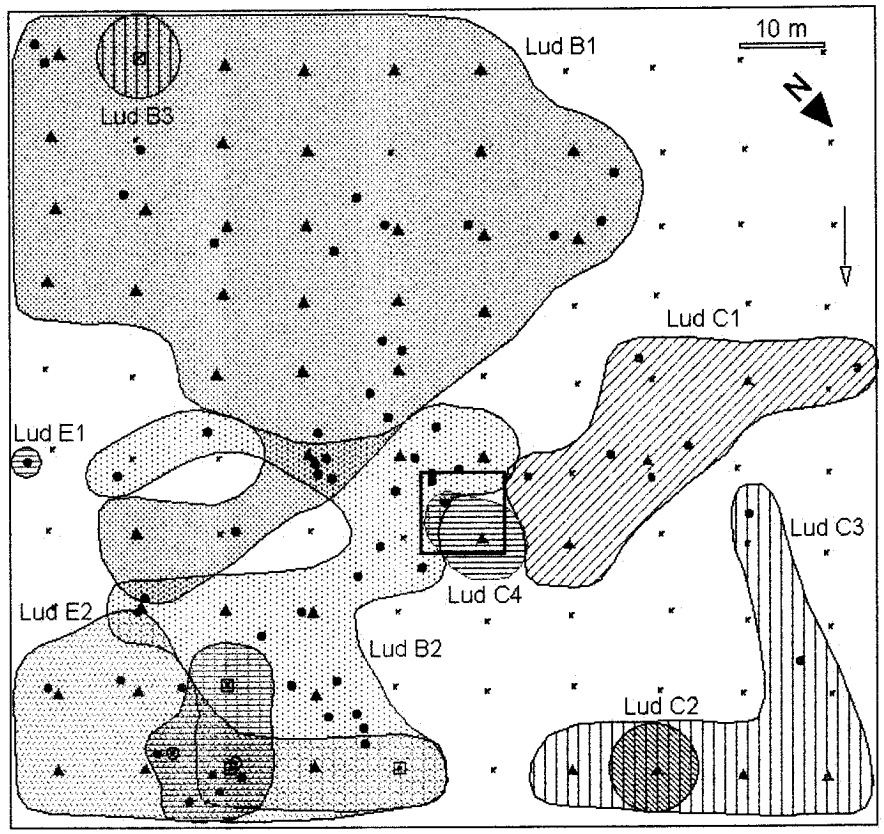
observed (i) between *A. cepistipes* and *A. ostoyae* and (ii) among the three plots. The *A. gallica* genets were not included in the analysis because of their reduced number (two) and their occurrence only in Ludiano.

In Ludiano, a total of nine genets were identified, three of *A. cepistipes*, four of *A. ostoyae*, and two of *A. gallica*. *A. cepistipes* and *A. ostoyae* occupied different sectors of the plot and interspecific spatial overlaps were very rare (Fig. 2.1a, Table 2.2). The two genets of *A. gallica* (Lud E1 and Lud E2) were confined to one edge of the plot and partially overlapped with the *A. cepistipes* genet Lud B2. The estimated sizes of the *A. cepistipes* genets ranged from 100 (Lud B3) to about 3000 m^2, whereas genets of *A. ostoyae* and *A. gallica* were smaller (100-700 m^2). The three *Armillaria* species showed little intraspecific spatial overlaps (Fig. 2.1a, Table 2.2).

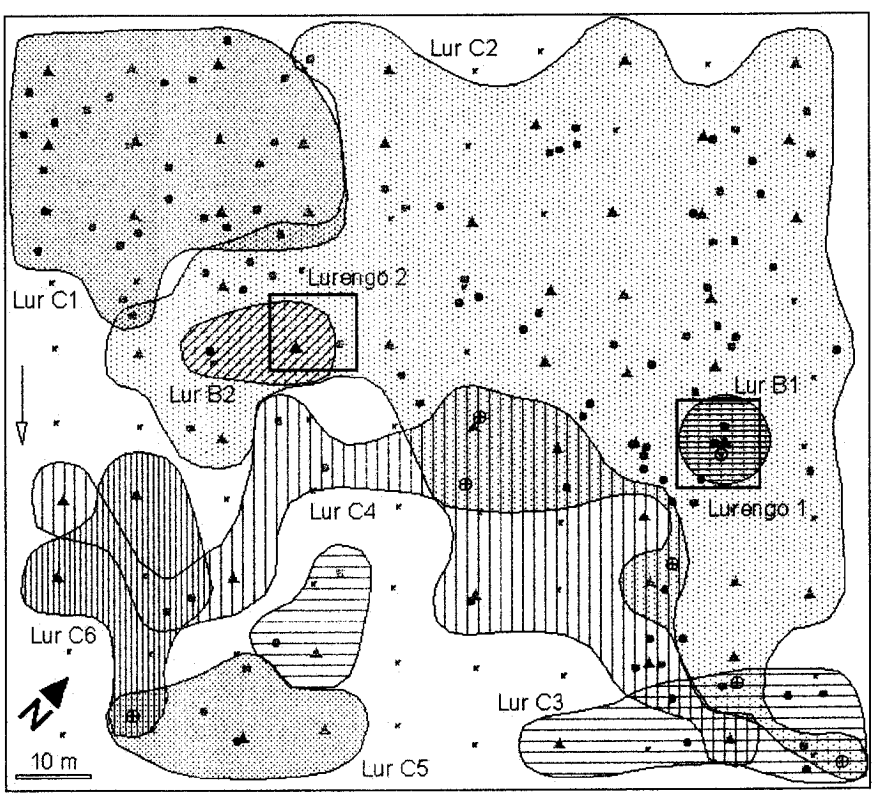
In Lurengo, six genets of *A. ostoyae* and two genets of *A. cepistipes* were found (Fig. 2.1b). The largest genet (Lur C2) had an estimated size of 4100 m^2 and a maximal linear extent of 105 m. Only one genet (Lur C3) showed a fragmented distribution. Spatial overlaps among *A. ostoyae* genets were rare (9.2% of the territory occupied by *A. ostoyae*, Tab. 2.2) and mainly limited to the border zone of the genets. The two small (100 and 150 m^2) *A. cepistipes* genets in Lurengo were located in two different sectors occupied by the *A. ostoyae* genet Lur C2 (Fig. 2.1b).

Three genets of *A. cepistipes* and four genets of *A. ostoyae* were detected in Dalpe (Fig. 2.1c and d). In contrast to the other plots, in Dalpe genets of the two species overlapped considerably. These interspecific overlaps were estimated to cover an area of 3200 m^2, which corresponds to 37.6% of the total territory occupied by both species. On the other hand, intraspecific overlaps were rare and limited to the borders of genets. Two genets of *A. ostoyae* (Da C2 and Da C3) and two genets of *A. cepistipes* (Da B1 and Da B3) showed a non-contiguous, fragmented distribution. The two largest genets (Da B2 and Da C1) had a maximal linear extent of 105 m and covered 4100 and 3230 m^2.

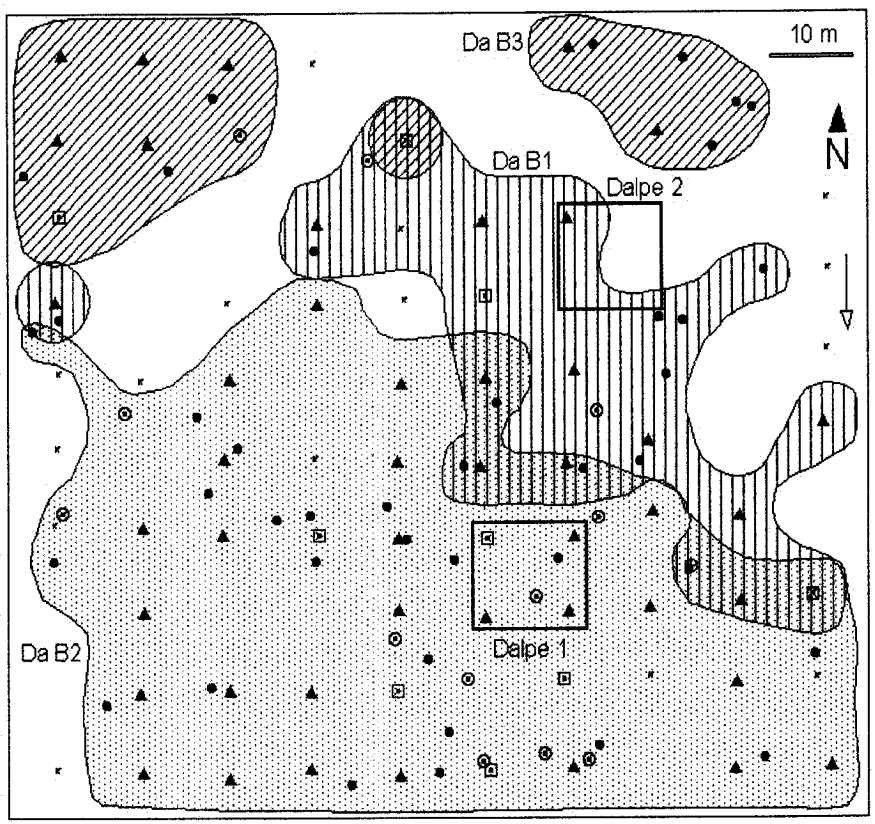
Figure 2.1. Spatial distribution of genets of *A. cepistipes* (B, green), *A. ostoyae* (C, red), and *A. gallica* (E, brown) in Ludiano **(a)**, Lurengo **(b)**, and Dalpe **(c and d)**: Triangular symbols indicate soil samples with one genet (▲); Square symbols indicate soil samples with two genets of the same species (⊠) or with genets of different species (⊡); Circular symbols indicate stumps colonised by one genet (●), by two genets of the same species (⊕), and by genets of different species (⊙); x, soil samples without rhizomorphs; Arrow, slope direction (downhill). The black rectangles indicate the location of the subplot(s) in each plot.


Lud B1
10 m
N
Lud B3
Lud C1
Lud E1
Lud C3
Lud C4
Lud E2
Lud B2
Lud C2

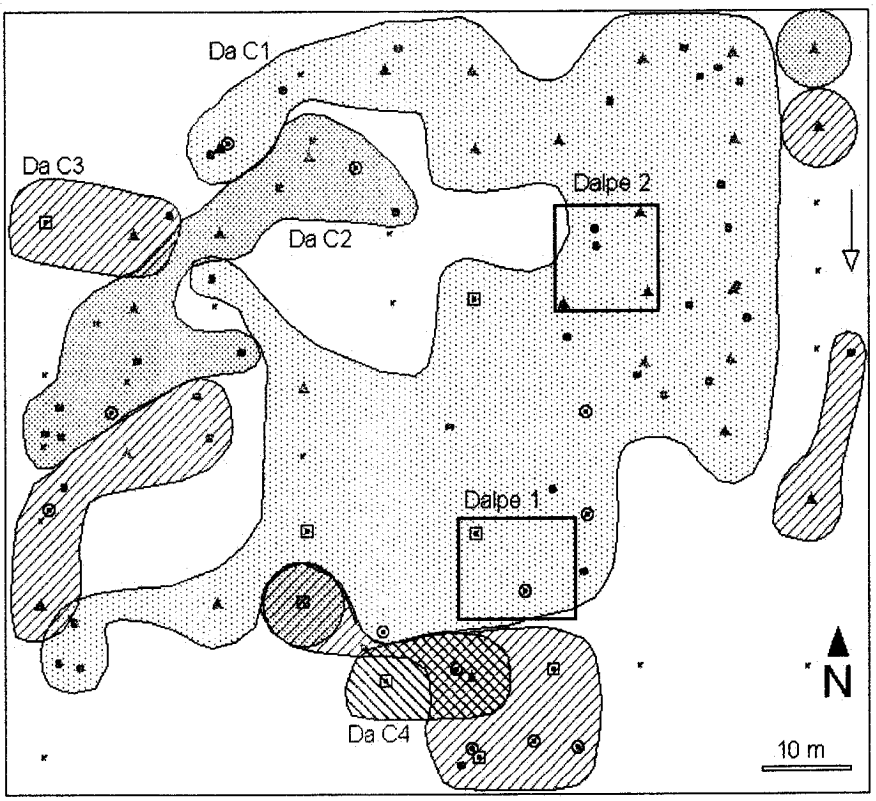
(a)


Lur C2
Lur C1
Lurengo 2
Lur B2
Lur B1
Lur C4
Lurengo 1
Lur C6
Lur C3
N
10 m
Lur C5

(b)


Da B3
10 m
N
Da B1
Dalpe 2
Da B2
Dalpe 1

(c)


Da C1
Da C3
Dalpe 2
Da C2
Dalpe 1
N
Da C4
10 m

(d)

2.3.2 Subplots

The five subplots were established in areas where a genet of *A. cepistipes* and *A. ostoyae* adjoined or overlapped in the soil (see Fig. 2.1a-d). The goal was to investigate the spatial patterns of genets along interspecific boundaries.

The incidence of soil samples with rhizomorphs varied from 59.7% (43 out of 72 in Lurengo 2) to 93.1% (67 out of 72 in Dalpe 1). Except in Dalpe 1 where about 90% of the territory was occupied by *Armillaria*, in the other four subplots contiguous areas without rhizomorphs in the soil were observed (Fig. 2.2a-e). In all subplots, positive samples contained on average two and a maximum of four (Ludiano 1, Lurengo 2) to six (Dalpe 1, Dalpe 2) rhizomorphs. The success of *Armillaria* isolation from rhizomorphs ranged from 91.6% (Dalpe 1) to 97.3% (Ludiano 1). Species identification showed that the incidences of *A. cepistipes* and *A. ostoyae* varied greatly among the subplots (Table 2.3). *A. cepistipes* was dominant in Ludiano 1 and Dalpe 1, whereas *A. ostoyae* was prevailing in the other three subplots. Most positive soil samples contained either rhizomorphs of *A. cepistipes* or *A. ostoyae* (Table 2.3). Nevertheless, overlaps between *A. cepistipes* and *A. ostoyae* genets, indicated by the finding of rhizomorphs of both species in a single soil sample, were detected in all five subplots (Table 2.3). The overlapping areas ranged from 4.2% (Lurengo 2) to 27.7% (Dalpe 2) of the total area occupied by *Armillaria*. Numerous genets of both *Armillaria* species showed a scattered and irregular distribution (e.g. Fig. 2.2c and d).

New *Armillaria* genets were found only in the subplots of Lurengo. One new genet (Lur C7) was located in the subplot Lurengo 1 (Fig. 2.2b) and five genets (Lur C8, Lur C9, Lur B3, Lur B4, and Lur B5) in the subplot Lurengo 2 (Fig. 2.2c). Four new genets (Lur B3, Lur B4, Lur B5, and Lur C9) were only found in a single soil sample, each with an estimated size of 2 m^2. Two new genets were larger. They were found in five and seven soil samples and covered an area of approximately 10 m^2 and 14 m^2.

2.3.3 PCR-RFLP analysis

The results of species identification with diploid-haploid pairings were confirmed with the PCR-RFLP analysis of a portion of the IGS region of the ribosomal DNA. Digestion of the PCR products with the enzyme *Alu* I showed that 10 out of 11 *A. cepistipes* genets belonged to the known restriction patterns type cep 1 or cep 2 (Pérez Sierra *et al.* 1999). All three isolates of the *A. cepistipes* genet Lud B1 showed a new *Alu* I restriction pattern with

Table 2.3. Incidence of *A. cepistipes* and *A. ostoyae* in the soil in the subplots, established along interspecific boundaries .

Subplot	N of samples		N of rhizomorphs per sample[b]	N of samples with rhizomorphs of[c]			Area covered by *Armillaria* spp.	
	Total	Positive[a]		*A. cep*	*A. ost*	*A. cep*+*A. ost*	Total (m^2)	Overlaps *A. cep.*+*A. ost*[d] (%)
Ludiano 1	61	39	1.9 ± 0.9	33	3	3	90	8.9
Lurengo 1	72	48	1.8 ± 1	18	27	3	105	19.0
Lurengo 2	72	43	1.7 ± 1	2	39	2	95	4.2
Dalpe 1	72	67	2.1 ± 1.1	46	6	15	148	27.7
Dalpe 2	57	41	2.3 ± 1.1	9	25	7	92	20.7
Total	334	238	2.0 ± 1.1	108	100	30	530	17.4

[a]Soil samples containing rhizomorphs.

[b]Number of rhizomorphs (mean value and standard deviation) in the positive soil samples.

[c]Number of positive samples containing rhizomorphs of *A. cepistipes* (*A. cep*), *A. ostoyae* (*A. ost*) or both species (*A. cep*+*A. ost*).

[d]Spatial overlaps between *A. cepistipes* and *A. ostoyae* genets given as percentages of the total area covered by *Armillaria*.

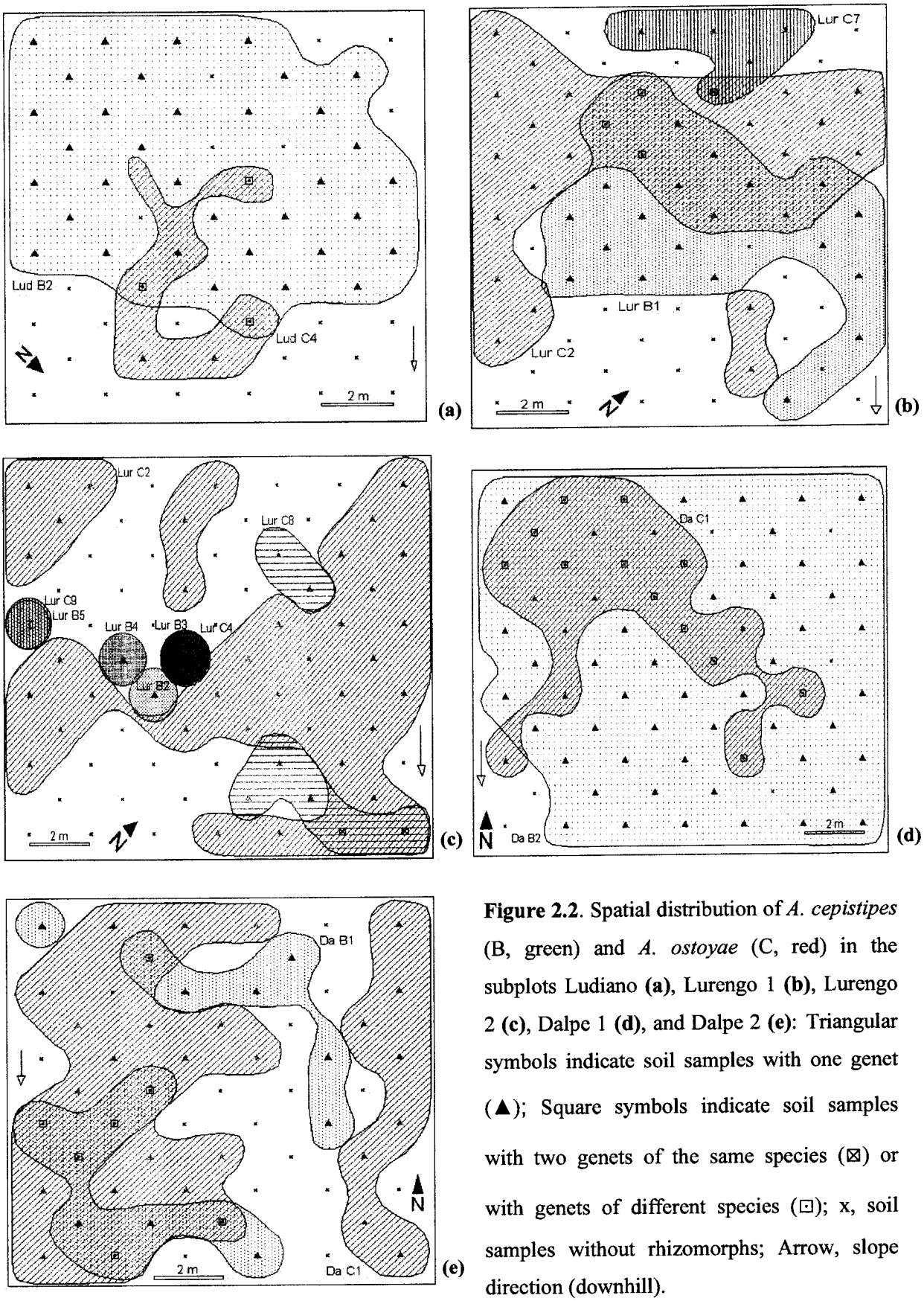


Figure 2.2. Spatial distribution of *A. cepistipes* (B, green) and *A. ostoyae* (C, red) in the subplots Ludiano **(a)**, Lurengo 1 **(b)**, Lurengo 2 **(c)**, Dalpe 1 **(d)**, and Dalpe 2 **(e)**: Triangular symbols indicate soil samples with one genet (▲); Square symbols indicate soil samples with two genets of the same species (⊠) or with genets of different species (⊡); x, soil samples without rhizomorphs; Arrow, slope direction (downhill).

fragment sizes of 600, 340, and 200 bp. This new pattern was characterised by a sum of the fragment sizes exceeding the size of the PCR product of 920 bp. This ambiguity could be due to an incomplete digestion or a microheterogeneity in the IGS regions of the ribosomal RNA genes. Within *A. ostoyae* and *A. gallica* no intraspecific variation was detected. The observed *Alu* I patterns corresponded with already published patterns (Harrington & Wingfield 1995, Pérez Sierra *et al.* 1999). The results with the enzymes *Hinc* II, *Mva 1269* I (*Bsm* I) and *Nde* I also confirmed those obtained in previous studies (Harrington & Wingfield 1995, Pérez Sierra *et al.* 1999).

2.4 Discussion

We conducted an intensive sampling in the soil and stumps in three comparable spruce stands to analyse the population structure of coexisting *Armillaria* species. *A. cepistipes* and *A. ostoyae* were shown to be the dominant species in all investigated spruce stands.

Based on somatic incompatibility, two to six genets of each *Armillaria* species were detected in each 1 ha plot. The resulting densities of seven to nine *Armillaria* genets per hectare are similar to those observed by Worrall (1994) in North America in plots with only *A. ostoyae*. In France, Legrand *et al.* (1996) found two study sites with *A. cepistipes*, *A. ostoyae*, and *A. gallica*, where the density was either five or six *Armillaria* genets per hectare. Most other species of wood-inhabiting basidiomycetes are generally characterised by more dense populations than those of *Armillaria*. For example, Kirby *et al.* (1990) found 19 genets of the wood-decay fungus *Resinicium bicolor* in a spruce stand of 1250 m^2 (152 genets per ha). Populations of the pathogenic *H. annosum* can be composed of up to 4800 genets per hectare (Piri *et al.* 1990, Swedjemark & Stenlid 1993). According to Hansen & Hamelin (1999), the density of a basidiomycete population, measured as the number of genets in a stand, is affected by substrate availability, frequency of opportunities for establishment, external disturbances (e.g. forest management), and intra- and interspecific competition.

The number of genets which can be detected probably depends on the intensity of sampling. The intensive main sampling conducted in our study has provided a representative view of the density of large and medium-sized *Armillaria* genets. However, the detection of additional very small genets in two subplots suggests that the actual number and density of genets is higher than estimated.

All *Armillaria* genets found on the stumps were also found in the soil. Thus, a systematic soil sampling seems to be sufficient to determine the occurrence and minimum density of *Armillaria* species and genets. Nevertheless, stump investigation can provide supplementary detailed information on the sizes and spatial distribution of genets. Delimiting the exact physical extent of genets, which is known to be difficult in studies of the population structure of soil-borne basidiomycetes (Anderson & Kohn 1995), was not a primary objective of this study. Nevertheless, the estimated sizes of the larger genets (0.3-0.4 ha) are comparable to those reported for *A. cepistipes* and *A. ostoyae* in coniferous and hardwood forests in central Europe (Legrand *et al.* 1996, Marxmüller & Holdenrieder 2000). Similar sizes of *A. ostoyae* genets were also observed by Smith *et al.* (1994) in a red pine (*Pinus resinosa* Aiton) seedling plantation in North America. The larger genets occurring in our plots probably further extend into the surrounding stands. Therefore, sampling on a larger scale would be necessary to determine the effective size of these genets. Some genets could also be smaller than estimated. Sampling in the five subplots demonstrated that, on a small scale, considerable gaps can exist within the territory occupied by *Armillaria*. These observations indicate that rhizomorphs of a genet are not evenly distributed in the soil, but considerably vary in density.

Several of the identified genets showed a non-contiguous, patchy distribution. The single patches can be considered as the physically and physiologically independent units of the genets, called ramets (Dahlberg & Stenlid 1994, Dettman & van der Kamp 2001b). Kile (1983) suggested that the probability of fragmenting increases with the size and age of a genet. The scattered genets observed in our plots could therefore represent remnants of larger genets. They could also be the result of an irregular vegetative growth from an initial point (e.g. colonised stump) through the production of rhizomorphs or spread via root contacts. Another possible explanation is that non-contiguous genets are inbred siblings (Kile 1983), which are not always distinguished by somatic incompatibility tests (Guillaumin *et al.* 1991). The highly scattered *A. ostoyae* genet Da C3 in Dalpe could have been created by past management operations. By dragging stems downhill it is possible that rhizomorphs or colonised woody debris were displaced and became established in another sector of the stand. Fragmented genets have also been observed in other rhizomorph- and cord-producing fungi, such as *Marasmius androsaceus* (Holmer & Stenlid 1991), *Tricholomopsis platyphylla* (Thompson & Rayner 1982), as well as in the ectomycorrhizal fungus *Suillus bovinus* (Dahlberg & Stenlid 1990). Fragmentation could be beneficial for the survival of a genet by impeding the spread of deleterious cytoplasmic elements. Fragmented genets could be the result of a famine-induced thinning of the rhizomorph network or mycelium because of

insufficient disposability of resources (Smith *et al.* 1994, Anderson & Kohn 1995).

The assessment of genet fragmentation is strongly affected by the specific criteria employed to identify non-contiguity between neighbouring isolates. In our study, two isolates of the same genet, distant $\leq$ 20 m, were considered to represent a contiguous genet. Decreasing this arbitrary distance would certainly increase the number of fragmented genets identified in our three plots. The quantitative estimates of spatial overlaps between genets also depend on the criteria adopted to define the limits of genets.

In our study, intraspecific spatial overlaps were rare and were generally limited to the borders of genets as has been observed in previous studies (Rizzo & Harrington 1993, Worrall 1994, Rizzo *et al.* 1995, Legrand *et al.* 1996). This suggests that genets of the same species are strongly antagonistic, probably because of somatic incompatibility and use of the same ecological strategy. Thus, the spatial distribution of a genet would be mainly affected by the position of adjacent genets of the same species (Rizzo & Harrington 1993, Smith *et al.* 1994). In Ludiano, no spatial overlaps between *A. cepistipes* and *A. ostoyae* were observed. The two species occupied different sectors of the plot and there is probably little interspecific interaction. In contrast, a genet of *A. cepistipes* partially overlapped with the two genets of *A. gallica*. The detection of *A. gallica* in Ludiano was unexpected, since this species typically occurs in hardwood forests at low altitudes (Guillaumin *et al.* 1993). Both *A. cepistipes* and *A. gallica* are preferentially saprotrophs and spatial overlaps among genets of the two species have been rarely observed (Legrand *et al.* 1996). In Lurengo, the two small genets of *A. cepistipes* were located within the territory occupied by an *A. ostoyae* genet, which could suggest a recent establishment. Whether these two *A. cepistipes* genets will gain size in the future at the cost of the *A. ostoyae* genet needs to be determined.

The third plot (Dalpe) was characterised by large overlaps between *A. cepistipes* and *A. ostoyae*. About 38% of the total territory covered by *Armillaria* was occupied by both species. Dalpe was also the plot with the highest incidence of fragmented and irregularly distributed genets. Therefore estimates of overlapping areas in this plot will strongly depend on the criteria adopted to define the boundaries and contiguity of a genet. Detailed sampling in the subplots, however, showed that spatial overlaps along interspecific boundaries are common in all three plots. Considerable overlaps between preferentially saprotrophic and pathogenic *Armillaria* species have been observed in other studies and have been assumed to indicate different colonisation strategies and resource partitioning between the species (Rizzo & Harrington 1993, Smith *et al.* 1994, Legrand *et al.* 1996, Baumgartner & Rizzo 2001). In our plots, this hypothesis is supported by the finding that *A. ostoyae* is more efficient in

primary stump capture than *A. cepistipes* (Chapter 3A).

Our results show that, in comparable Norway spruce stands where *A. cepistipes* and *A. ostoyae* coexist, the incidence of each species as well as the spatial patterns and sizes of genets can vary considerably. The factors determining this variation are not known. It can be assumed that site characteristics (e.g. exposition, soil, and topographical heterogeneity) and stand characteristics (e.g. history, management practise, and species composition) could influence the population dynamics of *Armillaria*. A general type of interaction between *A. cepistipes* and *A. ostoyae* is difficult to deduce from our results. On parts of the investigated areas, both species seem to exclude each other indicating competition, probably because of a similar ecological strategy. However, on other parts (e.g. in Dalpe) there were considerable interspecific overlaps, which could be explained by a more neutralistic co-existence with both species ignoring each other. Our study only gives a snapshot of a dynamic system. Further studies, focusing on the dynamic and experimental manipulation of local *Armillaria* populations are needed to examine the degree of competition between *A. cepistipes* and *A. ostoyae*.

3

Primary resource capture by *Armillaria cepistipes* and *Armillaria ostoyae*

A. Field investigation: Primary resource capture in two sympatric *Armillaria* species in managed Norway spruce forests*

*Prospero S, Holdenrieder O, Rigling D. 2003. *Mycological Research* 157: 329-338

Abstract

The ability of the preferentially saprotrophic fungus *Armillaria cepistipes* and the pathogenic *A. ostoyae* to capture fresh stump resources was investigated in managed Alpine Norway spruce (*Picea abies*) forests where both species occur sympatrically. The incidence of *Armillaria* species and genets as rhizomorphs in the soil as well as external and internal (heart rot) stump colonisation were determined in three comparable 1 ha plots. The results indicate that *A. cepistipes* and *A. ostoyae* have a very similar strategy to capture fresh stump resources. Both species produce dense networks of rhizomorphs in the soil enveloping living trees. After the felling of the trees, the stumps are rapidly captured through spread of mycelial fans in the cambial zone. Our study suggests that *A. ostoyae* is slightly more competitive than *A. cepistipes* in primary resource capture.

Key-words: *Armillaria cepistipes*, *Armillaria ostoyae*, stumps, primary resource capture.

3.1 Introduction

Competition is an important driving force in the biology of ecologically similar fungal species (Shearer 1995). In wood-decaying fungi, two stages of competition are commonly distinguished: primary resource capture, which describes the process of gaining initial access to an available resource, and secondary resource capture, referring to the combat for resources after their capture (Cooke & Rayner 1984). The consequences of combative competition can be either replacement of one fungus by the opponent or deadlock, where neither competitor gains headway (Boddy 2000).

Competition for available resources among wood-decaying fungi has the potential to be used for biocontrol of pathogenic wood-rotting fungi (Holdenrieder & Greig 1998, Boddy 2000). Many agents of root and butt rots of trees are facultative parasites, exhibiting a considerable saprotrophic ability. During their saprotrophic life phase, facultative parasites are vulnerable to competition from "aggressive" lignicolous saprotrophs (Holdenrieder & Greig 1998). In this context, the stumps created by forest management represent a specific and readily available resource. The colonisation of fresh stumps is important for the establishment, development, and survival of many root and butt rot fungi (Cooke & Rayner 1984). Therefore, stumps are privileged targets for biocontrol measures (Rayner & Todd 1979, Holdenrieder & Greig 1998).

Species of *Armillaria* occur worldwide and are ecologically significant fungi in forest ecosystems. They can behave as primary or secondary pathogens, causing root and butt rots on many species of woody plants in natural and planted forests, orchards, and gardens. The genus *Armillaria* also includes important wood-rotting saprotrophs (Shaw & Kile 1991). At present about 40 species of *Armillaria* are known (Watling *et al.* 1991, Pegler 2000). However, information about the biological and ecological characteristics, as well as the geographical distribution of each species, is still incomplete.

Current research is mainly focused on the highly pathogenic *Armillaria* species such as *A. ostoyae*, *A. mellea*, and *A. luteobubalina*. *A. ostoyae,* which occurs in Europe, North America, and Asia, attacks conifers especially (Guillaumin *et al.* 1993). Economic losses due to this species can be particularly serious in managed forests where stumps created by precommercial thinning can increase inoculum levels following the colonisation with *A. ostoyae* (Cruickshank *et al.* 1997). To date, the effective control of *A. ostoyae* is still problematic. A few methods are known, such as inoculation of stumps with antagonistic wood decay fungi (Chapman & Xiao 2000), removing stumps to reduce inoculum levels or specific

silvicultural strategies (e.g. planting less susceptible tree species) (Morrison & Mallett 1996), but their efficacy is not always satisfactory.

The preferentially saprotrophic species of *Armillaria* have received much less attention. For example, little information is available on the ecology of *A. cepistipes*, despite it being the most frequent rhizomorph-producing *Armillaria* species in the soil of mixed mountainous coniferous forests in central Europe (Legrand & Guillaumin 1993, Rigling *et al.* 1998, Marxmüller & Holdenrieder 2000). Above ground, however, *A. cepistipes* has been only rarely observed because it rarely forms fruiting bodies (Marxmüller *et al.* 1990).

Several *Armillaria* species can coexist in small plots in various forest types worldwide (e.g. Kile 1986, Worrall 1994, Legrand *et al.* 1996, Dettman & van der Kamp 2001a). In Europe, *A. cepistipes* and *A. ostoyae* are among the *Armillaria* species which can occur sympatrically within the same forest stand and may even colonise the same substrate (Legrand *et al.* 1996, Marxmüller & Holdenrieder 2000). Therefore, *A. cepistipes* might effectively compete with *A. ostoyae* for resources (Holdenrieder & Greig 1998). Understanding the interaction between *A. cepistipes* and *A. ostoyae in situ* will provide important information about the potential use of *A. cepistipes* for the biocontrol of *A. ostoyae* and possibly other root and butt rot fungi.

The study reported here aimed to determine the importance of fresh Norway spruce (*Picea abies*) stumps as a resource for *A. cepistipes* and *A. ostoyae* coexisting sympatrically in managed forests and to investigate the ability of these two species in primary stump capture. Specific questions addressed were: (1) to what proportion are stumps colonised by *Armillaria* spp.?; (2) what strategy do *A. cepistipes* and *A. ostoyae* use to capture fresh stump resources?; and (3) what is the mutual competitive ability of the two species to capture stumps along interspecific boundaries? In investigating these questions, we first determined the incidence of *Armillaria* species and genets as rhizomorphs in the soil and then followed the colonisation of fresh stumps over a period of three years.

3.2 Materials and Methods

3.2.1 Study sites

The three study sites are located at about 1400 m a.s.l. in the southern part of the Swiss Alps, 5-17 km apart (Table 3.1). This region is characterised by an acidic subsoil (gneiss) and a temperate climate with about 1600 mm annual precipitation. Each site is covered with a

naturally regenerated, managed spruce stand belonging to the vegetation type *Calamagrostio villosae-Abietetum* (Keller *et al.* 1998), which is the most widespread vegetation at the mountainous level (1100-1600 m a.s.l.) in southern Switzerland. All the stands are dominated by Norway spruce. Additional species are European larch (*Larix decidua*), several pioneer broadleaved species (*Sorbus aucuparia*, *Salix caprea* and *Betula pendula*) and, in one stand (Dalpe), Silver fir (*Abies alba*). The stands are comparable in age (140-160 yr) and past management practice. A preliminary soil sampling has revealed the presence of rhizomorphs of *A. cepistipes* and *A. ostoyae* in all three stands. No mortality on mature trees caused by *Armillaria* was observed in the three stands.

At each site, a 1 ha plot (100 x 100 m) was established in 1999 containing standing trees and a large number of one- to three-year old stumps (Table 3.1). Tree species, size (DBH of standing trees; diameter of stumps), and tree status (living or dead) were recorded. All trees and stumps over 12 cm diameter were numbered and mapped. The surface of the stumps was examined for visual signs of heart rot (grey-brown or yellow-brown discoloration or decay in the heartwood).

In summer 2000, five supplementary subplots, each with a size of 144-168 m^2 (12 x 12-14 m) and containing 1-4 stumps, were established in areas where *A. cepistipes* and *A. ostoyae* genets adjoined or overlapped in the soil. One subplot was in Ludiano (Ludiano 1), two were in Lurengo (Lurengo 1 and Lurengo 2), and two in Dalpe (Dalpe 1 and Dalpe 2).

3.2.2 Sampling

In each plot a 10 x 10 m square grid was established. Systematic sampling was conducted by taking a cube of soil (15 cm side length) at each grid point with a small spade. In the five subplots, soil samples were first taken on a 2 x 2 m square grid. Subsequently, additional samples were taken in the middle of each 2 x 2 m square. The soil samples were sieved through a 9 mm square mesh to separate the soil from the roots and rhizomorphs. All the rhizomorphs from a sample were put in a plastic bag together with approx. 400 cm^3 topsoil (0-20 cm). The soil was later used for pH measurement as described by Scheffer & Schachtschabel (1998). In the laboratory, the collected rhizomorphs were carefully washed under running tap water and blotted dry between paper towels. The number of individual rhizomorphs in each sample was recorded. The length and thickness of each rhizomorph from the 10 x 10 m sampling were measured from digital images of each rhizomorph with the

Table 3.1. Topographical and silvicultural characteristics of the three study plots.

Plot	Coordinates	Altitude[a] (m a.s.l.)	Topsoil pH[b]	Growing stock[c] (m^3 ha^{-1})	Living trees			Stumps			
					No.	DBH[d] (cm)	Spruce[e] (%)	No.	Diam[f] (cm)	Spruce (%)	Age[g] (Years)
Ludiano	46° 25' 26'' N 8° 56' 28'' E	1400	3.48 ± 0.05	300	98	31	77	217	45	92	1
Lurengo	46° 30' 5'' N 8° 44' 55'' E	1440	3.66 ± 0.03	300	90	33	96	203	46	99	2-3
Dalpe	46° 27' 50'' N 8° 47' 25'' E	1360	3.61 ± 0.03	350	129	42	43	138	50	65	1-2

[a]Altitude at the centre of the plot.

[b]Average value and standard error of the pH in the 100 soil samples.

[c]Before the last silvicultural intervention (Source: Forest service of the Canton Ticino).

[d]Average diameter at 1.3 m height. Only trees with DBH > 12 cm were considered.

[e]Percent of Norway spruce (*Picea abies* (L.) Karst.).

[f]Average diameter at 0.3 m height. Only stumps with diameter > 12 cm were considered.

[g]Age in 1999, at the beginning of the study.

computer program WinRHIZO (Régent Instruments, Quebec). The dry weight was determined after drying the rhizomorphs at 60°C for 15 h. The density of rhizomorphs in each plot was calculated as mean length of all soil samples given in metres of rhizomorphs m^{-2}. The densities of *A. cepistipes* and *A. ostoyae* rhizomorphs in the soil were estimated by considering: (1) all those samples containing rhizomorphs of only one *Armillaria* species, and (2) samples containing rhizomorphs of both species with all rhizomorphs identified to species as described below.

During autumns 1999, 2000, and 2001, all stumps were examined for external colonisation by *Armillaria*. In each year, one third of the sequentially numbered stumps were systematically (1999: 1,4,7, ...; 2000: 2,5,8, ...; 2001: 3,6,9, ...) selected from all over the plot. Portions of bark were removed from the collar region of three main lateral roots using an axe and the presence of subcortical mycelial fans or rhizomorphs was recorded. When necessary, ground vegetation and soil above the root collar were removed. A piece of wood with mycelial fans or rhizomorphs on the surface was removed from each colonised root collar and brought to the laboratory for isolations. All accessible (3-5) lateral roots on the stumps located in the subplots were examined for *Armillaria* colonisation as above. Additionally, in order to determine the depth of *Armillaria* penetration into the wood, core samples were taken from the same roots at a distance of 10-30 cm from the stump by means of an increment borer driven by hand. The cores had a length of 7-8 cm and 5 mm diameter. Before each sampling, the increment borer was dipped in 70% ethanol and dried with paper towels. For isolation, the increment cores were brought to the laboratory.

The incidence of *Armillaria* in heart rots, referred to as internal stump colonisation, was investigated by analysing 35-40 selected stumps in each plot. These stumps showed a beginning or an advanced decay in the heartwood and were distributed all over the plot. From one half of the stumps, a disk 5-10 cm thick was cut using a chain saw, after sawing off the top of the stump. From the second half of the stumps, two core samples (5 mm diameter) that penetrated the decayed heartwood were taken by means of an increment borer. The collected discs and core samples were taken to the laboratory for further analysis.

3.2.3 Isolation of *Armillaria* and other decay fungi

After washing, the collected rhizomorphs were first dipped in 50% ethanol for 15-20 seconds. Then, from each rhizomorph three segments of 1 cm length were surface sterilised in 30% hydrogen peroxide (H_2O_2) for 25-40 seconds (depending on the thickness of the rhizomorphs)

and placed on a Petri plate (8.5 cm diam) containing malt extract agar (12 g l^{-1} malt extract; 15 g l^{-1} Bacto Agar) amended with 2 mg l^{-1} benomyl and 100 mg l^{-1} streptomycin (Maloy 1974).

The pieces of wood with subcortical mycelial fans were washed under running tap water with a brush and blotted dry between paper towels. For each sample, six small pieces (2-5 mm x 2-5 mm) of mycelium were surface sterilised in sodium-hypochlorite (7% active chlorine; Chemische Fabrik Schweizerhalle, Basel) for 5-10 seconds and rinsed in sterile distilled water for 10-15 seconds (Prospero *et al.* 1998). The samples were dried between paper towels and placed on modified MATS plates (Legrand & Guillaumin 1993) consisting of 20 g l^{-1} malt extract, 15 g l^{-1} Bacto Agar, 230 mg l^{-1} thiabendazole in 1 ml concentrated lactic acid, and 100 mg l^{-1} streptomycin, but omitting polymyxin sulphate and sodic benzylpenicillin.

Fungi from the stump disks were isolated as follows. From the margin of each decay column, six small cubes approx. 5 mm side length were cut out and surface sterilised in sodium-hypochlorite (7% active chlorine) for 20-30 seconds. After rinsing in sterile distilled water for 10-15 seconds, the samples were blotted dry between paper towels and placed on MATS plates. The increment cores taken from the roots and stumps with heart rot were also placed on MATS plates according to the same surface sterilisation treatment. Each core sample taken from the roots was put on a single MATS plate and care was taken to indicate the bark side (external side) of the core. The depth of *Armillaria* penetration into the roots was indicated by the occurrence of typical brushes of aerial hyphae on the cores after 1-2 weeks incubation.

All isolation plates were incubated in the dark at 20-25°C. After 1-3 weeks, pure cultures were transferred to malt extract agar (15 g l^{-1} Bacto Agar; 20 g l^{-1} Diamalt, Hindelbank, Switzerland).

3.2.4 Identification of *Armillaria*-genets and -species

Genets were identified as somatic incompatibility groups by pairing isolates on Shaw and Roth's medium as described by Harrington *et al.* (1992). For species identification, three isolates of each genet were paired with haploid tester strains of the five European annulate *Armillaria* species (Korhonen 1978) according to Harrington *et al.* (1992). For each species, we used three different tester strains (provided by Jean-Jacques Guillaumin, INRA, Clermont-Ferrand), which were specifically selected for pairings with diploid mycelia. Mycelial plugs (3-4 mm side), cut from the margin of growing cultures of the unknown isolates and the

testers, were placed approx. 5 mm apart on malt extract agar. The Petri plates (8.5 cm diam), which each contained three pairings, were incubated in the dark at 20-25°C. Species identification was considered successful if, after 4-6 weeks, at least two of the three testers of the same species lost the original fluffy and white culture morphology and became flat and crustose (Guillaumin *et al.* 1991), while the testers of the other species remained unchanged. Representative isolates are deposited in the culture collection of the WSL.

3.2.5 Statistical analysis

Data were analysed using the program DataDesk Version 6 (Velleman, 1997). For the quantitative variables of the rhizomorphs and for the pH values, one-way analysis of variance (ANOVA) was performed. The abundance (dry weight) of rhizomorphs in a soil sample was analysed by linear regression using the distance from the nearest stump and the pH of the soil sample as the independent variables. The frequencies of *Armillaria* species in the soil and on the stumps were compared using a chi-square test. This test was also performed to analyse the *Armillaria* spp. colonisation rates of stumps with and without heart rot.

3.3 Results

3.3.1 Rhizomorphs in the soil

Rhizomorphs were found in 40 (Ludiano), 49 (Lurengo), and 63 (Dalpe) of the 100 soil samples collected in each plot. In all the plots, positive samples contained on average two, a minimum of one and a maximum of six individual rhizomorphs. The most extensive networks of *Armillaria* rhizomorphs in the soil were found in Dalpe (mean length 14 ± 1.7 m m^{-2} SEM) and Ludiano (mean length 13 ± 2.6 m m^{-2} SEM). In Lurengo, subterranean rhizomorphs were considerably less abundant (mean length 7 ± 0.9 m m^{-2} SEM).

The mean distance between a sample point and the nearest stump was 3.4 ± 0.2 m SEM in Lurengo, 4.4 ± 0.3 m SEM in Dalpe, and 4.7 ± 0.5 m SEM in Ludiano. In all three plots, the abundance (dry weight) of rhizomorphs in a soil sample did not significantly correlate with the distance between the sample point and the nearest stump (Ludiano: $R^2 = 0.01$, $P = 0.251$; Lurengo: $R^2 = 0.06$, $P = 0.101$; Dalpe: $R^2 = 0.005$, $P = 0.505$). There was also no correlation between the pH of the topsoil and the abundance of rhizomorphs in a soil sample (data not shown).

257 *Armillaria* isolates were obtained from 328 rhizomorphs with a success of isolation of 68% in Lurengo, 79% in Dalpe, and 88% in Ludiano. *A. cepistipes* and *A. ostoyae* were found in all three plots. In addition, 16% of the isolates in Ludiano belonged to *A. gallica*. Species identification with diploid-haploid pairings was unequivocal. Analysis of variance showed that the rhizomorphs of *A. cepistipes* were significantly thicker and heavier than those of *A. ostoyae* (Table 3.2). Rhizomorphs of *A. gallica* were not further characterised because of their limited number (14 individual rhizomorphs) and the fact that they occurred in only one plot. In all three plots, *A. cepistipes* produced a more dense network of rhizomorphs in the soil than *A. ostoyae*. For both *Armillaria* species, the variation in density among samples within a plot was considerable. Considering all plots, the mean density of *A. cepistipes* rhizomorphs (25.3 ± 2.4 m m^{-2} SEM) was significantly ($P < 0.001$) higher than those of *A. ostoyae* (14.9 ± 1.2 m m^{-2} SEM).

Table 3.2. Characteristics of the *Armillaria cepistipes* and *A. ostoyae* rhizomorphs.

Characteristics	*A. cepistipes*	*A. ostoyae*	*P*-Value
No. of rhizomorphs	141	102	
Diameter (mm)	1.1 ± 0.03[a]	0.9 ± 0.03	< 0.0001
Dry weight (mg cm^{-1})	5.1 ± 0.38	2.9 ± 0.16	< 0.0001
Density (m m^{-2})	25.3 ± 2.4	14.9 ± 1.2	< 0.001

[a]Mean values and standard errors are given.

3.3.2 External stump colonisation

In the first investigation in 1999, the proportion of Norway spruce stumps with subcortical mycelial fans or rhizomorphs of *Armillaria* ranged from 41.3% in Ludiano to 73.3% in Dalpe (Silver fir: 69.2%) and 78.9% in Lurengo (Table 3.3). Additional surveys in 2000 and 2001 revealed only little change in the proportion of stumps colonised in Ludiano and Lurengo. By contrast, an increase of 20.3% between 1999 and 2001 was observed in Dalpe (Silver fir: increase of 24.1%). Considering the results of all three investigations, the incidence of stump colonisation also varied among plots (Table 3.3). In Ludiano, 50.6% of the colonised stumps

Table 3.3. Incidence of external stump colonisation (i.e. subcortical mycelial fans or rhizomorphs) by *Armillaria*.

	Stumps						% colonised stumps with 1, 2 or 3 colonised roots[a]		
			Age[b]	*Armillaria* colonisation[c] (%)					
Plot	No.	Species	(Years)	1999	2000	2001	1 root	2 roots	3 roots
Ludiano	199	Norway spruce	1	41.3	36.7	36.2	50.6	31.2	18.2
Lurengo	202	Norway spruce	2-3	78.9	82.8	76.1	31.8	38.8	29.4
Dalpe	89	Norway spruce	1-2	73.3	92.9	93.6	27.3	20.8	51.9
	44	Silver fir	1-2	69.2	93.8	93.3	15.8	21.1	63.1

[a]All colonised stumps observed in 1999, 2000, and 2001 are included.

[b]Age in 1999, at the first examination.

[c]Colonised stumps were characterised by the presence of subcortical mycelial fans or rhizomorphs on one to three main lateral roots. One third of the stumps were investigated each year.

Table 3.4. Incidence of *A. cepistipes* and *A. ostoyae* in the soil and on the stumps.

	Ludiano			Lurengo			Dalpe		
	No.[a]	*A. cep* (%)	*A. ost* (%)	No.	*A. cep* (%)	*A. ost* (%)	No.	*A. cep* (%)	*A. ost* (%)
Soil[b]	33	81.8	18.2	49	4.1	95.9	61	73.8	37.7
Stumps[c]	53	83.0	17.0	111	1.8	99.1	95	55.8	56.8
Chi-square test[d]	$\chi^2 = 0.02$, $P = 0.887$			$\chi^2 = 0.742$, $P = 0.389$			$\chi^2 = 4.674$, $P = 0.03$		

[a]Number of soil samples or stumps from which *Armillaria* spp. could be isolated. In Ludiano, *A. gallica* was recovered from four soil samples and 10 stumps, which are not included in this table.

[b]Within a plot, a sum of the *A. cepistipes* and *A. ostoyae* incidence exceeding 100 % indicates the presence of soil samples containing rhizomorphs of both species (Dalpe: seven samples).

[c]Within a plot, a sum of the *A. cepistipes* and *A. ostoyae* incidence exceeding 100 % indicates the presence of stumps colonised by both species (Lurengo: one stump; Dalpe: 12 stumps).

[d]Chi-square statistics to test for differences between species incidence in the soil and on the stumps.

showed only one root externally colonised by *Armillaria*. In Lurengo and Dalpe most of the colonised stumps (68.2% and 76.5%) had 2-3 lateral roots with signs of *Armillaria*. Stumps with subcortical rhizomorphs were frequent in Ludiano (62.3%). In Lurengo and Dalpe, the majority (86.3% and 67.5%) of the *Armillaria* colonised stumps were characterised by the presence of subcortical mycelial fans.

Pure cultures of *Armillaria* were obtained from 76.1% of the 352 colonised stumps. *A. cepistipes* and *A. ostoyae* were isolated from the stumps in all the plots. In addition, in Ludiano 12 Norway spruce stumps were colonised by *A. gallica*. Stumps colonised by both *A. cepistipes* and *A. ostoyae* were observed in Lurengo (one of 111 stumps) and Dalpe (12 of 95 stumps). The incidence of each species on the stumps varied greatly among the plots and was generally related to the species frequency in the soil (Table 3.4). In Ludiano and Lurengo, the species which was dominant in the soil (*A. cepistipes* and *A. ostoyae*, respectively) also prevailed on the stumps. In Dalpe, *A. cepistipes* and *A. ostoyae* were observed with practically the same frequency on the stumps, even though *A. cepistipes* was more frequently found in the soil samples (Table 3.4). This difference in species frequencies between soil and stumps was statistically significant (chi-square test: $\chi^2 = 4.674$, $P = 0.03$).

Table 3.5. Percent of stumps colonised by an *Armillaria cepistipes* or *A. ostoyae* genet that occurs in the nearby soil.

	A. cepistipes		*A. ostoyae*	
Plot	Stumps[a] (No.)	Colonised by a genet occurring in the nearby soil (%)[b]	Stumps (No.)	Colonised by a genet occurring in the nearby soil (%)
Ludiano	44	93.2	9	77.8
Lurengo	2	50.0	110	86.8
Dalpe	53	86.8	54	72.2
Total	*99*	*88.9*	*173*	*81.8*

[a]Number of stumps colonised by the *Armillaria* species indicated. One stump in Lurengo and 12 stumps in Dalpe were colonised by both *Armillaria* species.

[b]Percent of stumps colonised by an *A. cepistipes* or *A. ostoyae* genet occurring in at least one of the four nearest soil samples. If no rhizomorphs of *Armillaria* were found in the four soil samples, the next nearest positive sample was considered.

In all three plots, the majority of the stumps were colonised by an *A. cepistipes* or *A. ostoyae* genet that occurred in at least one of the four nearest sample points in the soil (Table 3.5). The same situation was observed in Ludiano for the 12 stumps colonised by *A. gallica*. The number of stumps colonised by an A. *cepistipes* or *A. ostoyae* genet increased with genet size in the soil, expressed as the number of positive soil samples (Fig. 3.1). The relationship was a little stronger and the regression line steeper for *A. ostoyae* than for *A. cepistipes*.

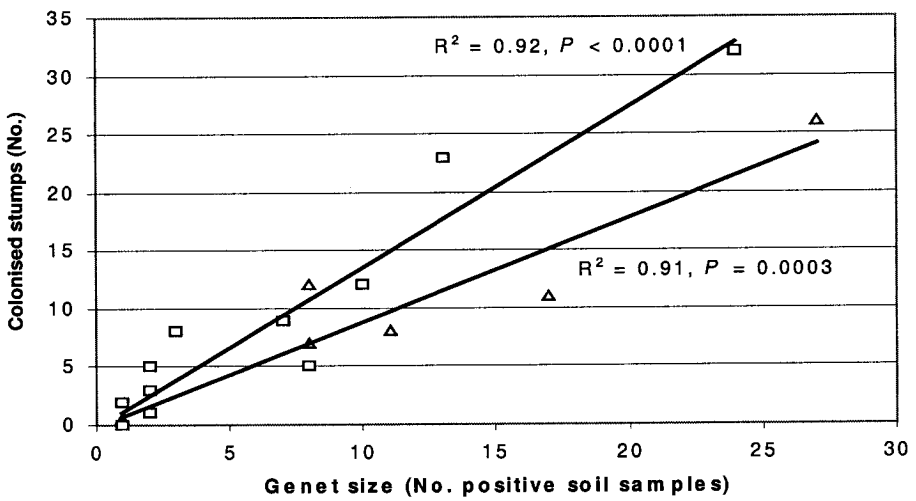


Figure 3.1. Relationship between genet size of *A. cepistipes* (triangles) and *A. ostoyae* (squares) and number of colonised stumps. To account for differences in stump density among plots, the observed number of stumps colonised by a genet was divided by the stump density (No. stumps 100 m^{-2}) in the plot.

3.3.3 Internal stump colonisation

In the three plots, the frequency of Norway spruce stumps with a visible decay in the heartwood varied from 21.7% (Lurengo) to 22.3% (Ludiano) and 41.4% (Dalpe; Silver fir: 23.9%). In Ludiano and Dalpe approximately a quarter (23.8% and 24.4%) of the stumps with heart rots showed signs of wounding, which was probably caused by falling rocks or logging before the trees were felled. Cultures were isolated from the heart rots in 79.8% of the 114 analysed stumps (Table 3.6). 35 isolates (38.5%) belonged to *A. ostoyae*, *A. cepistipes*, *Heterobasidion annosum*, *Climacocystis borealis*, or to the genus *Sistotrema*. *H. annosum* and *Sistotrema* spp. were isolated in all the plots from both initial and advanced decays with a frequency of 10.5% and 5.3%, respectively (Table 3.6). *C. borealis* was found with a

frequency of 5.3% in initial decays in Lurengo. *Armillaria* species were isolated from 13.2% of the heart rots and was almost exclusively associated with advanced decays. In Ludiano and Dalpe, only *A. cepistipes* was isolated from decayed heartwood, whereas in Lurengo only *A. ostoyae* (Table 3.6).

Table 3.6. Isolation of fungal species associated with heart rot (internal stump colonisation).

Plot	Stumps[a] (No.)	Isolated fungal species (No.)[b]						No fungi isolated (No.)
		A. cep	*A. ost*	*H*	*C*	*S*	*U*	
Ludiano	35	5	0	3	0	1	17	9
Lurengo	38	0	3	7	2	3	21	2
Dalpe	41	7	0	2	0	2	18	12
Total	*114*	*12*	*3*	*12*	*2*	*6*	*56*	*23*

[a]Number of analysed stumps with heart rot.

[b]*A. cep* = *Armillaria cepistipes*; *A. ost* = *Armillaria ostoyae*; *H* = *Heterobasidion annosum*; *C* = *Climacocystis borealis*; *S* = *Sistotrema* spp.; *U* = other unidentified species. *H. annosum*, *C. borealis*, and *Sistotrema* ssp. were identified using the key given in Stalpers (1978).

Among all the stumps showing heart rot, 43.2% in Ludiano, 65.1% in Lurengo, and 88.1% in Dalpe were externally colonised by *Armillaria*. In Ludiano and Dalpe no significant difference in the incidence of external *Armillaria* colonisation between stumps with and without heart rot was observed. By contrast, in Lurengo external stump colonisation by *Armillaria* was significantly (chi-square test: $\chi^2 = 7.345$, $P = 0.007$) lower on stumps with heart rot than on those without heart rot.

3.3.4 Subplots

To investigate the mutual competitive ability of *Armillaria cepistipes* and *A. ostoyae* to capture stump resources, subplots were established along interspecific boundaries. The proportion of soil samples containing rhizomorphs varied greatly among the five subplots, ranging from 59.7% (Lurengo 2) to 93.1% (Dalpe 1). Species identification showed that in all subplots both *A. cepistipes* and *A. ostoyae* were, as expected, present in the soil.

Table 3.7. Incidence of *A. cepistipes* and *A. ostoyae* in the soil and on the stumps in five subplots established along interspecific boundaries.

Plot	Stump	Soil		Stump colonisation		
		Samples[a] (No.)	Occurrence of *A. cep/A. ost*[b]	Subcortical[c] *A. cep/A. ost*	Wood *A. cep/A. ost*[d]	Wood Depth (cm)
Ludiano1	746	11	10/2	0/0	2/0	3.5
Ludiano1	748	13	12/1	0/1	1/0	2
Lud total	*2*	*24*	*22/3*	*0/1*	*3/0*	*3*
Lurengo1	1347	12	6/8	0/4	0/3	0.7
Lurengo1	1400	5	3/2	0/0	0/1	0.5
Lurengo1	1401	14	3/13	0/3	0/1	2.3
Lurengo1	1402	11	10/2	½	0/0	0
Lurengo2	1321	12	0/12	0/3	0/1	1.8
Lur total	*5*	*54*	*22/37*	*1/12*	*0/6*	*1.1*
Dalpe 1	2041	15	10/11	2/0	1/0	0.1
Dalpe1	2044	14	14/3	0/2	2/3	3.4
Dalpe 1	2046	12	11/2	2/0	3/0	0.8
Dalpe 2	1895	7	1/7	0/4	0/1	0.2
Dalpe 2	1896	11	5/8	0/4	0/4	1.7
Da total	*5*	*59*	*41/31*	*4/10*	*6/8*	*1.5*
Total	*12*	*137*	*85/71*	*5/23*	*9/14*	*1.4*

[a]Number of positive sample points located within a circle of 3 m radius around the stump (total samples: 13-16).

[b]Occurrence of *A. cepistipes* and *A. ostoyae* in the soil samples. A sum of soil samples with *A. cepistipes* and *A. ostoyae* exceeding the total number of positive soil samples indicates the presence of soil samples containing rhizomorphs of both species.

[c]Number of main lateral roots showing subcortical mycelial fans or rhizomorphs of *A. cepistipes* and *A. ostoyae*. For each stump, all accessible (three to five) roots were analysed.

[d]Number of increment cores with *A. cepistipes* and *A. ostoyae* taken from the roots.

All the 12 stumps analysed in the subplots were colonised by *Armillaria*. Ten stumps showed one to four main lateral roots with subcortical mycelial fans or rhizomorphs (Table 3.7). In two stumps, *Armillaria* was only found in the wood. The mean penetration depth of *Armillaria* into the wood of the roots varied from 1.1 cm (Lurengo) to 3 cm (Ludiano). In 53.6% of the roots with mycelial fans or rhizomorphs in the cambial zone, *Armillaria* was also detected in the wood. In all cases, the same *Armillaria* species and genet were isolated from mycelial fans or rhizomorphs and from the wood.

Only soil samples within a circle of 3 m radius from each stump were considered when comparing species incidence in the soil and on the stumps. Considering all subplots, the two species showed a similar incidence (ratio *A. cepistipes/A. ostoyae*: 85/71) in the soil samples (Table 3.7). Nevertheless, *A. ostoyae* was more frequent on the stumps, both in the cambial zone (ratio *A. cepistipes/A. ostoyae*: 5/23) and in the wood (ratio *A. cepistipes/A. ostoyae*: 9/14). This tendency was particularly evident in Dalpe and Lurengo (Table 3.7).

3.4 Discussion

The objectives of this study were to determine the importance of fresh Norway spruce stumps as a resource for *Armillaria cepistipes* and *A. ostoyae* and to investigate the ability of these two species in primary stump capture. For this purpose, the incidence of *Armillaria* species and genets in the soil and on stumps (external and internal colonisation) in three stands was compared.

Both *Armillaria* species produced rhizomorphs intensively in the soil, with *A. cepistipes* dominating in Ludiano and Dalpe and *A. ostoyae* in Lurengo. The ability of *A. cepistipes* to produce abundant rhizomorphs has been previously reported (Legrand *et al.* 1996, Rigling *et al.* 1998). In addition, our study shows that *A. ostoyae* can develop considerable networks of rhizomorphs in spruce forests even if the produced rhizomorphs are thinner and less abundant than those of *A. cepistipes*. The high density of rhizomorphs in the soil could have been stimulated by the past management of the stands. Following colonisation of stumps created by silvicultural operations, *Armillaria* can produce new rhizomorphs until the food-bases are completely decayed (Rishbeth 1972, Stanosz & Patton 1991).

The extent of external stump colonisation (i.e. subcortical mycelial fans or rhizomorphs) by *Armillaria* in these mountainous spruce forests was considerable and emphasises the ecological role of this fungal genus as a wood decomposer. Within 3-4 years after felling, 40-

90% of the Norway spruce stumps were captured by *Armillaria*. Most of the stumps had two to three lateral roots with signs of *Armillaria* spp. A similar extent of colonisation was observed for Silver fir stumps in Dalpe. High colonisation rates by *Armillaria* were also detected on two-year old aspen (*Populus tremuloides*) stumps in Wisconsin (Stanosz & Patton 1990). Cruikshank *et al.* (1997) found that, in four biogeoclimatic zones of British Columbia two years after thinning, 12-51% of the Douglas fir (*Pseudotsuga menziesii*) stumps had been colonised by *A. ostoyae*. Our study shows that when *Armillaria* occurs in the soil, it will colonise nearby stumps. Low incidence of stump colonisation was only observed in areas without *Armillaria* rhizomorphs in the soil, e.g. in certain areas in Ludiano.

As our data show, stump capture by *Armillaria* occurs primarily within one to three years following cutting. After this period, the proportion of colonised stumps tends to remain constant. It is generally assumed that a fresh stump can be captured in four ways (Redfern & Filip 1991) by (1) invasion from outside by newly arriving rhizomorphs; (2) invasion from already present epiphytic rhizomorphs which are sometimes abundant (Stanosz & Patton 1991, Marçais & Wargo 2000); (3) rapid extension from existing lesions on the roots; or (4) germination of basidiospores on the cut surface. According to Rishbeth (1988), this last mode of colonisation is rare, occurring only under particular conditions. In our plots, the high incidence of stumps colonised by an *Armillaria* genet occurring in the soil in the proximity of the stump suggests capture by invading rhizomorphs. Whether these rhizomorphs were epiphytic or newly arrived from the rhizomorph network surrounding the tree is not known. We can also not rule out that the rhizomorphs first initiated lesions on the roots of living trees, which extended after the trees were cut. In any case, the ability of *Armillaria* to produce an extensive network of rhizomorphs enveloping living trees as potential food-bases is probably decisive for rapid substrate capture. After penetration into the cambial zone of the stump, the effective colonisation mostly occurs through subcortical mycelial fans.

Both *A. cepistipes* and *A. ostoyae* were efficient stump colonisers. Thus, fresh stumps represent an important resource for the survival of the two species in managed forests. In two plots (Ludiano and Lurengo), the *Armillaria* species strongly prevailing in the soil was also the most frequent on the stumps. In Dalpe, the two species were found with a similar frequency on the stumps, although *A. cepistipes* was more frequent than *A. ostoyae* in the soil. This observation could indicate a better ability of *A. ostoyae* in capturing stumps. A prevalence of *A. ostoyae* on stumps and *A. cepistipes* in the soil was also observed by Legrand *et al.* (1996). The finding that, by a given size, a genet of *A. ostoyae* colonises more stumps than a genet of *A. cepistipes* also seem to confirm the above hypothesis. The results of the

subplots further support this conclusion. Stumps located along interspecific boundaries were more frequently captured by *A. ostoyae* than by *A. cepistipes*. It has been reported that *A. ostoyae* can cause lesions on the roots of living conifers (Morrison *et al.* 2000). In inoculation experiments on Norway spruce seedlings we have observed that *A. ostoyae* produce much more root lesions than *A. cepistipes* (Chapter 4). Therefore, one could hypothesise that *A. ostoyae* has a distinct advantage over *A. cepistipes* in capturing stumps because it is more frequently present in root lesions.

The unexpected detection of *A. gallica* on Norway spruce stumps in Ludiano indicates its ability to colonise conifer stumps as previously observed by Marxmüller *et al.* (1990). In Europe, *A. gallica* is common at low altitudes and generally associated with hardwoods (Guillaumin *et al.* 1993). We found *A. gallica* at 1400 m a.s.l. in Ludiano, which is one of the highest habitats of this species in Europe. Even at the edge of its distribution, *A. gallica* shows a similar strategy in primary resource capture as *A. cepistipes* and *A. ostoyae*.

Stumps with heart rot (internal colonisation) represent a special category of stumps since heartwood capture has occurred while the trees were still standing and alive. However, these stumps also contribute to the survival of facultative parasitic fungi and can act as an additional source of inoculum. *Armillaria* was isolated from 8 to 17% of the analysed heart rots of Norway spruce and Silver fir. This incidence is within the range observed in other studies in Europe (Schönhar 1993, Graber 1994, Holdenrieder *et al.* 1994). When comparing internal with external mode of stump capture, it becomes evident that the latter form is much more important for *Armillaria*.

In one plot (Lurengo), only *A. ostoyae* was isolated from decayed heartwood, whereas in the other two plots only *A. cepistipes* was recovered. *A. ostoyae* is well known as a causal agent of heart and root rot on conifers in Europe and Canada (Anselmi & Lanata 1989, Whitney 1995, Morrison *et al.* 2000). The role of *A. cepistipes* in heart rot is less clear. This species has been associated with heart rot in Norway spruce particularly in Scandinavia (Guillaumin *et al.* 1993, Piri *et al.* 1990). Holdenrieder *et al.* (1994) found that in Switzerland most *Armillaria* butt and root rot on Norway spruce were caused by *A. ostoyae*, and only a minority by *A. cepistipes* and *A. borealis*. The second most frequently isolated species in our study was *H. annosum*, a well-known causal agent of heart rot on Norway spruce (Hallaksela 1984, Piri *et al.* 1990, Graber 1994, Holdenrieder *et al.* 1994). Also *C. borealis* and *Sistotrema* spp. are often associated with butt and root rot in Norway spruce (Hallaksela 1984, Graber 1994). The presence of heart or butt rot in general seems not to reduce the probability

of an external colonisation of the stumps by *A. cepistipes* or *A. ostoyae*. However, this aspect should be further investigated since our results are not unequivocal.

Our study indicates that, in mountainous Norway spruce stands in the Alps, *A. cepistipes* and *A. ostoyae* are efficient stump colonisers. Stumps as a food-base are therefore an important resource for the survival of these two *Armillaria* species in managed spruce stands. *A. cepistipes* and *A. ostoyae* have a very similar strategy for capturing fresh stumps. Both species produce a dense network of rhizomorphs in the soil through which they are then able to rapidly capture newly created spruce stumps. Competition for available resources presumably occurs between *A. cepistipes* and *A. ostoyae*, particularly along interspecific boundaries. Our findings suggest that *A. ostoyae* is slightly more competitive than *A. cepistipes* in primary resource capture. However, the local dominance of *A. cepistipes* indicates that this species may be an interesting agent for biological control of *A. ostoyae*. Furthermore, by rapidly colonising the external part of the stumps and subsequently penetrating into the wood, it could also entrap other root and butt rot fungi, such as *H. annosum*, already present in the stump, thereby impeding their spread (Holdenrieder & Greig 1998). A potential drawback of using *A. cepistipes* as a biocontrol agent could be its apparent capacity to cause heart rot in living trees, and this needs further investigation.

3

Primary resource capture by *Armillaria cepistipes* and *Armillaria ostoyae*

B. Experimental study: Competitive ability of *A. cepistipes* and *A. ostoyae* in primary stump capture

Abstract

In managed Norway spruce (*Picea abies*) forests, *Armillaria cepistipes* and *Armillaria ostoyae* are efficient stump colonisers and may compete for these resources. The aim of this experimental study was to quantify the mutual competitive ability of the two *Armillaria* species in colonising Norway spruce stumps experimentally.

Five isolates of *A. cepistipes* and two isolates of *A. ostoyae* were inoculated pair-wise into pots containing 4-year-old spruce seedlings. The inoculations were conducted simultaneously and sequentially (*A. cepistipes* one year before *A. ostoyae*). As controls, the isolates were also inoculated alone. Six months after creation of the stumps, the rhizomorph production and the stump colonisation were assessed.

Armillaria spp. was identified from 627 rhizomorphs and 90 colonised stumps. In the simultaneous inoculations, *A. cepistipes* dominated both as rhizomorphs in the soil and on the stumps. Nevertheless, *A. ostoyae* was relatively more frequent on the stumps than in the soil and *A. cepistipes vice versa*. In both species, the ability to colonise the stumps in simultaneous inoculations was significantly reduced compared to the single inoculations. In respect to rhizomorph production, simultaneous co-inoculations had a slightly stimulatory effect on *A. cepistipes* and no effect on *A. ostoyae*. In the sequential inoculations, there were considerable differences between the two *A. ostoyae* isolates in their ability to produce rhizomorphs and to colonise stumps. *A. cepistipes* slightly inhibited stump colonisation by both *A. ostoyae* isolates.

Our study suggests a neutralistic co-existence of *A. cepistipes* and *A. ostoyae* as rhizomorphs in the soil. Concerning the ability to colonise stumps, the two species experience a mutual negative effect from the interaction, probably because of interspecific competition.

Key-words: *Armillaria cepistipes*, *Armillaria ostoyae*, *Picea abies*, rhizomorph production, stump colonisation, interaction.

3.1 Introduction

The basidiomycete genus *Armillaria* occurs worldwide in boreal, temperate, and tropical forests, as well as in horticultural and forest plantations. As facultative saprotrophs, *Armillaria* species contribute significantly to wood decomposition and mineral cycling. Moreover, some members of the genus are primary or secondary necrotrophic parasites (Kile *et al.* 1991). Armillaria root rot is a long known serious problem of several coniferous and broadleaved tree species (Shaw & Kile 1991).

Frequently, preferentially saprotrophic and pathogenic species of *Armillaria* co-exist sympatrically in the same forest. Such pairs of co-existing *Armillaria* species include *A. calvescens* Bérubé & Dessureault and *A. ostoyae* (Romagnesi) Herink (Rizzo & Harrington 1993), *A. gallica* Marxmüller & Romagnesi and *A. mellea* (Vahl: Fr.) Kummer (Baumgartner & Rizzo 2001), and *A. cepistipes* Velenovsky and *A. ostoyae* (Legrand *et al.* 1996). During their saprotrophic life phase, co-occurring species probably compete for available resources. Understanding the specific interactions between co-existing *Armillaria* species could provide important indications about the potential of saprotrophic species to be used for the biological control of the pathogenic species.

In managed forests, stumps are readily available resources, which are commonly exploited by *Armillaria* and other root rot pathogens (Rishbeth 1988, Legrand *et al.* 1996, Cruickshank *et al.* 1997). In managed spruce forests, we have observed that both *A. cepistipes* and *A. ostoyae* are efficient stump colonisers (Chapter 3A). Stumps located along interspecific boundaries were, however, preferentially colonised by *A. ostoyae*, suggesting that this species is slightly more competitive than *A. cepistipes* in primary capture of fresh stumps. According to Redfern & Filip (1991), *Armillaria* can capture fresh stumps by invasion from soil rhizomorphs, by extension from pre-existing root lesions, or by basidiospores. In *A. ostoyae*, stump colonisation may often occur through rapid extension from root lesions, which are sometimes frequent (Morrison *et al.* 2000). On the contrary, the preferentially saprotrophic *A. cepistipes* rarely causes lesions on the roots of living trees (Chapter 4). In this species, stumps are probably either captured by invasion of already present epiphytic rhizomorphs or by newly arrived rhizomorphs.

The experimental study reported here aimed to determine the competitive ability of *A. cepistipes* and *A. ostoyae* in colonising fresh Norway spruce (*Picea abies* (L.) Karst.) stumps created in pots. The specific questions that we addressed were: (i) Is the mutual ability to produce rhizomorphs affected by the presence of the other *Armillaria* species?, (ii) Which

Armillaria species preferentially colonise the stumps when both species are present as rhizomorphs in the soil?, and (iii) Does the presence of rhizomorphs of *A. cepistipes* in the soil prevent or reduce the stump colonisation by *A. ostoyae*? To investigate the first and second questions, *A. cepistipes* and *A. ostoyae* were simultaneously inoculated into the pots, whereas for the third question *A. cepistipes* was inoculated before *A. ostoyae*.

3.2 Materials and Methods

3.2.1 Plant and fungal sources

The experiment was conducted with four year-old Norway spruce seedlings from the Swiss provenance Bremgarten (47°21.2'N, 8°19'E; 425 m a.s.l.). In April 2000, the seedlings were planted in 10 L plastic pots containing a soil substratum (pH 3.7-3.8) composed of 80% wood fibres material (Toresa spezial, Intertoresa AG, Ittigen, CH) and 20% peat by volume, with 2 g L^{-1} of a controlled release fertiliser (Osmocote Plus, Scotts Europe B.V., Heerlen, NL) and 3 g L^{-1} horn powder. One seedling was planted in the centre of each pot and one (single inoculation) or two sections (double inoculation) of a plastic pipe (20 cm x 3 cm diameter) were placed near the plant with care to not wound the roots.

For inoculations, two isolates of *A. ostoyae* and five isolates of *A. cepistipes* were used. The two *A. ostoyae* isolates (C14 and C18) were recovered from mycelial fans collected from two infected Norway spruce and showed a high virulence on Norway spruce seedlings under experimental conditions (Chapter 4). All five *A. cepistipes* isolates (B2, B3, B6, B7, and B8) were obtained from rhizomorphs in the soil and were low virulent. Inocula of these isolates were produced according to the method described in Chapter 4.

3.2.2 Experimental design

The experimental design of this study is shown in the Figure 3.2. Isolates of *A. cepistipes* and *A. ostoyae* were inoculated pair-wise in two different ways, simultaneously and sequentially (i.e. *A. cepistipes* prior to *A. ostoyae*). The inoculations were carried out as described in Chapter 4.

For the simultaneous treatment, ten pots were inoculated with each of ten pairings of isolates in May 2000 (Fig. 3.2). For comparison, each *A. cepistipes* and *A. ostoyae* isolate was also inoculated alone into 14 and 15 pots, respectively. The pots were placed in the forest nursery of the Swiss Federal Research Institute WSL (47°21.75'N, 8°27.4'E; 550 m a.s.l.;

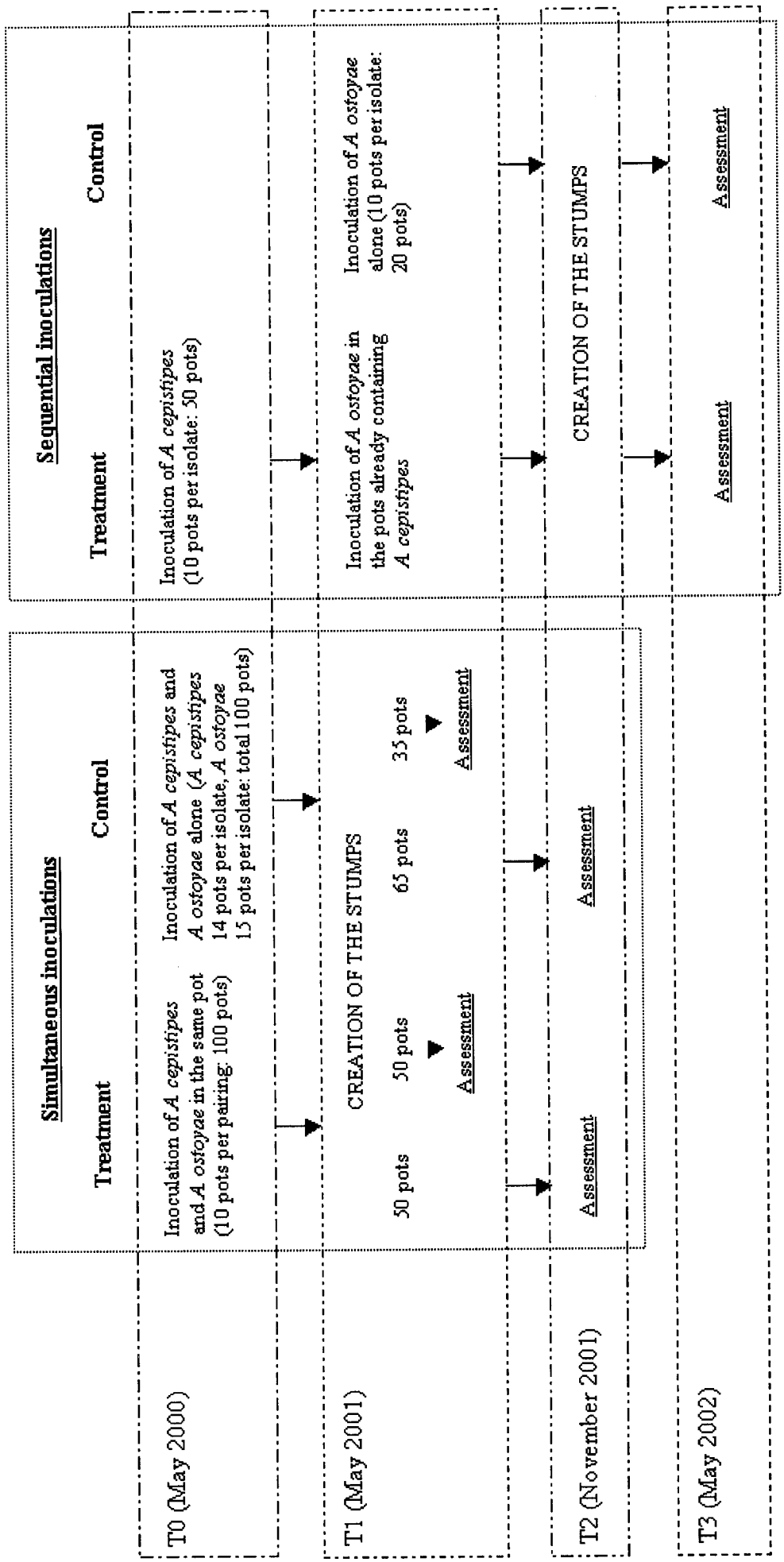


Figure 3.2. Experimental design of the study.

1074 mm mean annual precipitation; 8.2°C mean annual temperature) and watered if required. In May 2001 (i.e. one year after inoculation), stumps were created by cutting the seedlings about 5-10 cm above the soil. At this time, the seedlings had a mean diameter at the basis of 1.8 ± 0.3 cm and a mean height of 67.1 ± 8.5 cm. One half of the inoculated pots was harvested to assess rhizomorph production by *A. cepistipes* and *A. ostoyae* before the stump creation and to examine the roots for epiphytic rhizomorphs and lesions. The remaining pots were left in the forest nursery until the final assessment.

For the sequential treatment, each of the five *A. cepistipes* isolates was inoculated in ten pots in May 2000 (Fig. 3.2). One year later (May 2001), an *A. ostoyae* inoculum (C14 or C18) was inserted into each pot. Each pairing of isolates was replicated five times. As controls, ten pots were inoculated with each isolate of *A. ostoyae* alone. The pots were placed in the forest nursery as above. In November 2001 (i.e. six months after inoculation of *A. ostoyae*), the seedlings were cut to create the stumps. The pots were then placed in a greenhouse (temperature: day 20 ± 1 °C, night 15 ± 1 °C, watering once a week) until the final assessment in May 2002.

3.2.3 Assessment of rhizomorph production and stump colonisation

The rhizomorph production by the inocula was quantified by the total dry weight of all rhizomorphs found in a pot. To determine the dry weight, the rhizomorphs were lyophilised for 16 hours. In pots inoculated with both *A. cepistipes* and *A. ostoyae*, the incidence of rhizomorphs of each species was estimated as follows. After splitting of the pot ball into four sectors, in each sector all the rhizomorphs were separated by hand from the soil and the roots. Two individual rhizomorphs, if available, were then randomly selected for each sector. A segment of 1-2 cm length was cut from each of these rhizomorphs and brought to the laboratory for species identification.

To assess stump colonisation by *Armillaria*, portions of the bark were removed from the stem and the main roots (two to three) using a knife and the presence of subcortical mycelial fans was recorded. From each colonised root and stem, a 3-5 mm thick disk with mycelial fans was cut and brought to laboratory for *Armillaria* identification. A stump was considered colonised by an isolate that could be re-isolated from at least one sample. The roots were also investigated for the presence of epiphytic rhizomorphs and lesions with resin exudation.

3.2.4 *Armillaria* isolation and species identification

Armillaria isolation from the rhizomorphs was performed as described in Chapter 2. From the stem and root disks with subcortical mycelial fans, *Armillaria* was isolated as follows. The disks were first washed with tap water and dried between paper towels. Each disk was surface sterilised in sodium-hypochlorite (7% v/v active chlorine; Chemische Fabrik Schweizerhalle, CH-4013 Basel) for 30 seconds and rinsed in sterile distilled water for 10-15 seconds (Prospero *et al.* 1998). After drying with paper towels, the disks were placed in Petri plates (8.5 cm diameter) containing malt agar (20 g L^{-1} malt extract, 15 g L^{-1} bacto agar) amended with thiabendazole (230 mg L^{-1} in 1 ml concentrated lactic acid) and streptomycin (100 mg L^{-1}) (Chapter 2). The plates were incubated in the dark at 20-25 °C for two to three weeks. The *Armillaria* cultures obtained from the rhizomorphs and stumps were identified in somatic incompatibility pairings (Harrington *et al.* 1992) with the original isolates inoculated into the particular pot. If in a pot only one *Armillaria* species could be recovered from the rhizomorphs, the viability of both inocula was determined as described in Chapter 4. This was done in order to verify whether the absence of rhizomorphs of a species was due to a dead inoculum.

In November 2001 and May 2002, many attempts to isolate *Armillaria* from the rhizomorphs failed because of contaminations. Therefore, new rhizomorphs were randomly selected from the lyophilised rhizomorphs collected in the particular sector and their identity was determined by PCR-RFLP analysis (Harrington & Wingfield 1995). A segment (5-10 cm length, 10-20 mg) of a lyophilised rhizomorph was cut into pieces of 0.5-1 cm length. These pieces were put in a 2 ml Eppendorf tube and ground to a fine powder using a mixer mill (Schwingmühle MM 300, Retsch GmbH & Co. KG, D-42781 Haan). DNA was extracted from the mycelial powder essentially as described by Gardes & Bruns (1993). At the end of the extraction, the DNA pellet was resuspended in 50 µl of 1x TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Each DNA sample was diluted 10-fold with sterile distilled water and 2 µl were used for PCR amplification as described by Harrington & Wingfield (1995). The PCR-products were digested with the restriction enzyme *Hinc*II (MBI Fermentas). This enzyme allows to identify the two *Armillaria* species, since it cuts the PCR-product of *A. cepistipes* but not that of *A. ostoyae* (Harrington & Wingfield 1995).

3.2.5 Statistical analysis

The statistical analysis of data was performed with the software DataDesk, Version 6 (Data Description, Inc., Ithaca, NY, USA). Differences among isolates and pairings of isolates in the ability to produce rhizomorphs were investigated using one-way analysis of variance (ANOVA). The dry weight of the rhizomorphs was used to quantify the rhizomorph production. One-way analysis of variance was also used to compare the rhizomorph production of an isolate when inoculated alone and when inoculated in pairings. The quantity of rhizomorphs produced by each isolate in a pairing was calculated as follows. Based on field observations[1], it was supposed that the dry weight per unit length of *A. cepistipes* and *A. ostoyae* rhizomorphs was not significantly different. Therefore, for each pot (i.e. the experimental unit) the ratio between *A. cepistipes* and *A. ostoyae* in the number of rhizomorphs identified was also used to estimate the dry weight of each species. For each pairing of isolates, mean value and standard deviation were then calculated.

The frequencies of *A. cepistipes* and *A. ostoyae* in the soil as rhizomorphs and on the stumps were compared using the chi-square test. Only the qualitative (either *A. cepistipes* or *A. ostoyae*) and not the quantitative (number of isolates) data of stump colonisation were considered. Stumps colonised by both *Armillaria* species were counted for both species. Chi-square statistics was also used to compare the stump colonisation ability of *A. ostoyae* and *A. cepistipes* when inoculated alone and when co-inoculated with the other species.

3.3 Results

3.3.1 Simultaneous inoculations

Until the evaluation in May 2001, six seedlings had died because of an *Armillaria* infection. One was killed by *A. cepistipes* (isolate B7), three by *A. ostoyae* (one by the isolate C14 and two by the isolate C18) in pots inoculated only with this species, and two by *A. ostoyae* (one by each isolate) in pots inoculated with both *A. ostoyae* and *A. cepistipes*. The six pots containing the dead seedlings were excluded from the analysis.

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[1] Rhizomorphs collected in the plot of Dalpe (Chapter 2 and Chapter 3A): no significant difference ($P = 0.08$) in the dry weight per unit length between *A. cepistipes* and *A. ostoyae*.

In May 2001 (i.e. one year after inoculation), the rhizomorph production by the inocula and the status of the roots of the seedlings were assessed at the time of the creation of the stumps (Fig. 3.2). All the 48 pots inoculated with both *A. cepistipes* and *A. ostoyae* contained rhizomorphs. As shown in Table 3.8, the mean dry weight of the rhizomorphs produced in a pot ranged from 457 ± 243 mg (pairing B6/C18) to 1334 ± 727 mg (pairing B7/C18). The differences among the single pots (replicates) were considerable, as indicated by the large values of the standard deviations. Most isolates produced more rhizomorphs when inoculated in pairings than when inoculated alone. However, only in two *A. cepistipes* isolates in two pairings (B3/C14 and B7/C18) the differences were statistically significant. Considering all isolates together, *A. cepistipes* produced slightly more ($P = 0.048$) rhizomorphs when paired with *A. ostoyae* than when inoculated alone. *Armillaria* could be isolated from 246 of the 348 rhizomorphs. Species identification using somatic incompatibility pairings showed that 85% of the isolates belonged to *A. cepistipes* and 15% to *A. ostoyae*. The weighted ratio between *A. cepistipes* and *A. ostoyae* rhizomorphs in the single pairings varied from 2.9 (pairing B7/C18) to 19.8 (pairing B2/C14) (Table 3.8). In one pot only *A. ostoyae* was isolated from the rhizomorphs, in 23 pots only *A. cepistipes*, and in 24 pots both species. All the inocula contained in pots from which only one *Armillaria* species was recovered from the rhizomorphs were viable.

Rhizomorphs were found in all the 24 pots inoculated with *A. cepistipes* alone. The mean dry weight of the rhizomorphs produced in a pot ranged from 375 ± 131 mg (isolate B7) to 776 ± 197 mg (isolate B8) (Table 3.8). The differences among the isolates of *A. cepistipes* were not significant. Compared to *A. cepistipes*, the two isolates of *A. ostoyae* produced significantly ($P < 0.01$) less rhizomorphs.

Three seedlings (one inoculated with the isolate B6, one inoculated with the pairing of isolates B8/C18, and one with the pairing of isolates B7/C18) showed lesions with resin exudation on the roots but were still alive. Epiphytic rhizomorphs were found on the roots of 12 out of 24 seedlings inoculated with *A. cepistipes* alone and of 7 out of 48 seedlings co-inoculated with *A. cepistipes* and *A. ostoyae*. In this latter cases, five rhizomorphs belonged to *A. cepistipes* and two to *A. ostoyae*. None of the seedlings inoculated with *A. ostoyae* alone showed epiphytic rhizomorphs on the roots.

In November 2001, six months after production of the stumps, pots were assessed for (i) presence of rhizomorphs in the soil, (ii) stump colonisation by *Armillaria*, and (iii) presence of epiphytic rhizomorphs or lesions on the roots. All pots simultaneously inoculated with

Table 3.8. Comparison between the rhizomorph production of each *Armillaria* isolate when inoculated in pairings and when inoculated alone.

	May 2001[a]				**November 2001**[b]			
	Ratio	Rhizomorphs produced (mg)[c]			Ratio	Rhizomorphs produced (mg)		
Isolate(s)	*A. cep/A. ost*[d]	Total[e]	*A. cep*[f]	*A. ost*[f]	*A. cep/A. ost*	Total	*A. cep*	*A. ost*
B2/C14	19.8	750 ± 189	714 ± 119 [ns]	36 ± 80 [ns]	4.2	1123 ± 414	905 ± 336 [ns]	218 ± 310 [ns]
B3/C14	11.5	1287 ± 397	1184 ± 439 *	103 ± 147 [ns]	5.6	609 ± 241	517 ± 228 [ns]	92 ± 89 [ns]
B6/C14	3.4	714 ± 220	550 ± 221 [ns]	164 ± 195 [ns]	11.9	350 ± 189	323 ± 134 *	27 ± 60 *
B7/C14	10.9	875 ± 336	802 ± 356 [ns]	73 ± 101 [ns]	2.6	1017 ± 297	734 ± 161 [ns]	283 ± 184 [ns]
B8/C14	11.1	826 ± 109	758 ± 156 [ns]	68 ± 83 [ns]	6.2	1435 ± 378	1237 ± 268 [ns]	198 ± 225 [ns]
All B/C14	*9.1*	*893 ± 330*	*804 ± 342 * *	*89 ± 127 [ns]*	*4.5*	*908 ± 485*	*744 ± 388 [ns]*	*164 ± 202 [ns]*
B2/C18	3.3	870 ± 135	667 ± 231 [ns]	203 ± 156 [ns]	1.5	2377 ± 865	1407 ± 564 *	970 ± 498 [ns]
B3/C18	6.9	744 ± 151	650 ± 204 [ns]	94 ± 64 [ns]	4.3	737 ± 244	598 ± 325 [ns]	139 ± 211 [ns]
B6/C18	3.9	457 ± 243	363 ± 301 [ns]	94 ± 96 [ns]	1.2	1095 ± 658	594 ± 208 [ns]	501 ± 578 [ns]
B7/C18	2.9	1334 ± 727	996 ± 397 *	338 ± 488 [ns]	1.7	1060 ± 1000	660 ± 760 [ns]	400 ± 404 [ns]
B8/C18	5.1	769 ± 344	643 ± 387 [ns]	126 ± 125 [ns]	2.2	1911 ± 570	1306 ± 602 [ns]	605 ± 501 [ns]
All B/C18	*3.9*	*851 ± 457*	*676 ± 348 [ns]*	*175 ± 244 [ns]*	*1.7*	*1435 ± 903*	*913 ± 610 * *	*522 ± 500 [ns]*

All B/All C	*5.6*	*872 ± 395*	*740 ± 348 **	*132 ± 197 [ns]*	*2.4*	*1172 ± 765*	*829 ± 513 **	*343 ± 419 [ns]*
B2	-	-	649 ± 248	-	-	-	697 ± 276	-
B3	-	-	543 ± 141	-	-	-	400 ± 273	-
B6	-	-	518 ± 216	-	-	-	572 ± 228	-
B7	-	-	375 ± 131	-	-	-	513 ± 218	-
B8	-	-	776 ± 197	-	-	-	853 ± 391	-
All B	-	-	*581 ± 222*	-	-	-	*610 ± 315*	-
C14	-	-	-	67 ± 57	-	-	-	323 ± 280
C18	-	-	-	64 ± 71	-	-	-	373 ± 327
All C	-	-	-	*66 ± 57*	-	-	-	*348 ± 297*

[a]One year after inoculation.

[b]Eighteen months after inoculation.

[c]Mean value and standard deviation are given (n = 5).

[d]The species ratio was estimated by identifying a maximum of 8 randomly selected rhizomorphs per pot. Values represent average ratios weighted by the total amount of rhizomorphs for each pair.

[e]Total dry weight of the rhizomorphs found in a pot.

[f]The relative portion of rhizomorphs of each isolate in the total amount was estimated in each pot by using the species ratio (see above).
[ns] = not significantly different from the rhizomorph production of the particular isolate when inoculated alone, * = significantly different from the rhizomorph production of the particular isolate when inoculated alone.

A. cepistipes and *A. ostoyae* contained rhizomorphs ranging from 350 ± 189 mg (pairing B6/C14) to 2337 ± 865 mg (pairing B2/C18) dry weight (Table 3.8). In 8 out of 10 pairings, the two isolates produced similar amounts of rhizomorphs as when inoculated alone. In one pairing (B2/C18), the *A. cepistipes* isolate produced significantly more rhizomorphs than when inoculated alone, whereas in another pairing (B6/C14) both isolates produced significantly less rhizomorphs than when inoculated alone. Considering all pairings together, *A. cepistipes* produced significantly (P = 0.022) more rhizomorphs when paired with *A. ostoyae* than when inoculated alone. On the contrary, the rhizomorph production by *A. ostoyae* was not affected by the presence of *A. cepistipes*.

In November 2001, 347 of the 348 rhizomorphs collected could be identified, 235 in somatic incompatibility pairings and 112 by PCR-RFLP analysis. About 75% of the rhizomorphs belonged to *A. cepistipes* and 25% to *A. ostoyae* (Table 3.9). Rhizomorphs of both species were found in 34 pots and of *A. cepistipes* only in 16 pots. Pots containing only rhizomorphs of *A. ostoyae* were not observed. Epiphytic rhizomorphs were found on 17 stumps (34%); 14 belonged to *A. cepistipes* and three to *A. ostoyae*. Root lesions were only observed on two stumps inoculated with the pair of isolates B8/C18. All the 50 stumps in pots inoculated with both *Armillaria* species were colonised by *Armillaria*. A total of 111 isolates could be recovered from 48 colonised stumps. Species identification showed that 15 (31.3%) stumps were colonised by *A. ostoyae*, 28 (58.3%) by *A. cepistipes*, and five (10.4%) by both species. Considering all pots together, the frequencies of the *Armillaria* species in the soil and on the stumps were significantly different (χ^2 = 4.37, P = 0.037) (Table 3.9). *A. ostoyae* was more frequent on the stumps than in the soil and *A. cepistipes vice versa*.

In pots inoculated with a single *Armillaria* species, 97.8% (44 out of 45) of the *A. cepistipes* and 80% (16 out of 20) of the *A. ostoyae* inocula produced rhizomorphs. The isolates of *A. cepistipes* produced significantly (P < 0.01) more rhizomorphs than those of *A. ostoyae*. In *A. cepistipes*, a significant (P = 0.018) difference in rhizomorph production was observed between the isolate B3 (400 ± 273 mg per inoculum) and the isolate B8 (853 ± 391 mg per inoculum). There were no differences in rhizomorph production between May 2001 and November 2001 in the five *A. cepistipes* isolates. In the two *A. ostoyae* isolates, the mean rhizomorph dry weight increased considerably during this period.

Epiphytic rhizomorphs were found on the roots of 7 out of 45 (15.6%) seedlings inoculated with *A. cepistipes* whereas they were absent on the roots of seedlings inoculated with *A. ostoyae*. None of the seedlings inoculated either with *A. cepistipes* or *A. ostoyae* showed lesions with resin exudation on the roots. *A. cepistipes* colonised 97.8% (44 out of 45) of the

Table 3.9. Incidence of *A. cepistipes* and *A. ostoyae* in the soil and on the stumps, 18 months after simultaneous inoculation.

		Soil[a] (N)		Stump[b] (N)	
Pairing of isolates[c]	N[d]	*A. cepistipes*	*A. ostoyae*	*A. cepistipes*	*A. ostoyae*
B2/C18	5	24	16	2	2
B3/C18	5	26	6	4	2
B6/C18	5	22	10	5	1
B7/C18	5	25	12	3	3
B8/C18	5	26	12	0	4
All B/C18	*25*	*123*	*56*	*14*	*12*
Chi-square test[e]			$\chi^2 = 2.26, P = 0.132$		
B2/C14	5	29	7	5	0
B3/C14	5	28	5	4	2
B6/C14	5	22	1	4	2
B7/C14	5	28	10	3	2
B8/C14	5	33	5	3	2
All B/C14	*25*	*140*	*28*	*19*	*8*
Chi-square test			$\chi^2 = 2.55, P = 0.111$		
All pairings	*50*	*263*	*84*	*33*	*20*
Chi-square test			$\chi^2 = 4.37, P = 0.037$		

[a]Number of isolates of *A. cepistipes* and *A. ostoyae* obtained from the rhizomorphs in the soil.

[b]Number of stumps colonised by *A. cepistipes* or *A. ostoyae*. A sum of the stumps colonised by *A. cepistipes* or by *A. ostoyae* exceeding the total number of the analysed pots (N) indicates the presence of stumps colonised by both *Armillaria* species (five stumps).

[c]B = *A. cepistipes*, C = *A. ostoyae*.

[d]Number of replicates (pots).

[e]Chi-square test was conducted to compare the frequencies of *A. cepistipes* and *A. ostoyae* in the soil and on the stumps.

Table 3.10. Incidence of stump colonisation by *A. cepistipes* and *A. ostoyae* in single (*A. cepistipes* or *A. ostoyae*) and double (*A. cepistipes* and *A. ostoyae*) simultaneous inoculations.

	Incidence of stump colonisation		
Species, isolate	Single inoculation[a]	Double inoculation[b]	Chi-square statistics[c]
A. cepistipes, B2	9/10[d]	7/9	
A. cepistipes, B3	9/9	8/10	
A. cepistipes, B6	8/8	9/10	
A. cepistipes, B7	9/9	6/10	
A. cepistipes, B8	9/9	3/9	
Total A. cepistipes	*44/45*	*33/48*	$\chi^2 = 13.74$, $P = 0.0002$
A. ostoyae, C14	6/10	8/25	
A. ostoyae, C18	9/10	12/23	
Total A. ostoyae	*15/20*	*20/48*	$\chi^2 = 6.28$, $P = 0.012$

[a]*A. cepistipes* and *A. ostoyae* inoculated alone.

[b]*A. cepistipes* and *A. ostoyae* simultaneously inoculated in the same pot.

[c]Incidences of stump colonisation in single and double inoculations compared using chi-square test.

[d]Number of the stumps colonised by the particular isolate / Total number of the stumps.

stumps and *A. ostoyae* 75% (C14: 6 out of 10, C18: 9 out of 10). All isolates colonised proportionally more stumps when inoculated alone than when inoculated with an isolate of the other species (Table 3.10). These differences were statistically significant when combining the results of all isolates of each species (*A. cepistipes*: $\chi^2 = 13.74$, $P = 0.0002$, *A. ostoyae*: $\chi^2 = 6.28$, $P = 0.012$).

3.3.2 Sequential inoculations

In this treatment, the stumps were produced in November 2001, 18 months after inoculation of *A. cepistipes* and six months after inoculation of *A. ostoyae*. Rhizomorph production and stump colonisation were assessed in May 2002. One seedling had died because of an infection by *A. ostoyae* (pairing B2/C18; seedling killed by the isolate C18) before the creation of the

Table 3.11. Incidence of *A. cepistipes* and *A. ostoyae* in the soil and on the stumps, after sequential inoculation of *A. cepistipes* prior to *A. ostoyae*.

		Soil[a] (N)		Stump[b] (N)	
Pairing of isolates[c]	N[d]	*A. cepistipes*	*A. ostoyae*	*A. cepistipes*	*A. ostoyae*
B2/C18	4	15	8	0	3
B3/C18	5	16	10	1	4
B6/C18	5	15	16	0	4
B7/C18	5	20	13	2	3
B8/C18	5	24	10	1	4
All B/C18	*24*	*90*	*57*	*4*	*18*
Chi-square test[e]			$\chi^2 = 14.36, P = 0.0002$		
B2/C14	5	22	3	2	3
B3/C14	5	14	2	2	1
B6/C14	5	16	4	3	1
B7/C14	5	36	1	3	1
B8/C14	5	29	6	4	0
All B/C14	*25*	*117*	*16*	*14*	*6*
Chi-square test			$\chi^2 = 4.56, P = 0.03$		
All pairings	*49*	*207*	*73*	*18*	*24*
Chi-square test			$\chi^2 = 16.75, P = 0.0001$		

[a]Number of isolates of *A. cepistipes* and *A. ostoyae* obtained from the rhizomorphs in the soil.

[b]Number of stumps colonised by *A. cepistipes* or *A. ostoyae*.

[c]B = *A. cepistipes*, C = *A. ostoyae*.

[d]Number of replicates (pots). One pot (pairing B2/C18) containing a seedling already dead when the stump was produced is excluded from the analysis.

[e]Chi-square test was conducted to compare the frequencies of *A. cepistipes* and *A. ostoyae* in the soil and on the stumps.

stump and consequently this pot was excluded from the analysis.

Only one pot (pairing B3/C14) of the 49 inoculated with *A. cepistipes* and *A. ostoyae* contained no rhizomorphs. The total rhizomorph production of the different pairings of isolates ranged from 267 ± 122 mg (pairing B3/C14) to 1595 ± 373 mg (pairing B8/C18) dry weight per pot. Epiphytic rhizomorphs were found on the roots of 21 (42%) seedlings inoculated with both *Armillaria* species; all these rhizomorphs belonged to *A. cepistipes*. Resin exudation associated with lesions on the roots was observed on 11 seedlings (22%). From 287 rhizomorphs collected, 280 (97.6%) could be identified, 170 in somatic incompatibility pairings and 110 by PCR-RFLP analysis. A total of 207 (73.9%) isolates belonged to *A. cepistipes* and 73 (26.1%) to *A. ostoyae* (Table 3.11). Rhizomorphs of both species were found in 27 pots (55.1%). Only *A. cepistipes* rhizomorphs were found in 21 pots (42.9%) and only *A. ostoyae* in one pot (2%). A total of 45 stumps (91.8%) were colonised by *Armillaria* and pure cultures could be obtained from 42 of these stumps. *A. cepistipes* was identified on 18 stumps and *A. ostoyae* on 24 stumps. The *A. ostoyae* isolate C18 showed a considerable better ability to produce rhizomorphs and colonise stumps than the isolate C14 (Table 3.11). By considering the relative incidences of the two species, both *A. ostoyae* isolates were more frequent on the stumps than in the soil and *A. cepistipes vice versa.*

When inoculated alone, three out of 10 inocula of the *A. ostoyae* isolate C14 and 9 out of 10 of the isolate C18 produced rhizomorphs. In the 20 pots inoculated with *A. ostoyae*, only three seedlings (one inoculated with the isolate C14 and two with the isolate C18) had epiphytic rhizomorphs on the roots. Lesions with resin exudation were observed on one seedling inoculated with the isolate C14 and one with the isolate C18. The isolate C14 colonised 5 out of 10 stumps and the isolate C18 colonised 8 out of 10 stumps. In both *A. ostoyae* isolates, the ability to colonise the stumps was reduced when inoculated sequentially after *A. cepistipes*. The incidences of stump colonised dropped from 5 out of 10 (50%) in single inoculations to 6 out of 25 (24%) in sequential inoculations in the isolate C14, and from 8 out of 10 (80%) to 18 out of 24 (75%) in the isolate C18. These differences, however, were statistically not significant (C14: $\chi^2 = 2.24$, $P = 0.134$; C18: $\chi^2 = 0.1$, $P = 0.754$).

3.4 Discussion

The aim of this experimental study was to determine the competitive ability of *A. cepistipes* and *A. ostoyae* in colonising small Norway spruce stumps created in pots. For this purpose,

the two species were simultaneously and sequentially inoculated and their incidence as rhizomorphs in the soil and on the stumps was compared.

Our data show that *A. cepistipes* has a good ability to form vigorous rhizomorphs under experimental conditions, as previously reported by Guillaumin *et al.* (1989). *A. ostoyae* produced significantly less rhizomorphs than *A. cepistipes*. A low ability of this species to form rhizomorphs was also observed by other authors (Redfern 1975, Mohammed 1985, Guillaumin *et al.* 1989). It could be speculated that the specific soil substratum (particularly the low pH of 3.7-3.8) used in our experiment was more favourable for the rhizomorph production by *A. cepistipes* than by *A. ostoyae*. However, in Norway spruce stands rhizomorphs of *A. ostoyae* were preferentially found in acidic (pH 3-5) soils (Rigling *et al.* 1998). On the contrary, the frequency of *A. cepistipes* rhizomorphs decreased with increasing soil acidity. According to Mihail *et al.* (1995), the scarce rhizomorph production by pathogenic *Armillaria* species indicates a strategy of energy conservation to maximise the potential to attack living hosts. Saprotrophic species, on the other hand, compensate for the reduced virulence with an intensive production of rhizomorphs, resulting in a more widespread inoculum distribution and in a competitive advantage in exploiting forest stands, as discussed in Wargo & Carey (2001). In all our isolates, the rhizomorph production 18 months after inoculation (i.e. November 2001) was not significantly different than those observed six months before (i.e. May 2001). Nevertheless, the mean quantities of rhizomorphs produced by the two *A. ostoyae* isolates increased considerably from May 2001 to November 2001. These findings suggest that *A. cepistipes* rapidly initiated rhizomorphs from the inocula, whereas in *A. ostoyae* the rhizomorph growth was slower but more persistent. A more rapid rhizomorph production under experimental conditions by *A. cepistipes* compared to *A. ostoyae* was previously noted by Guillaumin *et al.* (1985).

Most isolates, when simultaneously inoculated in pairings produced similar quantities of rhizomorphs as when inoculated alone. Therefore, in the soil the two species seem to co-exist without or with little mutual influence. When considering all isolates together, the rhizomorph production by *A. ostoyae* was not affected by the presence of *A. cepistipes*. By contrast, *A. cepistipes* produced significantly more rhizomorphs when paired with *A. ostoyae* than when inoculated alone. Thus, the presence of the pathogenic *A. ostoyae* may have stimulated the rhizomorph production of the preferentially saprotrophic *A. cepistipes*, which could suggest that the latter may benefit from the interaction. However, the increased rhizomorph production by *A. cepistipes* may also be a stress reaction due to the presence of *A. ostoyae*, with *A. cepistipes* trying to escape to a new sector in the soil. In other studies, the production

of rhizomorphs by *Armillaria* spp. was also affected by the presence of other fungi. Mohammed & Guillaumin (1989) observed that in a rhizotron the presence of *A. cepistipes* and *A. gallica* had a stimulating effect on the rhizomorph production by *A. ostoyae*. The same effect on the rhizomorph production by *Armillaria mellea* (Vahl: Fr.) Kummer was caused *in vitro* by *Macrophoma* spp. (Watanabe 1986). In a study conducted by Kwasna (2001), the growth of *A. ostoyae* and *A. gallica* rhizomorphs from wood segments was stimulated by fungi living in the rhizosphere of common oak (*Quercus robur* L.). Investigations involving cord-forming basidiomycetes (e.g. Dowson *et al.* 1988) have also shown that the presence of competitors affects the development of the mycelial cord systems in the soil and litter. The stimulating or inhibiting effects on the development of rhizomorphs or mycelial cords could be due to secretion of fungal exudates or volatile metabolites (Sonnenbichler *et al.* 1997, Boddy 2000, Kwasna 2001).

When inoculated alone, both *Armillaria* species colonised the majority of the fresh stumps. This finding confirms (i) our field investigations indicating that when occurring in the soil, *A. cepistipes* and *A. ostoyae* will colonise most of the nearby Norway spruce stumps (Chapter 3A) and (ii) the assumption that pathogenic root rotting fungi such as *Armillaria* species rely on primary resource capture strategy (Holmer & Stenlid 1996). In May 2001, at the time of the stump creation, half of the seedlings had epiphytic rhizomorphs of *A. cepistipes* on the roots. Thus, it can be supposed that in this species, stumps were frequently colonised trough invasion from already present epiphytic rhizomorphs. In *A. ostoyae*, epiphytic rhizomorphs as well as lesions on the roots were rarely observed, suggesting that stumps were primarily colonised by newly arrived rhizomorphs.

In both *Armillaria* species, the ability to colonise the stumps was significantly inhibited by the presence of the other species. The inhibition was stronger in *A. cepistipes* than in *A. ostoyae*. Thus, the two species experienced a mutual negative effect from the interaction, probably because of interspecific competition. In fact, according to Culver (1992), true competition results in inhibition of all competitors. Both exploitation and interference competition could occur (Lockwood 1992, Wicklow 1992, Boddy 2000). Exploitation competition with one *Armillaria* species using the resource (i.e. stump) and consequently reducing its availability for the other species, and interference competition with mutual restriction of the access to the resource through some form of chemical interaction between the rhizomorphs.

In the pots simultaneously inoculated with both *Armillaria* species, *A. cepistipes* was dominating in the soil as rhizomorphs as well as on the stumps. Nevertheless, by considering

the relative incidences of the two species, *A. ostoyae* was more frequent on the stumps than in the soil, whereas for *A. cepistipes* the opposite applied. This finding indicates a better competitive ability of *A. ostoyae* in capturing fresh stumps, as previously observed in field investigations (Chapter 3A). The fresh stumps represent an intermediate stage between the living and dead substrates, since they still contain, for varying periods of time, living and functional woody tissues (Cooke & Rayner 1984). *A. ostoyae*, as parasitic species, may be advantaged over *A. cepistipes* in colonising these moribund tissues (Rayner & Todd 1979).

A significant difference between the relative incidences of *Armillaria* species in the soil and on the stumps was also observed in the sequential inoculations (*A. cepistipes* prior to *A. ostoyae*). *A. cepistipes* could not entirely benefit from the positional advantage, given by the rhizomorphs in the soil, and colonise the stumps before *A. ostoyae*. When the stumps were created, *A. cepistipes* may already had consumed most energy for rhizomorph production, which reduced the energy available for stump colonisation. In *A. ostoyae*, the rhizomorph production seemed to be an important requirement for the stump colonisation. This is indicated by the observation that the isolate (C18) with a better ability to produce rhizomorphs was also considerably better than the other isolate (C14) in stump capture. This finding also suggests that there are important differences among *A. ostoyae* genotypes in primary resource capture ability.

Our study shows that, when inoculated alone, *A. cepistipes* colonises most of the fresh Norway spruce stumps through the dense network of rhizomorphs produced in the soil. This could offer a chance for the artificial introduction of the saprotrophic *A. cepistipes* in *Armillaria*-free first-generation stands. By giving *A. cepistipes* a temporal advantage which allows the colonisation of most stumps, the resources available for *A. ostoyae* and other root and butt rot fungi would be considerably reduced. The long-term success of *A. cepistipes*, however, would be affected by its ability to defend the resources. Field and laboratory observations (Holdenrieder, unpublished) indicate that *Armillaria* spp. may exploit their substrates almost completely, thereby protecting them by forming pseudosclerotial tissues. Consequently, stumps colonised by *A. cepistipes* would become definitively unavailable for *A. ostoyae* and other root and butt rot fungi.

4

Comparison of the virulence of *Armillaria cepistipes* and *Armillaria ostoyae* on four Norway spruce provenances*

*Prospero S, Holdenrieder O, Rigling D. *Forest Pathology* (submitted)

Abstract

The basidiomycete *Armillaria cepistipes* frequently occurs in the same forest stand together with *Armillaria ostoyae*. In this study, we determined the virulence of 20 isolates of *A. cepistipes* and 16 isolates of *A. ostoyae* on 2-year-old Norway spruce (*Picea abies*) seedlings of four provenances.

Within 30 months after inoculation, 1.1% (22 of 1980) of the seedlings died or were dying because of an *A. cepistipes* infection and 19.1% (255 of 1335) because of an *A. ostoyae* infection. The incidence of dead and dying seedlings varied between 3% and 49% among the *A. ostoyae* isolates; the virulence of an isolate was positively correlated to its ability to produce rhizomorphs. One seedling provenance showed significantly lower susceptibility to *A. ostoyae* than the other three provenances. Both *Armillaria* species were able to attach rhizomorphs on the root surface. However, rhizomorphs of *A. ostoyae* caused significantly more lesions. In both *Armillaria* species, the virulence of the isolates was not correlated with their wood-degrading capability.

Key-words: *Armillaria cepistipes*, *Armillaria ostoyae*, *Picea abies*, host provenance, virulence, rhizomorph production, wood-degrading capability.

4.1 Introduction

Armillaria root rot is a major disease of woody plants, which may significantly affect the structure and function of forest ecosystems. In Europe, six *Armillaria* species can infect living trees: *A. borealis* Marxmüller and Korhonen, *A. cepistipes* Velenovsky, *A. ostoyae* (Romagnesi) Herink, *A. mellea* (Vahl: Fr.) Kummer, *A. gallica* Marxmüller and Romagnesi, and *A. tabescens* (Scop.: Fr.) Emel. These species differ in geographical and ecological distribution, host range, and pathogenicity (Guillaumin *et al.* 1993).

Previous investigations (Legrand *et al.* 1996, Rigling *et al.* 1998, Marxmüller & Holdenrieder 2000) have shown that *A. cepistipes* is one of the most common *Armillaria* species in Europe. Only few experimental data on pathogenicity are available for *A. cepistipes* (Redfern 1975, Morrison 1989, Rishbeth 1991) but circumstantial evidence suggests that it behaves preferentially as saprotroph (Guillaumin *et al.* 1993). *A. cepistipes* is generally considered to behave similar as *A. gallica*, a species which is categorised as a weak pathogen by field observations and inoculation experiments (Rishbeth 1982, Guillaumin *et al.* 1985). Frequently, *A. cepistipes* coexists sympatrically in the same stand with *A. ostoyae* (Legrand *et al.* 1996, Marxmüller & Holdenrieder 2000). *A. ostoyae* can act as a primary necrotrophic parasite, especially on conifers (Guillaumin *et al.* 1993). Tree mortality due to *A. ostoyae* is known from natural forests and plantations throughout Europe and North America (Morrison *et al.* 1991a, Kile *et al.* 1991). Besides mortality, *A. ostoyae* causes heart rot, which reduces the stability of the infected trees as well as the timber quality and production (Morrison *et al.* 1991b, Mallett & Volney 1999). In contrast to *A. cepistipes*, a number of inoculation trials involving isolates of *A. ostoyae* have been conducted (e.g. Shaw 1977, Rishbeth 1982, Gregory 1985, Mallett & Hiratsuka 1988, Mugala *et al.* 1989, Omdal *et al.* 1995, Morrison & Pellow 2002). *A. ostoyae* was found to be pathogenic on several species of conifers (e.g. *Pinus* species, *Abies concolor* (Gordon & Glend.), *Pseudotsuga menziesii* (Mirb.) Franco, *Larix occidentalis* Nutt.), sometimes exhibiting considerable variation in virulence among isolates. Norway spruce (*Picea abies* (L.) Karst) as the host has only limited been used in inoculation studies (Redfern 1975, Guillaumin & Lung 1985). This is surprising, as it is the most widespread coniferous species in central and northern Europe. Little is known about variation in susceptibility to infection by *A. cepistipes* and *A. ostoyae* within tree species.

In this study, we determined the virulence of a large number of isolates of *A. cepistipes* and *A. ostoyae* on Norway spruce seedlings of four provenances. The questions addressed were, (i) How does compare virulence of *A. cepistipes* with that of *A. ostoyae*?, (ii) What is the

variation in virulence among isolates of the same species, (iii) Are there differences in the susceptibility among different seedling provenances?, and (iv) Is there a relationship between the virulence of a fungal isolate and its wood-degrading capability?

4.2 Materials and Methods

4.2.1 Host plants

The virulence of the selected *Armillaria* isolates was tested on 2-year-old Norway spruce seedlings from four Swiss provenances (Table 4.1), two from low altitudes (Bremgarten and Burgdorf) and two from high altitudes (Gantrisch and Rüti). In April 1999, the field-grown, bare-root seedlings were planted in 3.5 L plastic pots containing a soil substratum (pH 3.7-3.8) composed of 20% peat and 80% wood fibres material (Toresa spezial, Intertoresa AG, Ittigen, CH) by volume, with 2 g L^{-1} of a slow releasing fertiliser (Osmocote Plus, Scotts Europe B.V., Heerlen, NL) and 3 g L^{-1} horn powder. Five plants were potted in each pot, one in the centre and four on the margin. Near the seedling in the centre, a section of a plastic pipe (20 cm x 3 cm diameter) was been placed with care to not wound the roots of the seedling. In July 2000, 3 g (1 g L^{-1}) Osmocote Plus were added to each pot.

Table 4.1. Provenance and height of the 2-year-old Norway spruce seedlings.

Location	Coordinates	Altitude	Height (cm)[a]		
		(m a.s.l.)	N[b]	1999	2001
Bremgarten	47°21.2'N, 8°19'E	425	820	13.2 ± 0.1a[c]	45.9 ± 0.3a
Burgdorf	47°2.75'N, 7°36.5'E	600	814	12.2 ± 0.1b	50.7 ± 0.3b
Gantrisch	46°42.75'N, 7°26.5'E	1600	847	5.1 ± 0.05c	34.9 ± 0.4c
Rüti	46°44'N, 7°27'E	1560	832	8.4 ± 0.1d	48.3 ± 0.4d

[a]Mean value and standard error are given.

[b]Non-inoculated (control) and dead/dying seedlings are not included.

[c]Numbers within a column followed by different letters differ significantly ($P < 0.05$).

4.2.2 *Armillaria* spp. isolates

Twenty isolates of *A. cepistipes* and 16 isolates of *A. ostoyae* described in Table 4.2 were tested. These isolates were collected from 1993 to 1998 from 30 mixed hardwood-coniferous forest sites distributed all over Switzerland. The sites were located at different altitudes (400-1570 m a.s.l.) and had a pH of the topsoil varying from 3.1 to 6.9. All *A. cepistipes* and 11 *A. ostoyae* isolates were recovered from rhizomorphs growing in the soil. Five isolates of *A. ostoyae* (C14, C15, C16, C17, and C18) were recovered from subcortical mycelial fans collected from infected conifers (C14, C18: *P. abies*; C15: *Pinus sylvestris* L.; C16: *P. menziesii*; C17: *Larix kaempferi* (Lamb.) Carr.). Six pairs of isolates comprising both *A. cepistipes* and *A. ostoyae* (B1-C1, B2-C2, B3-C3, B4-C4, B6-C6, and B7-C7) were collected from the same site. Species identification was performed by pairing the diploid isolates with three selected haploid tester strains of each of the five European annulate *Armillaria* species (Korhonen 1978, Harrington *et al.* 1992). The tester strains were kindly provided by J.-J. Guillaumin (INRA, Clermont-Ferrand, F). In addition, all isolates were also identified by PCR-RFLP analysis of a portion of the intergenic spacer (IGS) region of the ribosomal DNA according to Harrington & Wingfield (1995). The restriction patterns produced by digestion of the PCR-products with the enzyme *Alu* I were classified according to Pérez Sierra *et al.* (1999). Somatic incompatibility pairings conducted as described by Harrington *et al.* (1992), showed that all 36 isolates represented different genotypes.

4.2.3 Inoculum production

The inoculum was prepared as described by Rigling *et al.* (2003). Five fresh stem segments (10 cm x 3-4 cm diameter) of hazelnut (*Corylus avellana* L.) were placed on 200 ml chips of Norway spruce wood in a 1 L polypropylene box with a screwable lid. Demineralised water (200 ml) was added to each box and boxes were autoclaved (30 min, 120 °C and 1.1 bar) twice, at an interval of 24 hours. After the first autoclaving, sterile distilled water was added again until the wood chips were submerged. Each box was inoculated by placing two small pieces (3-4 mm side) of a growing *Armillaria* culture onto the wood chips. The boxes were incubated in the dark at 25 °C for four months. After two months incubation, distilled sterile water was added, if necessary, to replace water lost because of evaporation.

Table 4.2. Origin of the *A. cepistipes* and *A. ostoyae* isolates used in the virulence test.

Isolate	Species	RFLP-type[a]	Altitude (m a.s.l.)	Soil pH	Source	Year of isolation
B1	*A. cepistipes*	Cep 1	740	4.17	Rhizomorph	1997
B2	*A. cepistipes*	Cep 1	807	3.8	Rhizomorph	1998
B3	*A. cepistipes*	Cep 1	484	3.7	Rhizomorph	1996
B4	*A. cepistipes*	Cep 1	464	3.14	Rhizomorph	1998
B6	*A. cepistipes*	Cep 1	1364	3.95	Rhizomorph	1998
B7	*A. cepistipes*	Cep 2	1230	4.37	Rhizomorph	1997
B8	*A. cepistipes*	Cep 1	819	6.36	Rhizomorph	1993
B9	*A. cepistipes*	Cep 1	946	3.32	Rhizomorph	1993
B10	*A. cepistipes*	Cep 1	1515	3.25	Rhizomorph	1998
B11	*A. cepistipes*	Cep 1	570	6.08	Rhizomorph	1997
B12	*A. cepistipes*	Cep 1	655	6.92	Rhizomorph	1997
B13	*A. cepistipes*	Cep 1	882	5.8	Rhizomorph	1997
B14	*A. cepistipes*	Cep 1	690	5.3	Rhizomorph	1993
B15	*A. cepistipes*	Cep 1	775	5.56	Rhizomorph	1993
B16	*A. cepistipes*	Cep 1	1570	5.29	Rhizomorph	1997
B17	*A. cepistipes*	Cep 1	1180	5.83	Rhizomorph	1997
B18	*A. cepistipes*	Cep 1	1198	5.89	Rhizomorph	1993
B19	*A. cepistipes*	Cep 2	915	6.36	Rhizomorph	1993
B20	*A. cepistipes*	Cep 1	1440	5.14	Rhizomorph	1993
B21	*A. cepistipes*	Cep 1	940	4.52	Rhizomorph	1997
C1	*A. ostoyae*	Ost	740	4.17	Rhizomorph	1997
C2	*A. ostoyae*	Ost	807	3.8	Rhizomorph	1998
C3	*A. ostoyae*	Ost	484	3.7	Rhizomorph	1996

C4	*A. ostoyae*	Ost	464	3.14	Rhizomorph	1998
C5	*A. ostoyae*	Ost	642	3.76	Rhizomorph	1993
C6	*A. ostoyae*	Ost	1364	3.95	Rhizomorph	1996
C7	*A. ostoyae*	Ost	1230	4.37	Rhizomorph	1997
C9	*A. ostoyae*	Ost	950	3.75	Rhizomorph	1997
C10	*A. ostoyae*	Ost	985	4.21	Rhizomorph	1997
C12	*A. ostoyae*	Ost	890	5.26	Rhizomorph	1993
C13	*A. ostoyae*	Ost	480	3.1	Rhizomorph	1996
C14	*A. ostoyae*	Ost	485	-[b]	Mycelial fans	1997
C15	*A. ostoyae*	Ost	420	-	Mycelial fans	1996
C16	*A. ostoyae*	Ost	400	-	Mycelial fans	1997
C17	*A. ostoyae*	Ost	500	-	Mycelial fans	1998
C18	*A. ostoyae*	Ost	520	-	Mycelial fans	1995

[a]Cep 1 = 399, 200, 183 bp; Cep 2 = 310, 200, 135 pb; Ost = 310, 200, 135 bp according to Pérez Sierra *et al.* (1999).

[b]pH value not known.

4.2.4 Seedling inoculation and incubation

Inoculation was performed at the beginning of May 1999, one month after potting of the seedlings. The plastic pipe near the plant in the centre of the pot was removed and replaced with a hazelnut segment colonised by *Armillaria*. The top of the inoculum segment was covered with soil substratum. Five pots each containing five seedlings from the same provenance were inoculated with each *Armillaria* isolate. As control, ten pots of each seedling provenance were inoculated with autoclaved hazelnut segments. The pots were placed in a completely randomised block design in the forest nursery of the Swiss Federal Research Institute WSL (47°21.75'N, 8°27.4'E; 550 m a.s.l.; 1074 mm mean annual precipitation; 8.2 °C mean annual temperature). Each of five blocks contained four host provenances inoculated with 20 *A. cepistipes* and 16 *A. ostoyae* isolates. Watering was carried out, if required, by overhead sprinklers.

4.2.5 Virulence assessment

After inoculation, the seedlings were assessed monthly for symptoms of Armillaria root rot (chlorotic foliage and mortality) during three growing seasons (March-November), until November 2001. Seedlings that died were recorded and immediately checked for the presence of *Armillaria* mycelial fans in the cambial region of the root collar. Dead seedlings were not removed during the experiment.

At the end of the experiment, each seedling was assigned to one of the following categories: dead, dying (i.e. chlorotic foliage and subcortical mycelial fans on the root collar), and healthy (no visible above-ground symptoms of an *Armillaria* infection). The presence of rhizomorphs produced by the inoculum was verified both on the exterior and in the interior of the pot ball at the end of the experiment. For the exterior examination, the pot ball was removed from the pot and the frequency of rhizomorphs was quantified using the following scale: 0 = rhizomorphs absent, 1 = rhizomorphs visible in one spot of the pot ball, 2 = rhizomorphs visible in two to four spots of the pot ball, and 3 = rhizomorphs present everywhere. The pot ball was split into two parts using an axe to search for rhizomorphs in its interior. The rhizomorphs were quantified by: 0 = rhizomorphs absent, 1 = rhizomorphs growing out from one spot of the inoculum segment, 2 = rhizomorphs growing out from two to four spots of the inoculum segment, and 3 = rhizomorphs enveloping the whole inoculum segment. For each pot the mean value of the exterior and interior estimate was calculated and used for statistical analysis. If no rhizomorphs were found in the pot, the viability of the inoculum was verified as follows. The stem segment was washed under running tap water and blotted dry between paper towels. The segment was then split lengthwise and the two sections were incubated in a moist chamber at room temperature in the dark. Inocula were considered viable, if after three to seven days of incubation, the typical mycelial brushes of *Armillaria* developed on the cut surface.

4.2.6 Root investigation

At the end of the experiment, the roots of the seedlings from the low-altitude provenance Burgdorf and from the high-altitude provenance Rüti were thoroughly inspected. The roots were dipped in water to eliminate rests of soil substratum and subsequently checked for the presence of rhizomorphs attached to the root surface and lesions, identifiable by resin exudation. If a lesion was detected, the bark was removed to look for subcortical mycelial fans.

4.2.7 Wood-degrading capability

Fresh Norway spruce stem segments (10 cm length, 4-6 cm diameter) with bark were dried at 103 °C for 72 hours and the dry weight was recorded. Each segment was then placed in a 1 L polypropylene box with a screwable lid, containing 200 ml water. The boxes were autoclaved for 20 minutes at 120 °C and 1.1 bar. Inoculation was performed by placing two small pieces (3-4 mm side) of a growing *Armillaria* culture onto the top of the wood segments. Each *Armillaria* isolate was inoculated onto three stem segments. The boxes were incubated at 25 °C in the dark for one year. After four months of incubation, distilled sterile water was added, if necessary, to replace water loss. At the end of the experiment, the wood segments were cleaned from the mycelium growing on the surface using a knife, dried (103 °C, 72 hours) and weighed again. The wood-degrading capability was expressed as percent of weight loss of the stem segment.

4.2.8 Statistical analysis

All statistical analyses were performed using the program DataDesk, Version 6 (Data Description Inc., Ithaca, NY, USA). The means of the dependent variables seedling height, rhizomorph production, and wood degrading capability, were compared using one-way analysis of variance (ANOVA). The seedling provenance (for seedling height analysis) and the *Armillaria* species (for analysis of rhizomorph production and wood degrading capability) were considered as explanatory (independent) variables. For seedling heights analysis, only surviving seedlings without above-ground symptoms of *Armillaria* root rot at the end of the experiment were considered. Analysis involving binary characteristics of single seedlings, such as physiological status (dead or alive) and root status (with or without attached rhizomorphs and lesions), were performed with logistic regression. For each *Armillaria* species, the following regression model was used in the analysis of the seedling status:

$$\text{Log } it(\pi) = \log [P(Y_i = 1) * (1\text{-}P(Y_i = 1)^{-1}] = \beta_0 + \beta_1 x^{(1)} + \beta_2 x^{(2)} + \beta_3 x^{(3)}$$

Where $P(Y_i = 1)$ is the probability of a seedling to be dead (dead = 1, alive = 0), $\beta_{0\text{-}3}$ are the coefficients, and x the explanatory variables ($x^{(1)}$ = *Armillaria* isolate; $x^{(2)}$ = seedling provenance; $x^{(3)}$ = seedling position in the pot). In this analysis, dying seedlings due to *Armillaria* spp. were considered as dead. The same model was also applied for the analysis of the root status. In this case, $P(Y_i = 1)$ is the probability of a seedling to show attached rhizomorphs or lesions on the roots (present = 1, absent = 0) and $x^{(1)}$ = *Armillaria* species, the

considered explanatory variable. In the root analysis, only surviving seedlings without above-ground symptoms of *Armillaria* root rot at the end of the experiment were considered.

The relationship between virulence and (i) rhizomorph production, (ii) ability to attach rhizomorphs and cause lesions on the roots, and (iii) wood degrading capability of a fungal isolate was examined with linear regression.

4.3 Results

4.3.1 Growth of the seedlings

In 1999, at the time of inoculation, the heights of the 2-year-old seedlings from the four provenances were significantly different (Table 4.1). It is noticeable that the seedlings from the two low-altitude provenances (Bremgarten and Burgdorf) were higher than the seedlings from the two high-altitude provenances (Gantrisch and Rüti). At the end of the experiment, seedlings from the four provenances showed again significantly different heights. The smallest seedlings were produced by the provenance Gantrisch, followed by Bremgarten, Rüti, and Burgdorf (Table 4.1).

4.3.2 Rhizomorph production

At the end of the 30-month incubation, the pots were checked for rhizomorphs: 396 of 400 (99%) inocula of *A. cepistipes* and 267 of 320 (83.4%) inocula of *A. ostoyae* produced rhizomorphs. Viability checks showed that all inocula which failed to produce rhizomorphs were still viable at the end of the experiment. Three *A. cepistipes* isolates (B7, B14, and B18) and 10 *A. ostoyae* isolates (C3, C4, C5, C6, C7, C9, C12, C13, C15, and C17) had at least one inoculum not producing rhizomorphs. Two isolates of *A. ostoyae* were particularly poor rhizomorph producers (Table 4.3): the isolate C4 failed to produce rhizomorphs in 15 out of 20 pots and the isolate C9 in 12 out of 20 pots. Only three seedlings died or were dying in the 57 pots containing inocula which produced no rhizomorphs (each one in a pot inoculated with the *A. ostoyae* isolates C4, C7, and C9).

No significant difference between *A. cepistipes* and *A. ostoyae* was observed in the incidence of rhizomorphs on the exterior of the pot ball. By contrast, rhizomorphs of *A. cepistipes* were significantly ($P < 0.0001$) more frequent than those of *A. ostoyae* in the interior of the pot. Considering the total rhizomorph production in a pot as average of the

interior and exterior estimate, *A. cepistipes* produced significantly ($P < 0.0001$) more rhizomorphs than *A. ostoyae* (Table 4.3).

4.3.3 Virulence

The first seedlings killed by *A. ostoyae* were observed 11 months after inoculation (Fig. 4.1a). In *A. ostoyae*, disease progress strongly accelerated at the end of the second growing season (August 2000). During the first 15 months after inoculation, only four seedlings were killed by *A. ostoyae* whereas, in the second half of the experiment, four to 51 seedlings died every month. By contrast, no relevant changes in the disease progress were observed in the pots inoculated with *A. cepistipes* (Fig. 4.1b). A maximum of four seedlings died during one month (March 2001). By the end of the experiment (i.e. 30 months after inoculation), 1.1% (22 out of 1980) of the seedlings had died or were dying because of an *A. cepistipes* infection and 19.1% (255 out of 1335) because of an *A. ostoyae* infection (Table 4.3). Only pots containing an inoculum which produced rhizomorphs (663 out of 720) are considered in these numbers. All the dead and dying seedlings had subcortical mycelial fans of *Armillaria* on the root collar. Ten seedlings died because of attacks by *Botrytis* spp. or root weevils. No mortality was observed in the control pots.

Virulence expression (i.e. incidence of dead and dying seedlings) of the *A. ostoyae* isolates was positively correlated ($R^2 = 0.67$, $P < 0.0001$) with rhizomorph production. Isolates which were good rhizomorph producers (C1, C14, and C18) caused the most seedling infection. The virulence of *A. cepistipes* isolates was not affected ($R^2 = 0.03$, $P = 0.483$), however, by their capacity to produce rhizomorphs. Pots without rhizomorphs were also considered in the analysis.

The preliminary analysis performed in November 2000 (i.e. 18 months after inoculation) showed that seedling mortality was significantly higher with *A. ostoyae* than with *A. cepistipes* (Prospero *et al.* 2003). By the time of the final assessment, the difference between the two species had become even more marked ($P < 0.0001$). In addition, in *A. ostoyae* there was considerable intraspecific variation in the virulence expression (Table 4.3). One quarter of the isolates (C4, C12, C15, and C16) showed a very low (< 6%) ability to cause mortality. Five isolates (C2, C5, C6, C9, and C13) were moderately virulent (7-20%) and seven (C1, C3, C7, C10, C14, C17, and C18) highly virulent (> 20%). *A. ostoyae* isolates originating from subcortical mycelial fans were significantly ($P = 0.016$) more virulent than isolates obtained

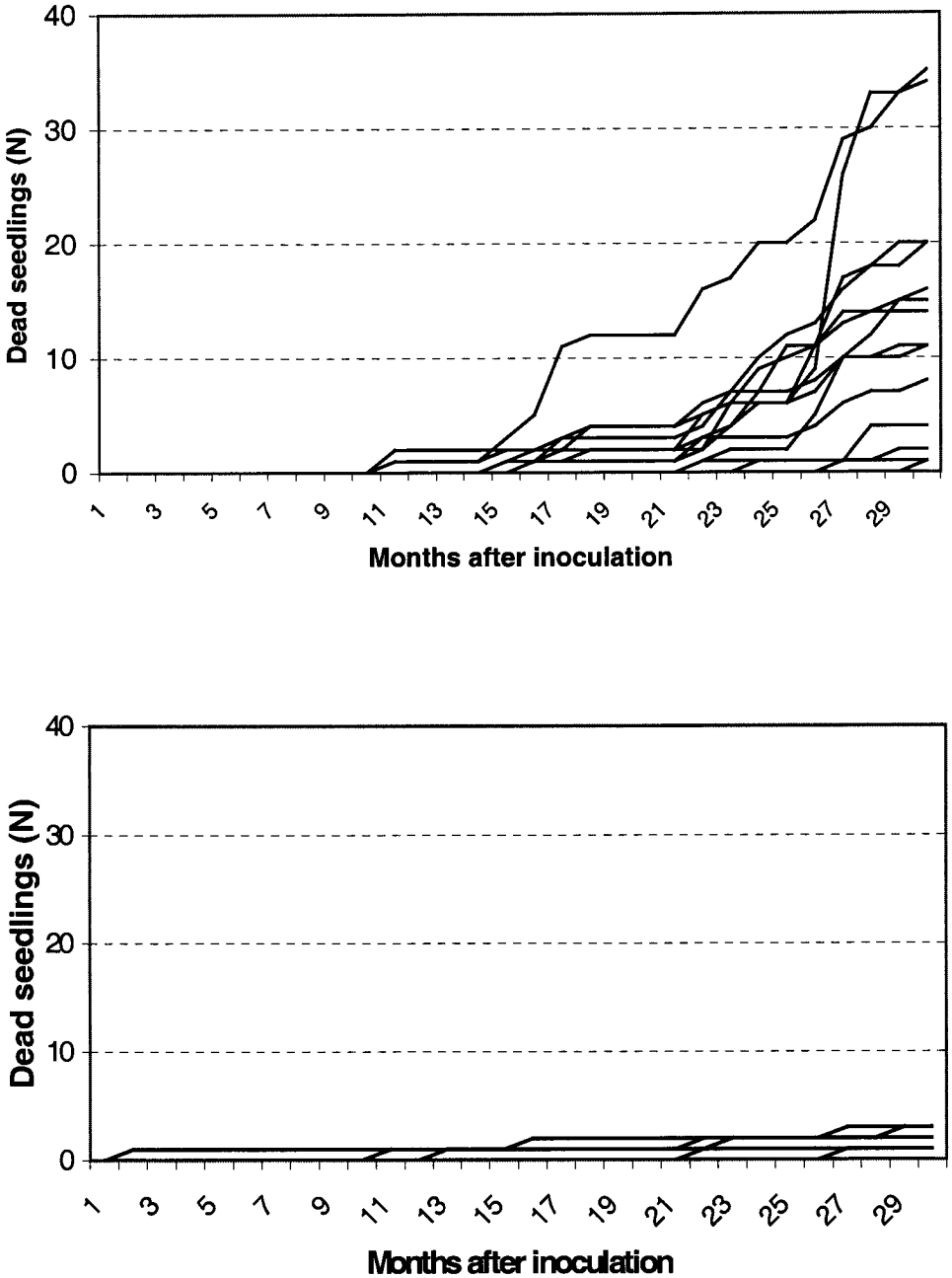

Figure 4.1. Mortality of 2-year-old Norway spruce seedlings during 30 months after inoculation with 16 isolates of *A. ostoyae* (a) and 20 isolates of *A. cepistipes* (b). Hundred seedlings were inoculated with each *Armillaria* spp. isolate.

Table 4.3. Virulence expression (i.e. number of dead and dying seedlings), rhizomorph production, and wood-degrading capability of the *A. cepistipes* and *A. ostoyae* isolates.

Isolate	Dead and dying seedlings in the four provenances (N)[a]				Total[b]	Rhizomorph production[c]	Wood-degrading capability (% weight loss)[d]
	Low-altitude provenances		High-altitude provenances				
	Bremgarten	Burgdorf	Gantrisch	Rüti			
A. cepistipes							
B1	1	0	0	0	1/100	1.1	19.9
B2	0	0	0	0	0/100	1.0	23.0
B3	0	1	2	0	3/100	1.2	16.4
B4	0	0	0	2	2/100	1.5	25.4
B6	0	0	0	0	0/100	1.2	22.0
B7	0	0	0	0	0/95	1.3	21.8
B8	0	0	0	0	0/100	1.4	18.5
B9	0	0	0	0	0/100	1.0	18.3
B10	1	0	0	0	1/100	1.2	18.5
B11	0	0	3	0	3/100	1.3	21.8
B12	2	0	0	1	3/100	0.9	23.7

B13	0	0	0	0	0/100	1.3	18.6
B14	0	0	1	0	1/90	1.7	-
B15	1	0	0	0	1/100	1.3	22.5
B16	0	0	0	0	0/100	1.3	19.9
B17	0	0	1	1	2/100	1.1	19.5
B18	0	0	0	0	0/95	1.2	9.3
B19	0	0	1	1	2/100	1.0	20.1
B20	0	0	0	0	0/100	0.8	-
B21	0	0	3		3/100	1.7	21.2
Total, mean[e]	*5/500*	*1/490*	*11/490*	*5/500*	*22/1980*	*1.2*	*19.7*
A. ostoyae:							
C1	10	12	7	9	38/100	1.5	-
C2	2	5	2	2	11/100	1.3	27.8
C3	7	2	5	5	19/85	1.0	21.8
C4	0	0	1	0	1/25	0.3	19.2
C5	3	1	0	0	4/60	0.6	17.6
C6	6	3	1	1	11/90	1.4	25.2
C7	7	7	3	5	22/95	1.1	19.3
C9	2	0	1	0	3/40	0.3	-
C10	6	14	1	3	24/100	1.3	-
C12	3	0	0	0	3/80	0.8	25.8

C13	4	4	3	4	15/95	1.0	16.7
C14	5	9	3	8	25/100	1.1	26.1
C15	1	0	0	1	2/70	0.6	20.9
C16	1	2	0	3	6/100	0.9	22.6
C17	4	8	0	10	22/95	0.9	23.8
C18	13	18	6	12	49/100	1.5	23.8
Total, mean[e]	*74/350a*	*85/340a*	*33/320b*	*63/325ab*	*255/1335*	*0.9*	*22.4*

[a]Every fungal isolate was inoculated into five pots containing totally 25 seedlings per provenance.

[b]Total = total number of dead seedlings by each isolate / total number of seedlings in pots in which rhizomorphs were produced.

[c]Mean estimates of rhizomorph production in all 20 pots per isolate (see Materials and Methods for details).

[d]Wood-degrading capability expressed as weight losses of autoclaved wood segments, one year after inoculation. - = no data obtained because isolates failed to develop on the wood segments.

[e]Total = total number of dead and dying seedlings in each provenance / total number of seedlings in pots in which rhizomorphs were produced. Numbers within the row followed by different letter differ significantly ($p < 0.05$). In *A. cepistipes* no statistical analysis could be performed because the number of dead and dying seedlings was too small. Mean = mean values for rhizomorph production and wood-degrading capability.

from rhizomorphs. However, if we exclude the most virulent isolate (C18) from this analysis, differences in virulence expression between the two sources of *A. ostoyae* isolate were not significant. In *A. cepistipes*, intraspecific variation in the virulence was low. At the end of the experiment, neither dead nor dying seedlings were observed in 9 out of 20 (45%) isolates (Table 4.3). The maximum number of dead and dying seedlings infected with *A. cepistipes* was three out of 100 inoculated seedlings, observed in the isolates B3, B11, B12, and B21. In both species, the position of the seedlings (centre or margin of the pot) did not influence their probability of being killed by *Armillaria*.

The four seedling provenances showed different susceptibility to *Armillaria* spp. The highest incidence of dead and dying seedlings was observed in the low-altitude provenance Burgdorf (10.4% of the seedlings), whereas the lowest incidence was found in the high-altitude provenance Gantrish (5.5% of the seedlings). *A. ostoyae* killed more seedlings from low-altitude provenances, but differences in mortality were only significant between the high-altitude provenance Gantrisch and the two low-altitude provenances. Interactions between *A. ostoyae* isolates and seedling provenance were significant ($P = 0.01$). In *A. cepistipes*, dead and dying seedlings were also observed in all four provenances. However, no statistical analysis could be performed because their number (totally 22) was too small.

4.3.4 Root analysis

At the end of the experiment, the roots of the seedlings without above-ground symptoms of an *Armillaria* infection were examined to determine the incidence of *Armillaria* lesions and rhizomorphs attached to the root surface. Only seedlings from Burgdorf (low altitude) and Rüti (high altitude) were considered in the analysis.

Considering both *Armillaria* species, 12.1% (92 out of 758) of the seedlings in the provenance Rüti and 18.7% (139 out of 744) in the provenance Burgdorf had at least one attached rhizomorph and/or a lesion on a main root or at the root collar. All the rhizomorphs were attached perpendicularly to the root surface. Typical epiphytic rhizomorphs growing on the root surface were not observed. For both provenances, no significant difference between the two *Armillaria* species was observed in the incidence of attached rhizomorphs. *A. ostoyae* caused significantly ($P < 0.0001$) more lesions on the roots than *A. cepistipes*. In *A. cepistipes*, only a few seedlings (Burgdorf: 6 out of 65, Rüti: 6 out of 26) with an attached rhizomorph showed lesions with resin flow (Table 4. 4). By contrast, in *A. ostoyae* more than 50% of the seedlings (Burgdorf: 21 out of 39, Rüti: 13 out of 22) with an attached rhizomorph had a

Table 4.4. Root status of the surviving seedlings from Burgdorf and Rüti without above-ground symptoms of *Armillaria* spp. infection.

Root status	Burgdorf		Rüti	
	A. cepistipes	*A. ostoyae*	*A. cepistipes*	*A. ostoyae*
Surviving seedlings (N)	489	255	495	263
With attached rhizomorphs (N)	*65*	*39*	*26*	*22*
- with lesions and mycelial fans (N)	1	21	1	13
- with lesions, without mycelial fans (N)	5	6	5	6
- without lesions, without mycelial fans (N)	59	12	20	3
Without attached rhizomorphs, with lesions (N)	*15*	*20*	*26*	*18*
- with mycelial fans (N)	0	4	0	10
- without mycelial fans (N)	15	16	26	8

lesion with resin flow and subcortical mycelial fans. In both *Armillaria* species, a number of seedlings with a lesion but without attached rhizomorphs were observed (Table 4.4); It is possible that in these cases the attached rhizomorphs were accidentally detached during the removal of the soil substratum from the roots. Except for the provenance Rüti inoculated with *A. ostoyae*, most of these seedlings had lesions without clearly visible mycelial fans.

The virulence of an *A. ostoyae* isolate was positively correlated with the incidence of lesions (Rüti: $R^2 = 0.39$, $P = 0.01$; Burgdorf: $R^2 = 0.36$, $P = 0.01$) and of attached rhizomorphs (Rüti: $R^2 = 0.33$, $P = 0.03$; Burgdorf: $R^2 = 0.48$, $P = 0.003$) on the roots of asymptomatic seedlings. No significant correlation was observed for *A. cepistipes*.

4.3.5 Re-isolations

Immediately after the death of the seedlings, re-isolations were attempted from the mycelial fans found on 10 of 14 seedlings killed by *A. cepistipes* and on 33 of 200 seedlings killed by *A. ostoyae*. Only from three seedlings it was not possible to re-isolate *Armillaria* because of contamination by other fungi or bacteria. The identity of the re-isolates was verified in somatic compatibility pairings (Harrington *et al.* 1992) with the original isolates inoculated in the particular pot. All the isolates recovered were somatically compatible with the original isolate.

4.3.6 Wood-degrading capability

Armillaria developed on 68.8% (33 out of 48) of the wood segments inoculated with *A. ostoyae* and on 71.7% (43 out of 60) of those inoculated with *A. cepistipes*. After one year incubation, the wood segments inoculated with *A. ostoyae* showed significantly ($P = 0.004$) larger weight losses (22.4% ± 0.7% standard error) than those inoculated with *A. cepistipes* (19.7% ± 0.6% standard error). The weight losses varied considerably among the isolates, ranging from 9.2 to 25.3% in *A. cepistipes* and from 16.6 to 27.8% in *A. ostoyae*. In both *Armillaria* species, the wood-degrading capability of an isolate was not correlated with its virulence expression (*A. cepistipes*: $R^2 = 0.04$, $P = 0.18$; *A. ostoyae*: $R^2 = 0.02$, $P = 0.39$).

4.4 Discussion

Our study shows not only that *A. ostoyae* can kill Norway spruce seedlings but also that there is a considerable variation in virulence among isolates. Intraspecific differences in virulence by *A. ostoyae* on other coniferous species (e.g. Douglas fir, Lodgepole pine, and Western larch) have been previously reported (Omdal *et al.* 1995, Morrison & Pellow 2002).

No significant differences in the virulence of *A. cepistipes* were observed among the tested isolates, mainly because virulence was generally very low. About half of the isolates (9 out of 20) did not incite any disease. The other isolates were comparable to the slightly virulent *A. ostoyae* isolates. For *A. cepistipes*, no extensive studies are available for comparison, since to our knowledge this was the first experiment involving a large number of isolates. The few data available also suggest low virulence of the tested *A. cepistipes* isolates (Redfern 1975, Morrison 1989, Rishbeth 1991). Our results confirm field observations, indicating that *A. cepistipes* is a weakly pathogenic species. It could be speculated that the virulence of *A. cepistipes* and some of the *A. ostoyae* isolates was underestimated due to the short (30 months) incubation time. However, two to three years is usually the maximum length of time for inoculation trials involving seedlings in pots (Shaw & Loopstra 1988, Morrison & Pellow 2002). After this period, seedlings begin to show symptoms of nutrient deficiency and competition stress, which may affect their susceptibility.

In our experiment, *A. cepistipes* produced more rhizomorphs than *A. ostoyae*. Weakly pathogenic *Armillaria* species (e.g. *A. cepistipes*, *A. gallica*, and *A. sinapina* Bérubé & Dessureault) typically form more abundant and vigorous rhizomorphs in the soil than highly pathogenic species (Rishbeth 1982, Guillaumin *et al.* 1985, Guillaumin *et al.* 1989, Cruickshank *et al.* 1997). Whereas in *A. cepistipes* no statistical analysis could be performed because the total number (22) of dead and dying seedlings was so small, the best rhizomorph producers of *A. ostoyae* were also the most virulent isolates. This result supports findings from other studies (Omdal *et al.* 1995, Desray *et al.* 1998, Morrison & Pellow 2002), and suggests that most infections by *A. ostoyae* are probably accomplished by rhizomorphs. Our study further indicates that rhizomorphs are more important for initiating infection than direct contact between the roots of the seedlings and the inoculum segment. The mortality of the seedlings planted in the centre of the pots, whose roots were in contact with the inoculum, was not higher than mortality of the seedlings on the margin of the pots. However, some infections through direct contact between the roots and the inoculum segment may also occur, as suggested by the few infected seedlings in pots containing no rhizomorphs.

The *A. ostoyae* isolates collected from diseased trees caused a higher seedling mortality than isolates obtained from rhizomorphs in the soil, suggesting that the virulence of the isolates was related to their source. However, when excluding the most virulent isolate (C18) obtained from an infected tree, the difference in virulence between the two sources becomes insignificant. Moreover, two isolates (C15 and C16) recovered from infected conifers showed a very low virulence. Low performance in inoculation trials of individual *A. ostoyae* isolates obtained from diseased conifers was also observed by other authors (Mallett & Hiratsuka 1988, Mugala *et al.* 1989). The ability of an *Armillaria* isolate to cause disease can possibly be modified by environmental factors as well as by the inoculum potential (Gregory *et al.* 1991). Furthermore, the low virulence of some isolates recovered from mycelial fans could indicate that they had secondarily colonised the trees, which had been already weakened or recently killed by other factors.

Mortality caused by *A. cepistipes* in the different provenances was too low to conduct a meaningful statistical analysis. Nevertheless, our experiment suggests that *A. cepistipes* is generally a weak pathogen on Norway spruce. In *A. ostoyae* significant differences in host susceptibility were observed between the high-altitude provenance Gantrisch and the two low-altitude provenances. The lower susceptibility of the provenance Gantrisch could be due to its slower growth in comparison to the other provenances. As a consequence of this slower growth, the roots came to contact with the pathogen (i.e. rhizomorphs, inoculum segment, or infected roots of other seedlings) later, resulting in a slowly progressing mortality. In addition, the fast growing plants may be less lignified and may contain less defence metabolites (Entry *et al.* 1991). Specific genetic traits could also be responsible for the reduced susceptibility of the provenance Gantrisch to *A. ostoyae*. Only few experimental data are available on variations in susceptibility and resistance to *Armillaria* within tree species. Most such studies have dealt with resistance of horticultural species to *A. mellea* (e.g. Proffer *et al.* 1988, Desray *et al.* 1998). For *A. ostoyae*, a large variation in susceptibility (0.9-30.7% mortality) has been observed among different provenances of *Pinus pinaster* Ait. (Lung-Escarmant & Taris 1989, Lung-Escarmant, INRA Bordeaux, personal communication). Nevertheless, differences in susceptibility of forest tree species to *Armillaria* spp. are frequently attributed to tree vigour and site conditions and not to host genetics (Singh 1983, Wargo & Harrington 1991). If a genetic component could be demonstrated, less susceptible provenances could be used in breeding programs or directly in forest plantations to reduce losses due to Armillaria root disease. Our data suggest that some genotypes of *A. ostoyae* are more virulent on particular

host provenances. Whether there are defined genetic determinants controlling *Armillaria*-host interactions needs further investigation.

All dead or dying seedlings infected by *A. cepistipes* or *A. ostoyae* had at least one lesion or attached rhizomorph on the tap root or at the root collar. This finding indicates that, in young Norway spruce, lethal infections occur mostly on the tap root or on the root collar, as observed by Shaw (1980) on ponderosa pine (*Pinus ponderosa* Laws) and by Robinson & Morrison (2001) on western larch (*Larix occidentalis* Nutt.). Both *Armillaria* species were able to attach rhizomorphs on the surface of the tap root or of the root collar of the seedlings. However, rhizomorphs of *A. ostoyae* caused significantly more lesions (necrosis). This result suggests that *A. ostoyae* is more efficient than *A. cepistipes* in penetrating the roots. A low efficiency of *A. cepistipes* and *A. gallica* to infect roots of healthy trees was previously reported by Rishbeth (1982) and Guillaumin *et al.* (1989). The ability of *A. ostoyae* to directly penetrate intact (non-wounded) root bark, on the other hand, was observed by Solla *et al.* (2002) on artificially inoculated Sitka spruce [*Picea sitchensis* (Bong.) Carr.] trees. This characteristic might distinguish pathogenic from preferentially saprotrophic *Armillaria* species, such as *A. cepistipes*.

Our data on wood deterioration show that *A. ostoyae* has a considerable saprotrophic ability, which does not correlate to the virulence of a genotype, and which exceeds that of *A. cepistipes*. However, this difference is small and the persistence of both species in Norway spruce stumps is probably very similar.

Our inoculation experiment showed a general low virulence of *A. cepistipes* isolates on Norway spruce seedlings. Under field conditions, *A. cepistipes* and *A. ostoyae* are competing for fresh tree stumps, but *A. ostoyae* is more successful in primary stump capture (Chapter 3A). Therefore the artificial introduction of *A. cepistipes* into a newly established forest on a site without or with only few *Armillaria* infections (i.e. on former agricultural land) may contribute to biological control of *A. ostoyae* and perhaps also other root rot pathogens which rely on stumps as food base.

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CURRICULUM VITAE

Simone Prospero

5th October 1972	Born in Locarno (TI), Switzerland
1987-1991	High school in Bellinzona, Scientific Matura
1991-1997	Study of Forest Sciences, Swiss Federal Institute of Technology (ETH) in Zurich (Switzerland)
1997	Diploma in Forest Sciences (Dipl. Forst. Ing. ETH)
1999-2003	PhD student at the Swiss Federal Institute of Technology in Zurich and research assistant at the Swiss Federal Research Institute WSL in Birmensdorf