



Mycorrhiza and Salt Tolerance of Trees

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Final Report of Partner 9

EU-project MYCOREM (QLK3-1999-00097)
The Use of Mycorrhizal Fungi in Phytoremediation Projects
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Preface

MYCOREM (QLK3-1999-00097) – “The Use of Mycorrhizal Fungi in Phytoremediation Projects” is an EU project of the specific program “Quality of Life and Management of Living Resources”. It comprises research and demonstration with participation of 6 public authorities and 5 SME's:

- 1 Botanisches Institut, Universität Köln, Germany
- 1c ICBGE, National Academy of Sciences, Kiev, Ukraine
- 1d Institut für Pflanzenkultur, Solkau - Schnega, Germany
- 2 CNRS-CPB, Vandoeuvre-les-Nancy, France
- 3 Limburgs Universitair Centrum, Diepenbeek, Belgium
- 4 RISSAC-MTA TAKI, National Academy of Sciences, Budapest, Hungary
- 5 Institute of Botany, Jagiellonian University, Krakow, Poland
- 6 Phytobacter GmbH, Wallmow, Germany
- 8 Triton Umweltschutz, Bitterfeld, Germany
- 9 Pius Floris Boomverzorging Vught BV, Netherlands
- 12 Robin Pepinières, Saint Laurent du Cros - Saint Bonnet, France

The objectives of Mycorem were the following:

1. Isolation of adapted mycorrhizal fungi from soils polluted by heavy metals, salt and PAH's (polycyclic aromatic hydrocarbons).
2. Selection of the most effective mycorrhizal fungal isolates conferring tolerances to plants suitable for phytoremediation.
3. Study of the mechanisms underlying stress tolerances of mycorrhizal fungi and plants on cellular level.
4. Large-scale production and quality control of the selected mycorrhizal fungi.
5. Demonstration of the possibilities for revegetation and phytoremediation of polluted land.

The project was divided in 12 work packages. Work package 1 was concerned with “Mycorrhizal fungi and salt stress” and put under responsibility of partner: 9, Pius Floris Boomverzorging, to meet in cooperation with partners 1, 1d, 3, 4, 5 and 12 the above listed objectives for the case of salt pollution.

This report focuses on “Mycorrhiza and salt tolerance of trees”, the studies carried out by partner 9. The results of the other partners concerning “Mycorrhizal fungi and salt stress” are taken into account for the discussion.

The author wants to acknowledge Prof. Hermann Bothe for the invitation to participate in this project and his coordination work, all Mycorem partners for the pleasant collaboration, Pius Floris and the staff of the tree care company and PHC Netherland as well as Klaas Poppinga and the staff of Bonte Hoek Nurseries for their support in designing and carrying out the experiments, Kees Rappoldt and Pieter Raats for advice on salinization and in situ salt measurement.

1. Introduction

Salt stress and salt tolerance

Salt in soil or spray is the most widespread chemical factor limiting plant growth. 7% of the soils on earth are saline, naturally in coastal and inland salt marshes and man-made by irrigation and de-icing salt. Alleviating plant salt stress and remediating saline soils is internationally of great economic interest.

How does salinity limit plant growth?

Increased concentrations of salt ions (Na^+ , Ca^{2+} , Cl^- , SO_4^{2-}) in the soil solution cause (Maas 1986, Marschner 1986):

1. Water deficit ('drought stress'): decreased water and nutrient availability due to increased osmotic pressure of the soil solution. This causes growth reduction, nutrient deficiencies and wilting.
2. Ion toxicity: excessive uptake of Na^+ and Cl^- affects cell membrane functioning and cell metabolism by reducing enzyme activities. This leads to growth inhibition and injury of the foliage (marginal chlorosis and necrosis on mature leaves).
3. Ion imbalance: ion competition diminishes the uptake, transport and internal distribution of nutritional elements such as K, Mg, Ca, P, and N. This causes nutrient deficiencies and growth reduction.
4. Soil compaction: high concentrations of Na^+ increase pH, deflocculate humic colloids and disperse clay particles. This leads to a destruction of soil structure with impaired drainage and root growth.

With regard to urban trees, it became clear in the early 1980s that salt applied to roads in winter was a serious cause of damage (Dobson 1991b,d, Gibbs 1994)).

Progressive above-ground salt injury symptoms of trees/shrubs (Leland 1980, Dobson 1991a):

1. Marginal yellowing/browning of leaves or needles (scorch, burn)
2. Reduced growth, stunted plants
3. Premature fall coloration and leaf drop
4. Twig and branch dieback
5. Loss of vigor
6. Death

How do plants deal with salt stress?

Different mechanisms of adaptation to high salt concentrations are more (salt tolerant plants) or less (salt sensitive plants) developed by different genotypes (Maas 1986, Marschner 1986). It is distinguished between salt excluders and salt includers.

Excluders (e.g. *Robinia*, *Quercus*) are mainly affected by internal water deficit due to the high osmotic pressure of the soil solution. They can adapt by enhanced synthesis of organic solutes (osmoregulation), by increased root surface or decreased shoot/leaf surface.

Includers are mainly affected by ion toxicity and imbalance. They can adapt by avoidance of high internal ion concentration via salt excretion, leaf drop, increase in tissue water content (succulence) or retranslocation in phloem. Another way of adaptation is to increase tissue tolerance via compartmentation of salts within individual leaf cells, synthesis of compatible solutes, maintenance of a high K/Na ratio in growing tissue and salt deposition in nonphotosynthetic tissue (e. g. *Platanus* accumulates sodium in roots without showing leaf damage; Balder 1998).

There is quite some literature available classifying tree/shrub species according to their sensitivity to salt (Francois and Clark 1978, Dobson 1991c, Matheny and Clark 1999). Below some examples concerning soil salt tolerance (can be different from spray salt tolerance):

Salt sensitive tree/shrub species:

Platanus x hispanica
Acer spp.
Fagus sylvatica
Carpinus betulus
Aesculus hippocastanum
Tilia spp.
Rosa spp.
Larix decidua
Picea abies
Pseudotsuga menziesii

Salt tolerant tree/shrub species:

Robinia pseudoacacia
Quercus spp.
Populus spp.
Rosa rugosa
Acacia spp.
Eucalyptus spp.
Pinus halepensis
Pinus nigra
Eleagnus angustifolia

How do mycorrhizal fungi deal with salt stress?

Soil microorganisms face similar problems to plants in saline soils. However, the effects of salinity on soil microbionts and their symbiotic relationships with plants are much less investigated. Dixon *et al.* (1993) reported that *in vitro* growth and *in situ* symbiosis of ectomycorrhizal fungi generally declined with increasing substrate salinity. However, salt tolerance of the tested fungi varied significantly between species and between isolates within a species. The genera *Pisolithus*, *Laccaria* and *Suillus* appeared more tolerant of sodium salts than *Thelephora* or *Cenococcum*. Reddell *et al.* (1986) and Dixon (1988) observed that *Frankia* and *Suillus* species compartmentalized salt and toxic metals in vacuoles and cell walls, thus partially excluding these agents from metabolic pathways. Already Mexal and Reid (1973) have demonstrated that ectomycorrhizal fungi have metabolic activity at water potentials, which wilt the host plant. This suggests an efficient cytoplasmatic osmoregulation. Most of the eighteen isolates of three Australian *Pisolithus* species were found to be resistant to NaCl concentrations of very saline soils (Chen *et al.* 2001).

Also the development of arbuscular mycorrhizal fungi from spore germination till root colonization is generally reduced by increasing salt concentrations (Juniper and Abbott 1993). However, AM fungal colonized halophytes like *Aster tripolium* occur in salt marshes world-wide and the content of AM fungal spores in saline soils can be high (Mason 1928, Rozema *et al.* 1986, Carvalho *et al.* 2001, Hildebrand *et al.* 2001). Carvalho *et al.* (2001) reported low AM fungal diversity with *Glomus geosporum* dominant in salt marshes of the Portuguese Tagus estuary. They concluded that the distribution of mycorrhizas in salt marsh is more dependent on host plant species than on environmental stresses. Most halophyte species are non-mycorrhizal. Molecular biological techniques revealed that 80%, on average, of the AM spores isolated from a range of European saline soils belonged to one single species, *Glomus geosporum*, which occurred much less in the surrounding non-saline habitats (Hildebrand *et al.* 2001, Landwehr *et al.* 2002). The authors speculate that specific AM ecotypes may be particularly adapted to saline conditions and that they could have a great potential in conferring salt tolerance to plants. On the other hand, Cantrell and Linderman (2001) reported that AM fungi from saline soil were not more effective than those from nonsaline soil in reducing growth inhibition of lettuce and onion plants by salt. In another study (Copeman *et al.* 1996) AM fungi originating from saline soil, in contrary to fungi from nonsaline soil, did not promote growth of tomato under saline conditions. However, reduction in leaf chloride concentrations mediated by these fungi may have beneficial implications for plant survival in saline soil. Increasing salinity decreased the hyphal development of *Glomus sp.* from saline soil to a higher extent than that of *Glomus deserticola* from nonsaline soil (Ruiz-Lozano and Azcon 2000). Though both AMF protected

host plants against salinity, they differed in their symbiotic efficiencies and mechanisms to mediate plant salt tolerance. Rosendahl en Rosendahl (1991) demonstrated large variations in salt tolerance of AM fungal species and isolates.

Mycorrhiza and alleviation of plant salt stress

Mycorrhizas are mutualistic associations of certain soil fungi and the roots of most crop plants and trees. Due to an extended network of fine hyphae the fungi can considerably improve the uptake of mineral nutrients to their host plant, whereas the plant supports the fungus with assimilation products (Harley and Smith 1983, Smith and Read 1997).

There are two major types of mycorrhiza:

Endomycorrhizas: the fungus grows inside root cells; *arbuscular mycorrhizas* forming so-called arbuscules inside root cells are the most widespread endomycorrhizas.

Ectomycorrhizas: the fungus grows between root cells and forms a mantel around the root surface; they occur mainly on woody plants.

With trees and shrubs both types of mycorrhiza can occur depending on tree species and habitat. Several tree taxa (e.g. *Salix*, *Populus*, *Alnus*, *Eucalyptus*) can form both endo- and ectomycorrhiza.

As written above, mycorrhizal fungi can more or less cope with salinity as doe plants, or even better. What is their role in improving plant fitness under saline conditions?

There is considerable evidence that arbuscular mycorrhizal (AM) fungi can enhance plant growth and vigor under salt stress conditions (Pond et al. 1984, Pfeiffer and Bloss 1988, Juniper and Abbott 1993, Ruiz-Lozano et al. 1996, Tsang and Maun 1999, Al-Karaki et al. 2001). This has been attributed to a more efficient nutrient uptake, particularly phosphorus (Hirrel and Gerdemann 1980, Ojala et al. 1983, Marschner 1986, Pfeiffer and Bloss 1988, Al-Karaki 2000), since addition of P fertilizer had similar effects as AM inoculation. P is the macronutrient with the lowest mobility in soil and thus often limiting plant growth, particularly when soil water potential and P diffusion rate is lowered in dry or saline soils.

However, there is growing evidence that the alleviating effect of mycorrhiza on plant salt stress may not be limited to improved P-nutrition, since addition of P to salinized soil reduced plant salt stress to a lesser extent than AM inoculation (Ruiz-Lozano et al. 1996, Cantrell and Linderman 2001, Feng et al. 2000). Increased potassium uptake may help to maintain a high K/Na ratio in the plant tissue (Poss et al. 1985). Differential nutrient uptake may positively affect cation/anion balance (Azcon and El-Atrash 1997). Mycorrhiza may change physiological processes such as water use efficiency, stomatal conductance, transpiration and carbon dioxide exchange rate (Ruiz-Lozano et al. 1996). AM may improve osmotic adjustment via accumulation of soluble sugars and electrolyte concentrations in root cells (Feng et al. 2002).

On the other hand, Graham and Syvertsen (1989) found that *Glomus intraradices* did not influence the tolerance of citrus seedlings to salinity, but enhanced Cl uptake.

Little is known about AM fungal trees on saline soils. Guttay (1976) reports on the impact of deicing salt on the endomycorrhizas of roadside sugar maples (*Acer saccharum*): trees damaged from continuous salt application had a diminished root system and particularly a loss in the surface roots, a decreased AM colonization, increased root Na and Cl, reduced root K and P, and reduced leaf Ca and Mg. However, the direct effects of salinity on fungal growth could not be separated from plant-mediated effects. Jain et al. (1991) reported AM improved productivity of multipurpose trees on substandard soils in India. In another study with trees, double inoculation of *Acacia cyanophylla* with rhizobia and AM fungi significantly increased salt tolerance (Hatimi 1999).

In contrast with AM fungi, we know much less about the impact of ECM fungi on trees in saline environments. Only recently Muhsin and Zwiazek (2002) demonstrated that *Hebeloma crustuliniforme* alleviated salt stress from white spruce (*Picea glauca*) seedlings. They identified the reduction of shoot Na uptake while increasing N and P absorption and maintaining high transpiration rates and root water conductance as important salt tolerance mechanisms related to ectomycorrhizal symbiosis.

The potential role of mycorrhiza in alleviating salt stress to their host plant can be summarized as follows:

1. Enhancing mineral nutrition to compensate for nutrient deficiencies
2. Compensating for nutritional imbalances
3. Improving plant water status
4. Reducing salt uptake into the host plant

Tree planting is regarded as a leading solution in controlling salinity (Dunn et al. 1994, Ghassemi et al. 1995). It is thought, for example, that hydrological imbalances may be considerably restored by replacement of shallow agricultural grasses with deep-rooted trees, thereby reversing the casual process of salinization (Hussain and Gull 1991, Sun and Dickinson 1995). Despite worldwide activities in this field there is still little knowledge, particularly in Europe, on the use of mycorrhizal fungi in facilitating tree establishment and survival on saline or salt-affected sites.

Hypothesis

1. Mycorrhizal fungi can increase salt tolerance of their host plants.
2. Mycorrhizal fungi from saline soils are more salt tolerant and better confer salt tolerance to plants.
3. Inoculation with selected mycorrhizal fungi can improve salt tolerance of plants.

Objective

The aim of this study was to test the hypothesis with trees under nursery and field conditions using artificial salinization (de-icing salt) and available inocula from different project partners.

2. Container experiments under nursery conditions

Material and methods

The container experiments were carried out at Bonte Hoek Nurseries (Meentweg 18, 9756 Glimmen, The Netherlands) with the kind support of owner K. Popping in the period of April 2000 until November 2002.

During the summer season (April - September) plants were kept outside and were included in the automatic sprinkling irrigation system of the nursery.

During the winter season (October – March) plants were kept in a glasshouse (2000/2001) at temperatures above 0° or in a plastic tunnel (2001/2002).

The tree species chosen for the experiments are commonly used in urban surroundings in the Netherlands (and Europe). The adopted criteria for the choice were economical importance, suitability for the available experimental conditions, salt sensitivity and mycorrhizal symbionts.

Aesculus hippocastanum: AM only; low soil salt tolerance, but moderately tolerant to salt spray, often planted in urban areas.

Tilia cordata: ECM mostly, low salt tolerance, often planted in urban areas in the Netherlands and elsewhere.

Populus canescens: ECM and/or AM, high salt tolerance (soil and spray), fast growing tree.

Ulmus x hollandica: AM mostly, typical Dutch tree which is almost the only tree that can withstand the strong salty winds along the coast; particularly interesting because of the Dutch elm disease (*Ceratocystis ulmi*), which causes big losses of these trees.

Aesculus pavia: AM only, ornamental tree planted in urban areas, low salt tolerance.

Fagus sylvatica: ECM only, low salt tolerance, popular tree for lanes and hedgerows.

Aesculus hippocastanum, *Tilia cordata*, *Populus canescens* and *Ulmus x hollandica* were purchased as one-year old bare-root seedlings from a nursery, where they were elevated in a nursery bed on field soil, thus under non-axenic conditions. *Aesculus pavia* and *Fagus sylvatica* were elevated in containers under semi-axenic conditions by Robin Pepinières (partner 12).

Mycorrhizal inoculation was carried out with inocula provided by MYCOREM partners (EU treatment) and with commercial inocula from Plant Health Care (PHC treatment).

EU inocula: were kindly provided by the Mycorem partners 1/1d, 5 and 12. However, not all desirable inocula were available in sufficient amount at the time of planting. Well available and also suitable inocula were used instead.

AM: *Glomus geosporum* ecotype from central European salt marshes (isolated by partner 1 and scaled up by partner 1d). This inoculum was available only in June 2001 and was used for experiment 6 (IFP 2000). For all other experiments we used *Glomus etunicatum* / *intraradices* IFP 1/99 from partner 1d.

ECM: No isolates from salt ecosystems were available. Prof. Turnau (partner 5) supplied us with *Paxillus involutus* strain nr. 59 (isolated from polluted site in Belgium by J. Colpaert, partner 3) and strain nr. 64 (zinc industrial waste, Poland) and *Scleroderma citrinum* strain nr. 67 (scots pine forest, Poland) as mycelium. *Paxillus involutus* is a generalist, very common on young trees in many places. It is regarded as stress tolerant, however this does not automatically mean that it is salt tolerant (J. Colpaert, pers. communication). *Fagus sylvatica* seedlings mycorrhizal with *Laccaria bicolor* were provided by Robin Pepinières (partner 12).

PHC inocula: The commercial mycorrhizal inocula of PHC are routinely applied by 'Pius Floris Boomverzorging' and 'Bonte Hoek Nurseries' with (trans)planting and restoring of trees in urban environments to support plant performance. There are different products available regarding the composition of mycorrhizal fungi, other components (beneficial bacteria, wetting agents, organic compounds) and the formulation (powder, granules, liquid root dip). With regard to nursery/tree care practice we decided to use the ready formulated commercial products instead of using the mycorrhizal component only. However, we chose the inocula with the least other components.

AM: *Mycor®VAMCocktail®*: contains *Entrophospora columbiana*, *Glomus etunicatum*, *Glomus clarum* and *Glomus sp.*, 88000 spores/kg, of which each of the four AM species represents at least 15%; dry yucca plant extract and humic acids. *Mycor®FlowerSaver®*: same strain composition as above, 22000 spores/kg, rhizosphere bacteria, dry yucca plant extract and humic acids.

ECM: *MycorTree® TreeSaver Injectable Pt*: contains *Pisolithus tinctorius*, minimum 3.9 billion (10^9) spores/kg; rhizosphere bacteria; dry yucca plant extract and humic acids.

AM/ECM: *MycorTree® TreeSaver Injectable*: contains *Entrophospora columbiana*, *Glomus etunicatum*, *Glomus clarum* and *Glomus sp.*, minimum 88000 spores/kg, of which each of the four AM species represents at least 15%; *Pisolithus tinctorius*, minimum 3.9 billion (10^9) spores/kg; rhizosphere bacteria; dry yucca plant extract and humic acids.

To suppress mycorrhizal development on the non-inoculated control trees the fungicide Benlate (Benomyl) was applied several times at the recommended dose during the growing season of 2001 with *Ulmus*, *Aesculus hippocastanum* (only the part which got salt later) and *Aesculus pavia*.

The **growth substrate** was a mix of 18 liter moderately fertilized (50% of the normal concentration) peat-based nursery substrate, 6 liter sand and 2 liter Biovin®, an organic fertilizer. For more details on the used products see Annex. The pH of this substrate was around 6. During the active growth season (April till July) according to the demand of the specific tree species additional fertilizer was added as NPK (12-4-6, Wuxal) solution and as granular slow release organic fertilizer (DCM5:10-4-8, DCM2:7-6-12-4MgO). In the second season (2001) slow release lime granules (DCM Groenkalk) were added to avoid decrease of pH. This lead to a pH around 7.

Tree seedlings were planted in 5 liter pots and inoculated at the same time by adding the inoculum in the planting hole and on the roots. The roots of the bare-root seedlings were cut back to a minimum and washed thoroughly under running water before planting. The container-grown seedlings were planted with the root ball. At the beginning of their third growing season (April 2002), *Fagus sylvatica* and *Tilia cordata* were transplanted in 20 liter pots with fresh substrate. At this occasion the PHC treated trees were reinoculated with *Pisolithus tinctorius*.

Deicing salt (NaCl, Akzo, Delfzijl, NL) was used to establish and maintain the different salt stress levels throughout the experiment. For the **salinization of the substrate and the *in situ* monitoring of salt concentrations** we developed the method described in detail in the Appendix of this report. The EC (electrical conductivity) of the container substrate was controlled regularly *in situ* using a dielectric soil moisture sensor. The choice of the salinity levels was based on literature values of maximum tolerable EC values and salt tolerance categories of plants. Under the nursery conditions (rainfall, automatic sprinkling irrigation) it was not possible to establish constant salt concentrations. This problem was faced by using a range of logarithmically increasing salinity levels. Depending on the experiment, salinization was started immediately after planting and/or several months later, allowing roots to be colonized without salt stress.

Below the experimental design is described per tree species:

Experiment 1 – *Aesculus hippocastanum*

inoculation treatments (AM): C non-inoculated control
EU *G.etunicatum* IFP 1/99
PHC Mycor®VAM Cocktail®

salinity levels: EC1, EC2, EC4, EC8, EC16
repetitions: 3 + 3
total number of trees: 90
start of experiment: April 2000
start of salinization: may 2000 + January 2001
end of experiment: April 2002

Experiment 2 – *Populus canescens*

inoculation treatments (AM/ECM): C non-inoculated control
EU *G.etunicatum* IFP 1/99 + *Paxillus involutus* 64 +
Scleroderma citrinum 67
PHC Mycortree® TreeSaver Injectable

salinity levels: EC1, EC2, EC4, EC8, EC16
repetitions: 3 + 3
total number of trees: 90
start of experiment: April 2000
start of salinization: may 2000 + January 2001
end of experiment: April 2002

Experiment 3 – *Ulmus x hollandica*

inoculation treatments (AM/ECM): C non-inoculated control
EU *G.etunicatum* IFP 1/99 + *Paxillus involutus* 64 +
Scleroderma citrinum 67
PHC Mycortree® TreeSaver Injectable

salinity levels: EC1, EC2, EC4, EC8, EC16
repetitions: 6
total number of trees: 90
start of experiment: April 2000
start of salinization: January 2001
end of experiment: April 2002

Experiment 4 – *Tilia cordata*

inoculation treatments (ECM): C non-inoculated control
EU *Paxillus involutus* 59
PHC Mycortree® TreeSaver Injectable Pt.

salinity levels: EC1, EC2, EC4, EC8
repetitions: 7
total number of trees: 84
start of experiment: may 2000
start of salinization: January 2001
end of experiment: November 2002

Experiment 5 – *Fagus sylvatica*

inoculation treatments (ECM): C non-inoculated control
 EU *Laccaria bicolor* (mycorrhizal seedlings)
 PHC Mycortree® TreeSaver Injectable Pt.

salinity levels: EC1, EC2, EC4, EC8

repetitions: 5

total number of trees: 60

start of experiment: may 2000

start of salinization: may 2001

end of experiment: November 2002

Experiment 6 – *Aesculus pavia*

inoculation treatments (AM): C non-inoculated control
 EU *Glomus geosporum* IFP 2000 (salt ecotype)
 PHC Mycor® FlowerSaver®

salinity levels: EC1, EC8, EC16

repetitions: 7

total number of trees: 63

start of experiment: June 2001

start of salinization: August 2001

end of experiment: September 2002

The following **parameters** were evaluated:

Leaf appearance at the beginning of the growing season using a 5-class system:

0: buds closed

1: buds swollen, green tips

2: buds open, leaves folded

3: leaves partially unfolded

4: leaves fully unfolded

Salt stress symptoms throughout the growing season using a 5-class system:

0: no symptoms

1: burned leaf edges

2: > 20% burned leaf surface

3: > 50% burned leaf surface, dieback of twigs

4: all leaves dropped, severe dieback or tree dead

Tree growth by measuring at the beginning and at the end of the growing season tree length in cm and stem diameter at the stem basis in mm. Based on these measurements an estimation of tree volume was carried out according to the formula: (stem diameter)² x stem length.

Mycorrhizal root colonization: AM as percentage root length colonized by the gridline intersect method after clearing and staining. ECM as number of mycorrhizal root tips per cm root length by gridline intersect and by rating system (5: high, 3: moderate, 1: low).

Foliar chloride concentration (Koch Bodemtechnik/Eurolab, Deventer, NL).

The pots were arranged per tree species completely randomized. All experiments involved two factors: salinity level and inoculation treatment. All data were analyzed by ANOVA. Means were compared based on least significant differences ($p < 0.05$).

Results and Discussion

Experiment 1 – *Aesculus hippocastanum*

Tree growth was not significantly affected by mycorrhizal inoculation across all salt levels (Table 2.1.1). Stem height and tree volume (data not shown) gave the same result.

Table 2.1.1 Growth of *Aesculus hippocastanum* trees with different mycorrhizal inoculation treatment (MycInoc), measured as increase in stem diameter (mm). Values are means (n = 30) with standard deviation. Means in each column followed by the same letter are not statistically different (p < 0.05).

MycInoc	2000	2001	Total growth (2000 + 2001)
Control	1.2 ± 1.6 a	6.3 ± 3.3 a	7.5 ± 3.9 a
EU	1.6 ± 1.4 a	6.1 ± 3.1 a	7.7 ± 3.6 a
PHC	1.7 ± 1.5 a	5.9 ± 3.2 a	7.6 ± 3.5 a

In the first year (2000) a growth reducing effect of salt was observed only with the Sd (salt direct) treatment and only with the highest salt level (Table 2.1.2). This reflects the cautious salt application in the beginning of the experiment due to lacking experience with salinization. In addition, the reserves that the tree seedlings had build up before the experiment could have masked toxic salt effects. In the second year (2001), when salinization was optimized, a clear growth decrease with increasing salt level could be observed with both Sd and SL treatments (Table 2.1.2). Already at the lowest salt addition EC2 burned leaf edges as salt stress symptoms were observed and tree growth was reduced significantly. This corresponds to the low soil salt tolerance of *Aesculus hippocastanum* reported in literature. At the highest salt concentration EC16 trees showed severe leaf burn, premature defoliation, stunted growth and dieback.

Table 2.1.2 Growth of *Aesculus hippocastanum* trees measured as increase in stem diameter (mm) at different salt concentrations. Values are means (n = 9) with standard deviation. Means in each column followed by the same letter are not statistically different (p < 0.05). Sd = salt directly applied, SL = salt later applied.

Salt	2000	2001	Total growth (2000 + 2001)
Sd EC1	2.0 ± 1.6 a	10.0 ± 2.2 a	12.0 ± 2.4 a
Sd EC2	1.4 ± 1.2 ab	8.2 ± 1.5 b	9.5 ± 1.7 b
Sd EC4	1.9 ± 1.0 a	7.4 ± 2.1 b	9.3 ± 1.6 b
Sd EC8	1.4 ± 1.2 ab	4.3 ± 1.5 c	5.8 ± 2.3 ce
Sd EC16	0.4 ± 1.6 b	1.9 ± 1.4 d	2.3 ± 1.9 d
SL EC1	1.7 ± 0.8 ab	9.8 ± 1.4 a	11.5 ± 1.5 a
SL EC2	2.1 ± 0.8 a	6.9 ± 1.0 be	9.0 ± 1.1 b
SL EC4	1.4 ± 0.8 ab	5.7 ± 1.4 ce	7.0 ± 2.4 c
SL EC8	1.8 ± 1.2 ab	4.4 ± 2.0 c	5.8 ± 2.2 ce
SL EC16	1.7 ± 1.4 ab	2.1 ± 1.8 d	3.6 ± 2.5 de

Mycorrhizal inoculation did not increase the salt tolerance of *A. hippocastanum* trees in this experiment (Fig. 2.1.1). Direct or later salt application did not make a difference.

Foliar chloride concentration did not increase proportionally with soil salt concentration (Fig. 2.1.2). Already at the lowest salt addition (EC2) foliar concentration increased considerably (30-50%) to almost the values at the higher soil salt concentrations (EC4/8). At the high salt levels accumulation of Cl in the leaves might have been limited due to root damage and reduced physiological activity of the tree. Mycorrhizal inoculation did not substantially influence foliar chloride concentration, which corresponds to the data on tree growth and mycorrhizal development. In literature salt stress symptoms are reported from 0.14% to 3.20% foliar chloride in dry matter. The non-salt treated trees of this experiment (EC1) had Cl concentrations clearly above 0.14%, whereas the salt stressed trees of treatment EC8 had Cl concentrations far below 3.20%. Therefore this parameter seems not to be very useful to determine salt stress in an absolute manner.

Fig. 2.1.1 Growth of *Aesculus hippocastanum* trees in 2001 with different inoculation treatment and at different salt concentrations, measured as increase in stem diameter (mm), Sd and SL data are combined, n = 6, means + SD.

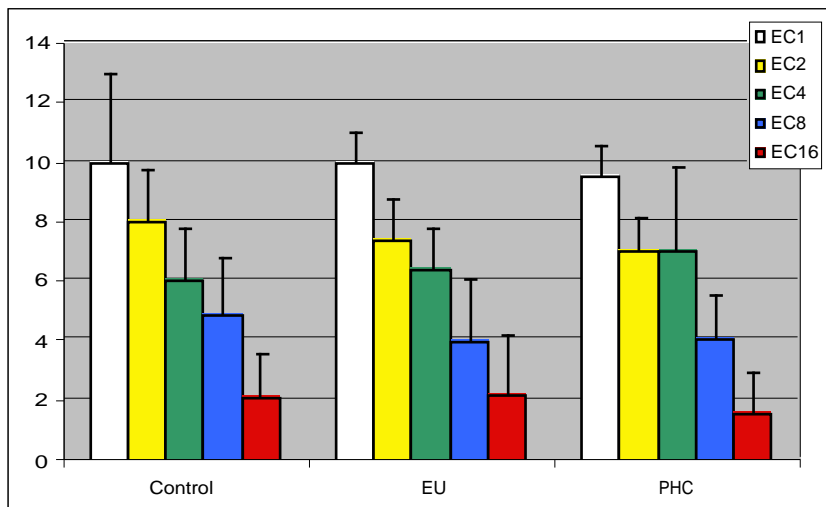
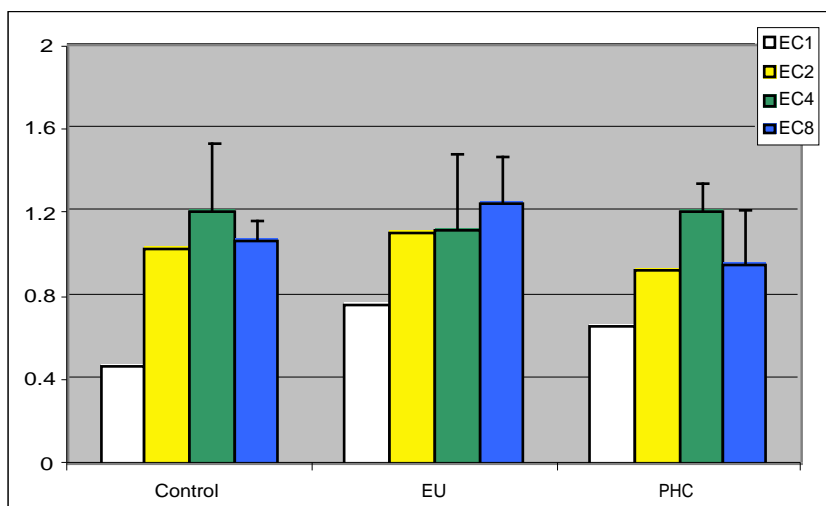


Fig. 2.1.2 Foliar chloride (% dry matter) of *Aesculus hippocastanum* SL trees in 2001 with different inoculation treatment and at different salt concentrations, n = 3, means + SD.



At the end of the first season (2000) arbuscular mycorrhizal colonization of *A. hippocastanum* trees was quite variable with sometimes high deviations from the average values (Table 2.1.3). Non-inoculated control trees were just as well colonized by AM fungi as inoculated trees. Only molecular analysis could reveal if inoculated fungi were competitive with indigenous fungi. There was no substantial difference in percentage root colonization between the inoculation treatments. A decrease of AM colonization with increasing salt stress (only Sd) was notable. It cannot be distinguished, if this decrease is due to a direct toxic effect of salt on the fungal development or to an indirect effect of less available assimilates from the salt stressed plant. However, tree growth was reduced only at the highest salt level (Table 2.1.2).

AM colonization at the end of the second season (2001) gave a similar picture than in 2000 (Table 2.1.4). The reduction by increasing salt concentrations was more pronounced with Sd than with SL, which received salt only in this second season, indicating a long-term effect of high salt stress. Correspondingly tree growth was now reduced already at EC2 (Table 2.1.2).

Table 2.1.3 Mycorrhizal root colonization of *A. hippocastanum* trees in October 2000 (% root length colonized), Sd = salt directly applied: values are means (n = 3) with standard deviation, SL = salt later applied: values refer to pooled samples of 3 repetitions per treatment, since salt was not yet applied.

Salt	C	EU	PHC
Sd EC1	48 ± 13	18 ± 25	44 ± 11
Sd EC2	-	-	-
Sd EC4	27 ± 23	22 ± 17	46 ± 8
Sd EC8	27 ± 21	17 ± 11	29 ± 25
Sd EC16	14 ± 15	19 ± 12	17 ± 13
SL EC1	36	26	72
SL EC2	58	58	68
SL EC4	72	54	52
SL EC8	7	51	61
SL EC16	60	36	48
<i>Average of SL (n=5)</i>	46.6	45.0	60.2
<i>Standard deviation</i>	25.7	13.5	10.2

The generally lower colonization with SL control can be probably accounted to the effect of the fungicide Benomyl that was applied several times in 2001 in order to create a proper non- or low-mycorrhizal control. However, this was only partially achieved and was not reflected in tree growth or foliar chloride accumulation. Thus higher root colonization did not confer higher salt tolerance to the trees.

Table 2.1.4 Mycorrhizal root colonization of *A. hippocastanum* trees in August 2001 (% root length colonized), Sd = salt directly applied, SL = salt later applied, values are means (n = 3) with standard deviation.

Salt	C	EU	PHC
Sd EC1	45 ± 9	35 ± 0	40 ± 9
Sd EC2	28 ± 12	40 ± 9	28 ± 6
Sd EC4	35 ± 26	27 ± 23	42 ± 14
Sd EC8	22 ± 15	18 ± 12	22 ± 15
Sd EC16	15 ± 17	12 ± 6	18 ± 12
SL EC1	22 ± 6	45 ± 9	45 ± 9
SL EC2	22 ± 15	33 ± 18	50 ± 0
SL EC4	15 ± 17	37 ± 13	18 ± 12
SL EC8	12 ± 12	25 ± 0	37 ± 13
SL EC16	15 ± 10	28 ± 12	28 ± 6

With *A. hippocastanum* application of two different AM inocula did not significantly increase root colonization above control values and did not increase the low salt tolerance of this tree species.

Experiment 2 – *Populus canescens*

Tree growth was not significantly affected by mycorrhizal inoculation across all salt levels (Table 2.2.1). Stem height and tree volume (data not shown) gave the same result.

Table 2.2.1 Growth of *Populus canescens* trees with different mycorrhizal inoculation treatment (MycInoc), measured as increase in stem diameter (mm). Values are means (n = 30) with standard deviation. Means in each column followed by the same letter are not statistically different (p < 0.05).

MycInoc	2000	2001	Total growth (2000 + 2001)
Control	2.9 ± 1.2 a	7.7 ± 3.2 a	10.6 ± 3.2 a
EU	3.2 ± 1.2 a	7.2 ± 3.3 a	10.4 ± 3.8 a
PHC	3.1 ± 0.9 a	6.8 ± 3.0 a	9.9 ± 3.1 a

In the first year (2000) no substantial growth reducing effect of salt (Sd treatment) was observed (Table 2.2.2). This might be due to the cautious salt application in the beginning of the experiment due to lacking experience with salinization. In addition, the reserves that the tree seedlings had build up before the experiment could have masked toxic salt effects. In the second year (2001), when salinization was optimized, a significant growth decrease with increasing salt level could be observed with both Sd and SL treatments (Table 2.2.2). However, only at the highest salt concentration EC16 trees showed clear salt stress symptoms and a substantial growth reduction. This corresponds to the high soil salt tolerance of *Populus canescens* reported in literature.

Table 2.2.2 Growth of *Populus canescens* trees measured as increase in stem diameter (mm) at different salt concentrations. Values are means (n = 9) with standard deviation. Means in each column followed by the same letter are not statistically different (p < 0.05). Sd = salt directly applied, SL = salt later applied.

Salt	2000	2001	Total growth (2000 + 2001)
Sd EC1	2.7 ± 1.5 ab	10.2 ± 2.7 a	12.9 ± 2.6 a
Sd EC2	3.5 ± 1.0 b	8.7 ± 1.4 ab	12.2 ± 1.1 ab
Sd EC4	2.6 ± 0.5 ab	8.2 ± 2.0 b	10.8 ± 1.9 b
Sd EC8	3.2 ± 0.5 ab	7.0 ± 1.7 b	10.2 ± 1.7 b
Sd EC16	2.3 ± 1.5 a	2.4 ± 2.5 c	4.7 ± 3.6 c
SL EC1	3.4 ± 0.7 b	8.4 ± 1.5 ab	11.5 ± 1.7 ab
SL EC2	3.4 ± 0.7 b	8.9 ± 1.6 ab	9.0 ± 2.1 ab
SL EC4	3.2 ± 0.9 ab	7.8 ± 2.0 b	7.0 ± 1.6 ab
SL EC8	2.8 ± 1.5 ab	8.3 ± 2.4 b	5.8 ± 2.6 ab
SL EC16	3.3 ± 1.2 ab	2.4 ± 1.5 c	3.6 ± 1.9 c

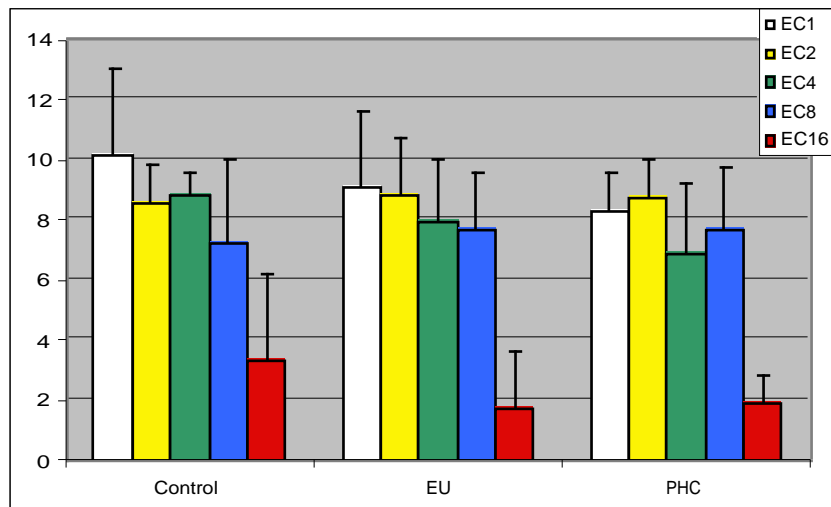
Mycorrhizal inoculation did not increase the salt tolerance of *Populus canescens* trees in this experiment (Fig.2.2.1). Direct or later salt application did not make a difference.

Though *Populus* trees are known to be colonized by ECM and/or AM fungi and both types were inoculated, only ECM colonization was observed in this experiment.

Root analysis in January and in November 2001 and February 2002 revealed abundant ECM colonization in all inoculation treatments (C, EU, PHC) with diverse morphotypes: *Thelephora*, *Hebeloma* (also fruitbodies), *Tuber* and a brown *Ascomycete*.

The inoculated *Paxillus*, *Scleroderma* and *Pisolithus* fungi were not found colonizing the roots. Apparently, they could not compete with the indigenous poplar mycorrhizas under this nursery conditions. The relatively high pH (6.5 to 7) might have played an important role. Also the relatively careful salt application during the first season might not have been effective to select the more salt or stress tolerant inoculated species. Particularly *Pisolithus* is considered to be more salt tolerant than for example *Thelephora* (Chen et al. 2001).

Fig. 2.2.1 Growth of *Populus canescens* trees in 2001 with different inoculation treatment and at different salt concentrations, measured as increase in stem diameter (mm), Sd and SL data are combined, n = 6, means + SD.



At the end of the second season, there was no obvious shift in abundance of morphotypes with increasing salt concentration, but a general reduction of mycorrhization.

Whereas at the end of the first season no differences could be observed between treatments, ECM colonization at the end of the second season was substantially decreased at EC8 together with a decrease in root density and vitality. At EC16 mainly dead roots and old mycorrhizas were observed. Root systems of plants, which received salt later (in the second season), were slightly less damaged than with direct salt application (in the first season). However, the difference between salt levels (EC8 and EC16) was more important.

The substantial decrease in root condition at EC8 was not yet visible with aboveground tree growth (Table 2.2.2), which probably would be affected with a delay of one season.

Experiment 3 - *Ulmus x hollandica*

Tree growth was not significantly affected by mycorrhizal inoculation across all salt levels in both years of the experiment (Table 2.3.1). Growth in the first year was clearly less than in the second year.

Table 2.3.1 Growth of *Ulmus x hollandica* trees with different mycorrhizal inoculation treatment (MycInoc), measured as increase in stem diameter (mm). Values are means (n = 30) with standard deviation. Means in each column followed by the same letter are not statistically different (p < 0.05).

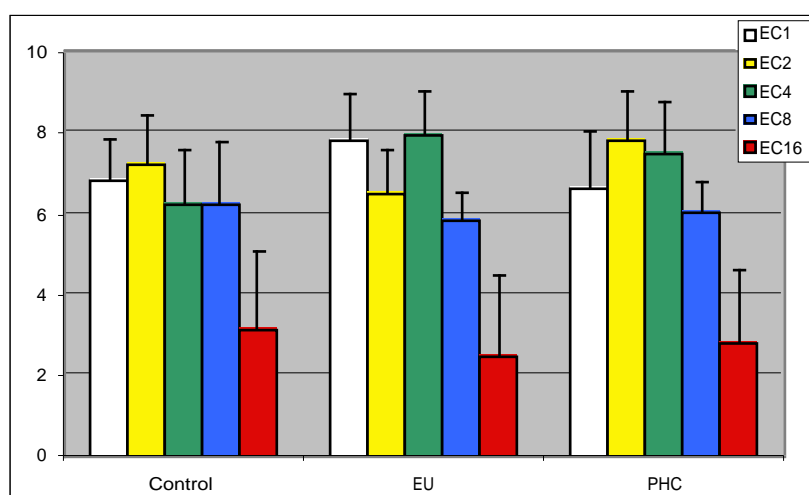
MycInoc	2000	2001	Total growth (2000 + 2001)
Control	1.98 ± 1.09 a	6.02 ± 1.98 a	8.00 ± 2.07 a
EU	1.38 ± 0.89 a	6.18 ± 2.35 a	7.57 ± 2.40 a
PHC	1.84 ± 1.03 a	6.14 ± 2.24 a	7.98 ± 2.22 a

Table 2.3.2 Growth of *Ulmus x hollandica* trees measured as increase in stem diameter (mm) at different salt concentrations. Values are means (n = 18) with standard deviation. Means in each column followed by the same letter are not statistically different (p < 0.05).

Salt	2000	2001	Total growth (2000 + 2001)
EC1	1.7 ± 0.8 a	7.2 ± 1.2 a	8.9 ± 1.0 a
EC2	2.1 ± 0.8 a	7.2 ± 1.2 a	9.3 ± 1.2 a
EC4	1.4 ± 0.8 a	7.3 ± 1.4 a	8.8 ± 1.3 ab
EC8	1.8 ± 1.2 a	6.1 ± 1.0 b	7.9 ± 1.5 b
EC16	1.7 ± 1.4 a	2.8 ± 1.8 c	4.6 ± 1.9 c

There was a clear reducing effect of increasing salt concentration on tree growth in the second year (2001), when salt was applied (Table 2.3.2). A significant growth decrease was observed at the two highest salt levels EC8 and EC16. Only at these high salt levels, salt stress symptoms such as leaf burn, premature defoliation (EC16) and stem dieback (EC16) could be observed.

Fig. 2.3.1 Growth of *Ulmus x hollandicas* trees in 2001 with different inoculation treatment and at different salt concentrations, measured as increase in stem diameter (mm), n = 6, means + SD.



Mycorrhizal inoculation did not increase the salt tolerance of *Ulmus x hollandica* trees in this experiment (Fig. 2.3.1).

At the start of the experiment with the one-year old seedlings a very low indigenous AM colonization was observed. One year after planting and inoculation and before the start of salinization (in January 2001) the average percentage AM root colonization was still low

with no significant difference between inoculation treatments (6.8%, 6.6% and 8.0% respectively for Control, EU and PHC).

In May 2002, after more than one year of salinization, AM colonization was increased to around 40% on the trees without salt addition, but decreased steeply with increasing salt concentration (Table 2.3.3). Still there were no differences between inoculation treatments. The application of the fungicide Benomyl on the control trees apparently was not effective.

Table 2.3.3 AM root colonization of *U. x hollandica* trees in May 2002 (% root length colonized), values refer to pooled samples of 6 repetitions per treatment.

Salt	C	EU	PHC
EC1	43	42	41
EC2	-	-	-
EC4	21	19	21
EC8	4.2	3.5	5.7
EC16	0	0	0

No ectomycorrhizal development could be observed despite inoculation with ECM fungi. This is in accordance with literature (Harley and Harley 1987; Balder 1998).

Application of two different AM fungal inocula did not significantly increase mycorrhizal root colonization above control values. Possible qualitative changes of mycorrhizal status by inoculation were not monitored during this study. However, corresponding to the results on root colonization, no differences in growth or salt tolerance of the trees could be observed between inoculation treatments.

According to literature, *Ulmus x hollandica* is a fairly salt tolerant tree species, particularly with regard to salt spray. The results of this study confirm that this tree species, like *Populus canescens*, also can tolerate relatively high soil salt concentrations. The role of mycorrhiza in the salt tolerance of *Ulmus* could not be elucidated with this experiment due to a lacking non-mycorrhizal control. However, the fact that increasing salt concentrations drastically reduced mycorrhizal colonization, suggests that mycorrhizal fungi are rather excluded by the plant with growing salt stress than contributing to its alleviation. The colonization data also suggest that *Ulmus* is less mycotrophic than for example *Aesculus*.

Experiment 4 - *Tilia cordata*

At the beginning of the season inoculated trees showed clearly less salt stress symptoms than control trees, particularly at the lower salt levels EC2 and EC4 (Fig. 2.4.1). However, later in the season (August) the differences between inoculation treatments became less important. Similar observations have been made in 2001 (data not shown).

Fig. 2.4.1 Salt stress symptoms of *Tilia cordata* trees in 2002 evaluated by a 5 class system (0, 1, 2, 3, 4), n = 7, means + SD.

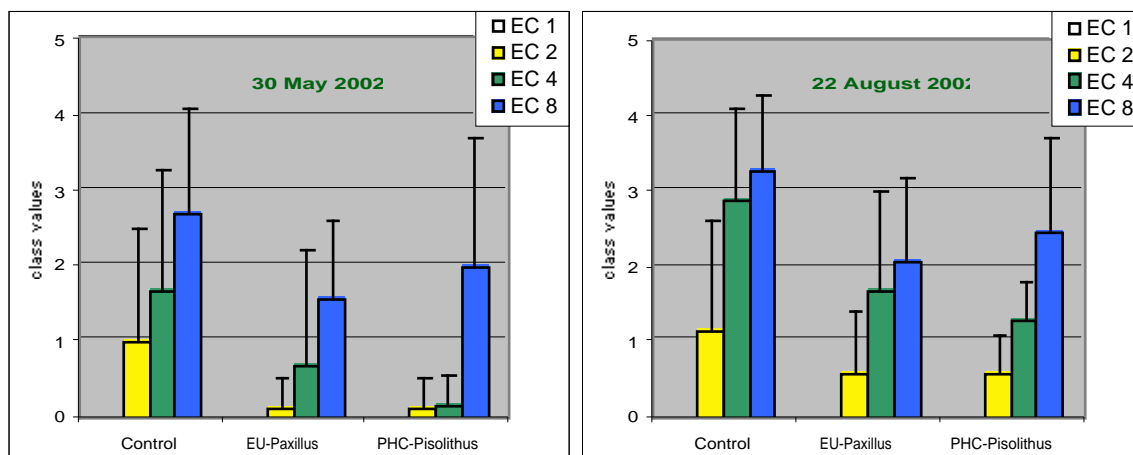
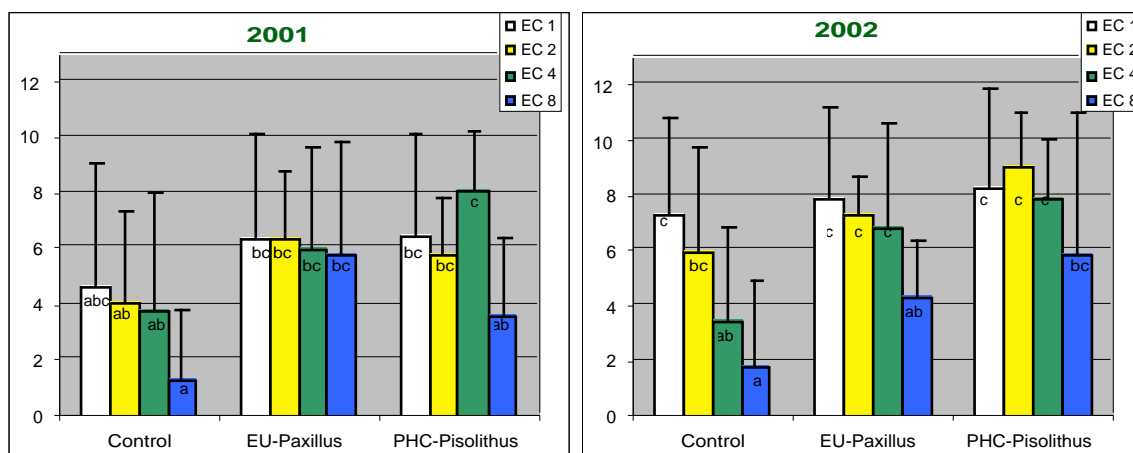


Fig. 2.4.2 Growth of *Tilia cordata* trees in 2001 and 2002 with different inoculation treatment and at different salt concentrations, measured as increase in stem diameter (mm), values are means (n = 7) with standard deviation. Means indicated by the same letters are not statistically different (p < 0.05).



Corresponding with the severeness of the salt stress symptoms, the growth of non-inoculated control trees was more affected by increasing salt concentrations than inoculated EU and PHC trees (Fig. 2.4.2). In average, growth of control trees was significantly lower than growth of inoculated trees (Table 2.4.1a) and the percentage of lost trees was much higher with control (Table 2.4.1b). Only at the highest salt concentration EC8, average tree growth was significantly reduced (Table 2.4.2.a) and tree loss was highest (Table 2.4.2.b). *Tilia cordata* is known as salt sensitive tree species, which corresponds with the strong salt affection of the control trees. Inoculation, however considerably increased salt tolerance of *Tilia* to a level comparable with more salt tolerant tree species such as *Populus canescens* and *Ulmus x hollandica* in this study.

Table 2.4.1a Growth of *Tilia cordata* trees with different mycorrhizal inoculation treatment (MycInoc), measured as increase in stem diameter (mm). Values are means (n = 28) with standard deviation. Means in each column followed by the same letter are not statistically different (p < 0.05).

MycInoc	2001	2002	Total growth (2001 + 2002)
Control	3.7 ± 3.7 a	4.7 ± 3.9 a	8.4 ± 6.9 a
EU- <i>Paxillus</i>	6.2 ± 3.3 b	6.7 ± 2.9 b	12.9 ± 5.4 b
PHC- <i>Pisolithus</i>	6.1 ± 3.0 b	7.9 ± 3.3 b	14.0 ± 4.6 b

Table 2.4.1b Percentage of lost (dead or severely stunted) *Tilia cordata* trees with different mycorrhizal inoculation treatment (MycInoc), n = 28.

MycInoc	2001	2002	Total (2001 + 2002)
Control	21.4	21.4	42.8
EU- <i>Paxillus</i>	3.6	14.3	17.9
PHC- <i>Pisolithus</i>	7.1	3.6	10.7

Table 2.4.2a Growth of *Tilia cordata* trees at different salt concentrations, measured as increase in stem diameter (mm). Values are means (n = 28) with standard deviation. Means in each column followed by the same letter are not statistically different (p < 0.05).

Salt	2001	2002	Total growth (2001 + 2002)
EC1	5.9 ± 3.8 a	7.9 ± 3.2 a	13.9 ± 5.5 a
EC2	5.5 ± 2.7 ab	7.5 ± 2.8 a	13.0 ± 4.8 a
EC4	6.0 ± 3.7 a	6.1 ± 3.6 a	12.0 ± 6.8 a
EC8	3.8 ± 3.6 b	4.0 ± 3.7 b	7.9 ± 6.3 b

Table 2.4.2b Percentage of lost (dead or stunted) *Tilia cordata* trees at different salt concentrations, n = 28.

Salt	2001	2002	Total (2001 + 2002)
EC1	0.0	14.3	14.3
EC2	3.6	7.1	10.7
EC4	10.7	10.7	21.4
EC8	17.9	10.7	28.6

When *Tilia* roots were first checked for colonization in January 2001 (7 months after planting), inoculated trees had substantially more ectomycorrhizas (0.53 and 1.32 mycorrhizal root tips/cm root length respectively for EU and PHC) than non-inoculated control trees (0.20 MRT/cm RL).

Root analysis at a later stage of the experiment, in March 2002, showed no substantial difference in root system development and degree of mycorrhization between control and inoculated trees at EC1 (Table 2.4.3). However, with increasing salt concentration, root and mycorrhizal development declined considerably with non-inoculated control trees, in contrast to EU and PHC inoculated trees, which showed no remarkable decline.

Table 2.4.3 Root analysis of *Tilia cordata* trees in March 2002; RS = root system: density and vitality; Myc = mycorrhizae: number, size and vitality; Rating scale: 5 = high, 3 = moderate, 1 = low; values are averages of 7 repetitions.

	Control		EU- <i>Paxillus</i>		PHC- <i>Pisolithus</i>	
	RS	Myc	RS	Myc	RS	Myc
EC1	3.6	3.6	3.9	3.3	5.0	4.4
EC2	3.3	3.0	4.1	3.6	4.7	4.1
EC4	2.4	1.9	3.9	3.3	4.7	4.0
EC8	2.7	1.9	4.4	3.6	3.7	3.3

However, the inoculated fungi *Paxillus* (EU) and *Pisolithus* (PHC) were not found colonizing the roots. Apparently they could not compete with the two dominant ectomycorrhizal morphotypes observed, *Hebeloma* sp. and *Tuber* sp., both common in tree nurseries. The relatively high pH (6.5 to 7) might have played an important selective role. The appearance of *Hebeloma* fruitbodies in autumn 2001 and 2002 (Table 2.4.4)

corresponded with the observed mycorrhization: inoculated trees supported substantially more fruitbodies also at high salt concentrations.

Table 2.4.4 Percentage of *Tilia cordata* trees with *Hebeloma* fruit bodies in autumn 2001 and 2002, n = 7.

MycInoc	Salt	2001	2002
Control	EC1	42.9	28.6
	EC2	0.0	14.3
	EC4	0.0	0.0
	EC8	0.0	0.0
EU- <i>Paxillus</i>	EC1	14.3	14.3
	EC2	14.3	42.9
	EC4	28.6	28.6
	EC8	14.3	28.6
PHC- <i>Pisolithus</i>	EC1	57.1	57.1
	EC2	28.6	14.3
	EC4	50.0	50.0
	EC8	16.6	33.3

These results on root analysis suggest that inoculation, though it did not lead to the establishment of the inoculated fungi, stimulated the development of indigenous nursery fungi, possibly due to growth stimulating components of the inoculum. This led to an accelerated development of a vital mycorrhizal root system in the first year of the experiment when salt was not yet applied (2000). When salt application started in the second year (2001), inoculated trees could cope much better with the stress and continued to grow. The less mycorrhizal control trees were affected by salt stress with impaired root growth and photosynthesis and consequently an impaired mycorrhizal development. Only in the salt-free control (EC1) root and mycorrhizal development reached the same level as with inoculated trees (Table 2.4.3). These results, supported by the results on tree growth and leaf symptoms, also suggest that the indigenous *Hebeloma* and *Tuber* fungi were more salt tolerant than *Tilia cordata* trees itself and that they could confer salt tolerance to the trees once they had sufficiently colonized the root system.

Experiment 5 - *Fagus sylvatica*

Leaf appearance in spring 2001 and 2002 was delayed with about 2 weeks at the highest salt level EC8. However, *Laccaria bicolor* inoculated trees (EU treatment) were clearly less delayed than non-inoculated control trees, PHC treated trees laying between EU and control (Photo nr.1). Independently of salt level EU treated trees broke earlier into leaf than PHC and untreated trees, suggesting a higher vitality (Photo nr.2).

Photo nr.1

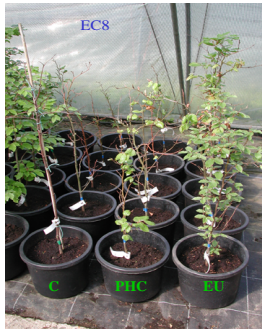


Photo nr.2

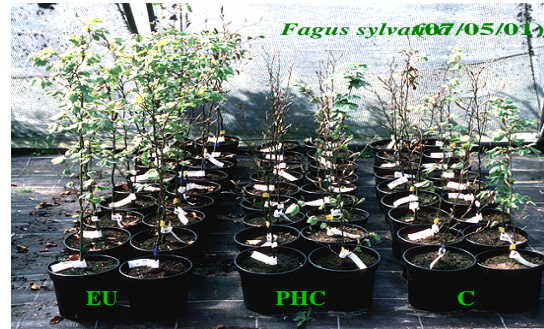
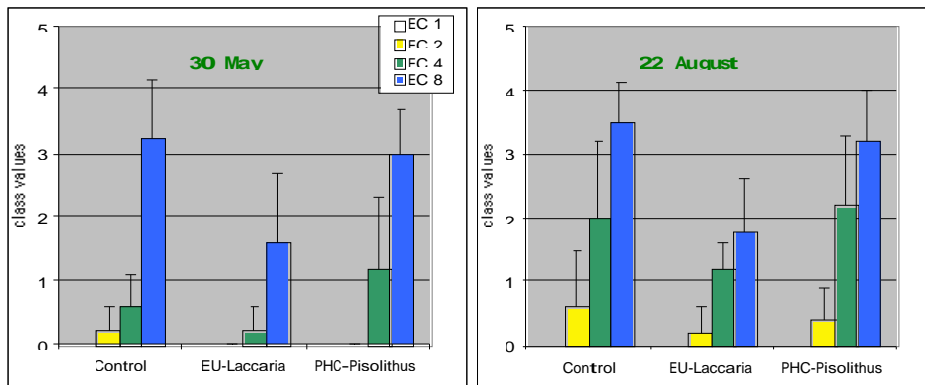
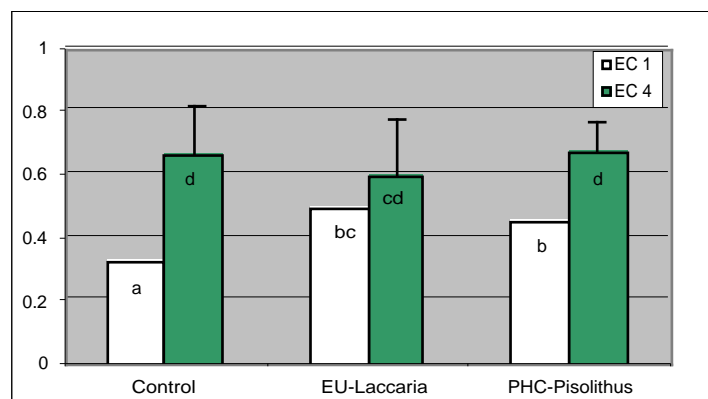


Fig. 2.5.1 Salt stress symptoms of *F. sylvatica* evaluated by a 5 class system (0, 1, 2, 3, 4), n = 5, means + SD.



EU treated trees also showed the least salt stress symptoms, particularly at the highest salt level EC8 (Fig. 2.5.1). Chloride accumulation at the end of the season (Fig. 2.5.2) did not differ significantly between mycorrhizal treatments at EC4 and was only little higher than the literature threshold value for leaf symptoms of 0.50 % of dry matter. However, with EU treated trees foliar chloride at EC4 was lowest and did not differ significantly from foliar chloride at EC1 (without salt addition) in contrast to PHC and Control trees, where chloride concentrations correlated better with soil salt concentrations.

Fig. 2.5.2 Foliar chloride of *Fagus sylvatica* trees on 30 August 2001 (% of dry matter), n = 5, means + SD. Means indicated by the same letter are not statistically different ($p < 0.05$).

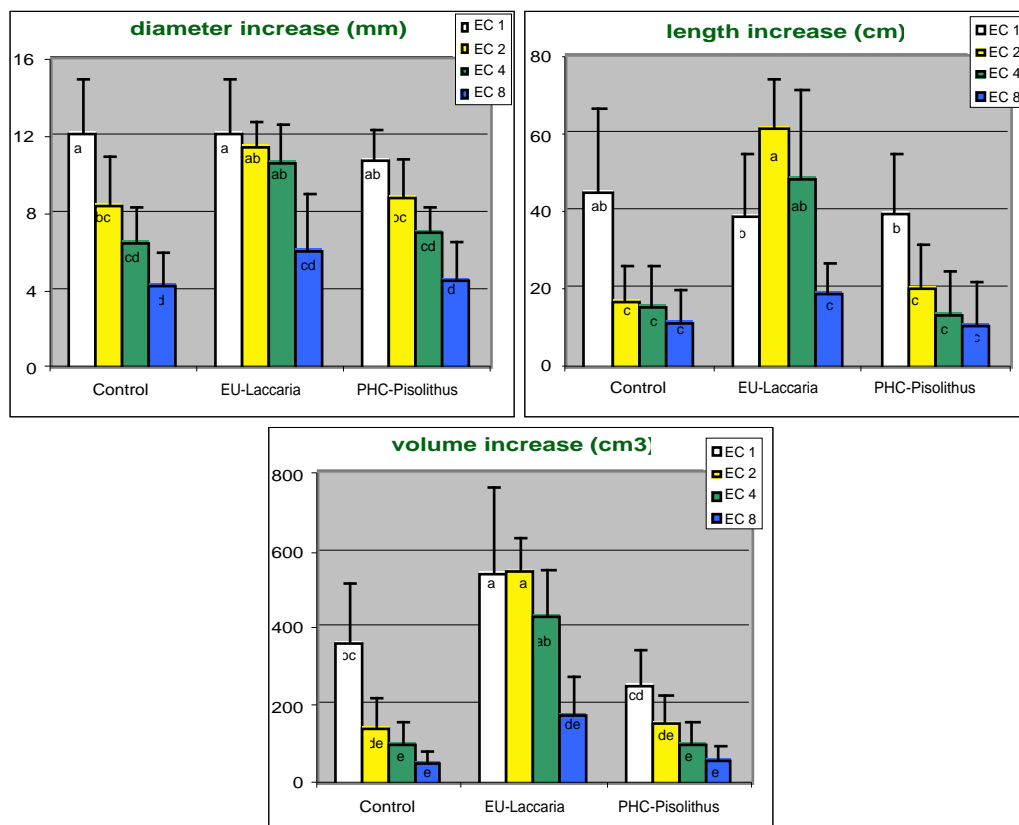


In the second (2001) and third (2002) year of the experiment a significant effect of salt concentration on tree growth was to observe (Table 2.5.1). With Control and PHC trees, growth was reduced with increasing salt level beginning at the low EC2 level, confirming the low salt tolerance of *Fagus sylvatica* as reported in literature. EU trees, in contrast, were less affected by increasing salt concentration and significantly decreased in growth only at the highest salt level EC8. Total tree growth of three years, based on stem diameter and stem length (Fig. 2.5.3), confirms the higher salt tolerance of EU trees.

Table 2.5.1 Growth of *Fagus sylvatica* trees with different mycorrhizal inoculation treatment (MycInoc) and at different salt concentrations (Salt), measured as increase in stem diameter (mm) in three subsequent growing seasons. Values are means (n = 5) with standard deviation. Means in each column followed by the same letter are not statistically different (p < 0.05).

MycInoc	Salt	2000	2001	2002
Control	EC1	2.0 ± 0.6 a	6.4 ± 0.5 a	3.8 ± 2.4 ab
	EC2	0.8 ± 0.4 b	5.4 ± 1.8 abc	2.3 ± 1.6 bc
	EC4	1.2 ± 0.6 ab	3.9 ± 1.0 cd	1.4 ± 0.9 c
	EC8	1.3 ± 0.6 ab	1.8 ± 1.9 e	1.3 ± 1.3 c
EU-Laccaria	EC1	1.4 ± 1.1 ab	6.8 ± 2.0 a	4.1 ± 1.5 a
	EC2	1.9 ± 1.1 a	6.7 ± 1.0 a	2.9 ± 1.2 ab
	EC4	1.3 ± 0.3 ab	6.3 ± 1.2 a	3.0 ± 1.7 ab
	EC8	1.2 ± 0.8 ab	2.8 ± 1.6 de	2.1 ± 1.8 bc
PHC-Pisolithus	EC1	1.3 ± 0.7 ab	5.9 ± 0.5 ab	3.6 ± 0.8 ab
	EC2	1.2 ± 0.6 ab	5.7 ± 1.8 ab	2.0 ± 0.5 bc
	EC4	1.3 ± 1.0 ab	4.3 ± 0.6 bc	1.5 ± 1.1 c
	EC8	1.2 ± 0.6 ab	2.3 ± 1.8 de	1.1 ± 0.7 c

Fig. 2.5.3 Growth of *Fagus sylvatica* trees during three years (may 2000 until October 2002) with different inoculation treatment and at different salt concentrations, as increase in stem diameter (mm), as increase in stem length (cm) and as increase in estimated tree volume (cm³). Values are means (n = 5) with standard deviation. Means indicated by the same letter are not statistically different (p < 0.05).



At the end of the experiment after three years no EU-Laccaria trees were lost, whereas 75% and 20% of Control trees and 40% and 20% of PHC-Pisolithus trees were lost respectively at salt level EC8 and EC4.

Table 2.5.2 Root analysis of *Fagus sylvatica* trees in April 2002; RS = root system: density and vitality; Myc = mycorrhizae: number size and vitality; Rating scale: 5 = high, 3 = moderate, 1 = low; values are averages of 5 repetitions.

	Control		EU-Laccaria		PHC-Pisolithus	
	RS	Myc	RS	Myc	RS	Myc
EC1	5.0	4.2	5.0	4.6	5.0	4.6
EC2	5.0	4.2	5.0	4.2	5.0	3.8
EC4	3.0	2.2	3.0	2.6	3.0	1.8
EC8	1.2	1.2	3.0	1.8	2.6	1.8

When *Fagus* roots were first checked for colonization in May 2001 (1 year after planting), inoculated trees had substantially more ectomycorrhizae than non-inoculated control trees at the lowest (EC1) as well as the highest (EC8) salt level (0.52/0.47, 0.39/0.38 and 0.16/0.14 mycorrhizal root tips/cm root length respectively for EU, PHC and Control for EC1/EC8). However, at EC8 mycorrhizae seemed less vital and older with thinner mantel and darker color.

Root analysis at a later stage of the experiment, in April 2002, showed no substantial difference in root system development and degree of mycorrhization between control and inoculated trees at EC1 (Table 2.5.2). With increasing salt concentration, root and mycorrhizal development declined most with non-inoculated control trees and least with EU inoculated trees.

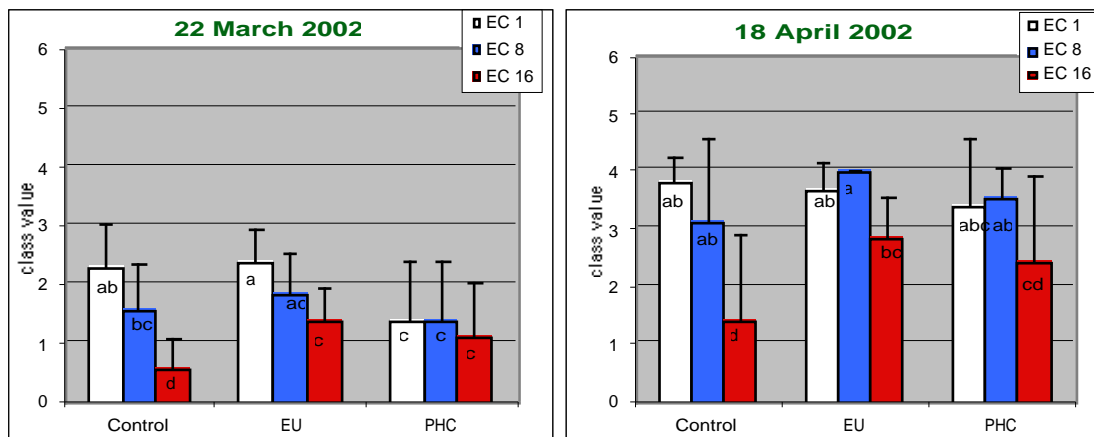
The inoculated fungus *Pisolithus tinctorius* (PHC) was not found colonizing the roots. Also the fungus *Laccaria bicolor* (EU), which was colonizing the *Fagus* seedlings at the beginning of the experiment when they arrived from Robin nurseries, disappeared in the course of the experiment. The relatively high pH (6.5 to 7) of the experimental substrate, which is not optimal for these two fungal species, might have played an important selective role. Apparently they could not compete with *Thelephora* sp., *Hebeloma* sp. and *Tuber* sp., the dominant ectomycorrhizal morphotypes observed in all treatments. In the third year (2002) *Hebeloma* fruitbodies were observed in some of the pots.

However, as described above, mycorrhizal status, tree growth and salt tolerance were higher with inoculated trees, particularly with EU trees, which were already well colonized at planting. PHC inoculation, which was carried out at planting tended to improve tree performance above control (higher mycorrhization at high salt level, less trees lost), but did not achieve the same significant effect as with *Tilia cordata*. This might be due to direct salt application after planting with *Fagus*, whereas with *Tilia* salt application was started one season later.

Experiment 6 - *Aesculus pavia*

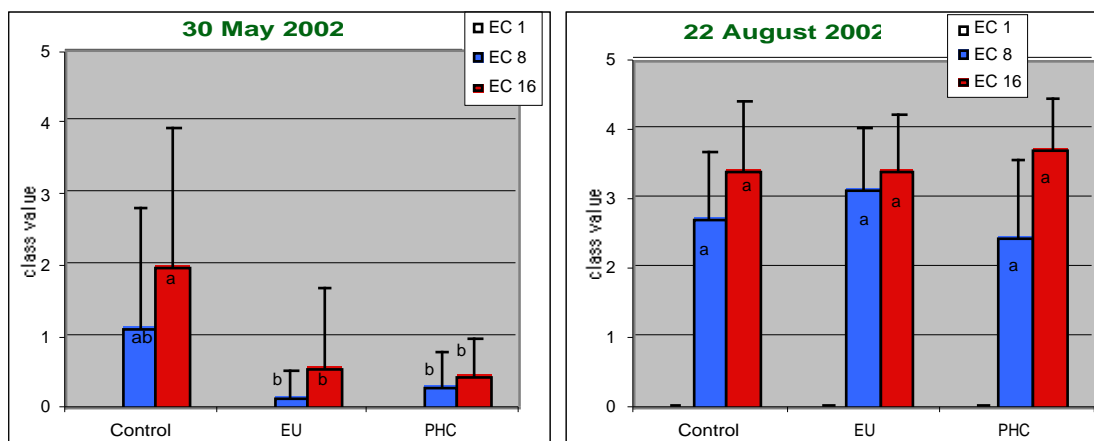
Leaf appearance of *Aesculus pavia* trees in 2002 was significantly delayed at the highest salt level EC16 with the non-inoculated Control, whereas with both inoculated treatments (EU and PHC) and at the lower salt level EC8 no significant delay was to observe (Fig. 2.6.1).

Fig.2.6.1 Leaf appearance of *Aesculus pavia* trees in 2002 evaluated by a 5 class system (0, 1, 2, 3, 4), n = 7, means + SD. Means indicated by the same letter are not statistically different ($p < 0.05$).



Also salt stress symptoms occurred significantly earlier with non-inoculated Control trees (Fig. 2.6.2). At the end of the season, however, all inoculation treatments showed severe salt stress at both salt levels EC8 and EC16.

Fig.2.6.2 Salt stress symptoms of *Aesculus pavia* trees in 2002 evaluated by a 5 class system (0, 1, 2, 3, 4), n = 7, means + SD. Means indicated by the same letter are not statistically different ($p < 0.05$).



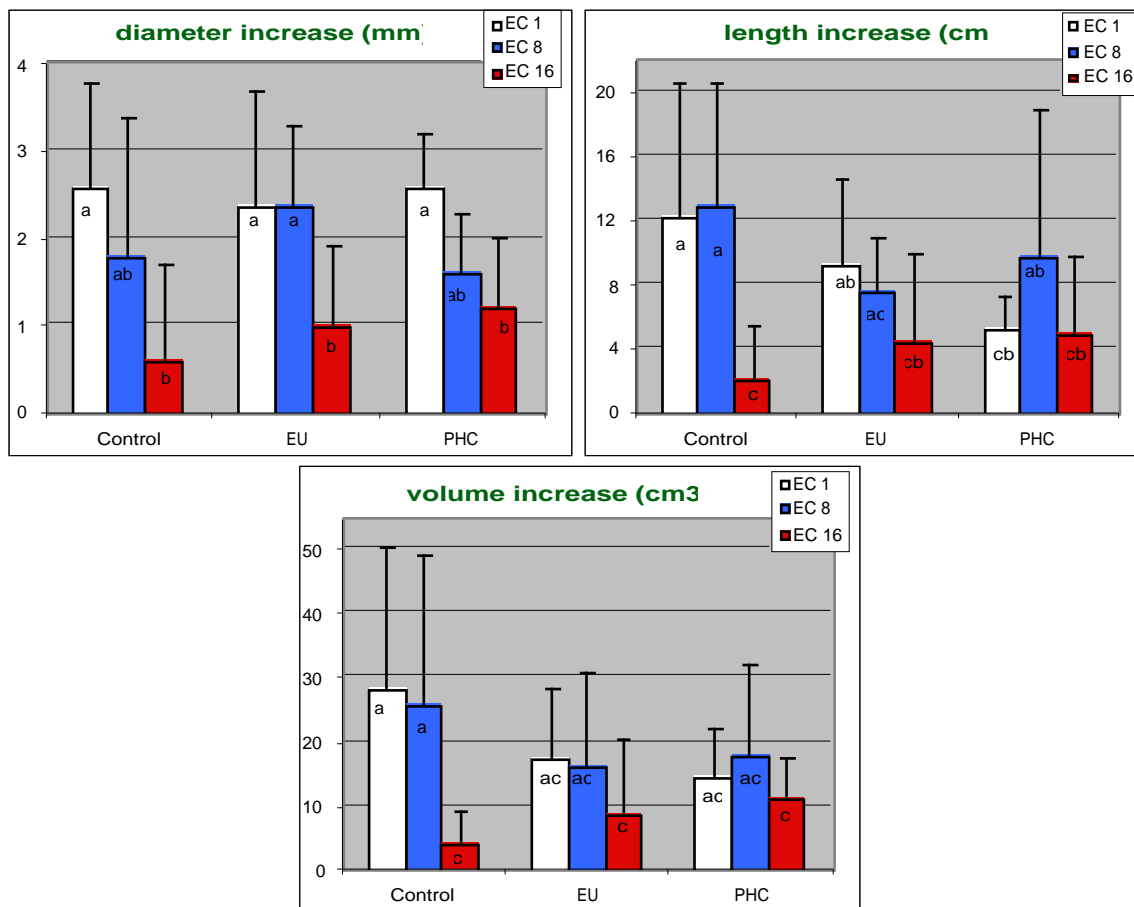
Tree growth reflects the results of leaf appearance and salt stress symptoms (Fig. 2.6.3). Control trees showed the biggest growth decrease with increasing salt concentration.

The container grown seedlings had 50% root length colonized with AM when received from Robin Pepinieres in June 2001 before planting and inoculation. At the end of the experiment in September 2002 AM colonization was around 70% with no difference between treatments (Table 2.6.1). Benomyl application on the Control in summer 2001 did apparently not effectively suppress AM. Neither did high salt concentrations. Molecular analysis of the colonizing fungi (carried out by partner 1) revealed that the inoculated *Glomus geosporum* from saline soils was not present in *A. pavia* roots meaning that it could not compete with the indigenous fungi even under high salt concentrations. In greenhouse experiments of partner 1 *Glomus geosporum* did not colonize plants at salt concentrations above 1% (~ EC16).

Table 2.6.1 AM root colonization of *A. pavia* trees in September 2002 (% root length colonized), values refer to pooled samples of 7 repetitions per treatment.

Salt	C	EU	PHC
EC1	74	71	73
EC8	71	74	75
EC16	71	73	72

Fig. 2.6.3 Growth of *Aesculus pavia* trees in 2002 with different inoculation treatment and at different salt concentrations, as increase in stem diameter (mm), as increase in stem length (cm) and as increase in estimated tree volume (cm³). Values are means (n = 7) with standard deviation. Means indicated by the same letter are not statistically different (p < 0.05).



Growth parameters and symptoms suggest that inoculation of the *A. pavia* trees improved their salt tolerance compared with non-inoculated trees. However, data deviation was high and thus the results are statistically not very significant. *Glomus geosporum* (EU) inoculum, ecologically adapted to high salt concentrations, was not more effective than the commercial PHC inoculum. The lower salt tolerance of the Control, if considered at all, cannot simply be explained by arbuscular mycorrhizal effects since the degree of colonization was just as high with Control trees and was not influenced by salt addition. Other factors associated with the inocula such as microbes, nutrients, organic substances, which could stimulate root development and improve plant vitality have to be considered.

Summary

The container-experiment with trees showed that mycorrhizal inoculation is not necessarily effective under non-sterile nursery conditions. However, depending on tree species, time and modus of inoculation, a higher salt tolerance of inoculated trees could be observed.

Inoculation with AM fungi did not affect percentage root length colonized of *Aesculus hippocastanum*, *Aesculus pavia* and *Ulmus x hollandica* under the non-sterile nursery conditions regardless the level of salt stress. Indigenous AM fungi were abundantly colonizing the trees. High salt concentrations reduced AM colonization depending on tree species suggesting a plant-mediated effect. *Aesculus* was more salt sensitive than *Ulmus*. Inoculation improved tree salt tolerance only in the case of *Aesculus pavia*: inoculated plants (*Glomus geosporum* IFP2000, PHC Mycor Flower Saver) showed a smaller delay in leaf appearance, less leaf injury, tree loss and tree growth reduction with increasing salt stress than non-inoculated control plants. However, this positive effect of inoculation seems rather due to unidentified stimulating factors in the inoculum than to a direct mycorrhizal effect, since root colonization was not changed by inoculation and the inoculated fungi could not be traced in the tree roots using molecular methods.

The inoculated ECM fungi (*Paxillus*, *Scleroderma*, *Pisolithus*, *Laccaria*) were not found colonizing *Tilia cordata*, *Fagus sylvatica* and *Populus canescens* trees under non-sterile nursery conditions. Indigenous ECM fungi (*Tuber*, *Humaria*, *Hebeloma*, *Thelephora*) were abundantly colonizing the trees. Colonization together with root and shoot development was reduced with high salt stress. This seemed to be rather a plant-mediated effect, since well-developed mycorrhiza was observed at the highest salt levels when plants were well colonized before salt addition. Inoculated *Tilia* and *Fagus* showed better colonization (with indigenous ECM) and higher salt tolerance (better growth, less leaf burn) than the non-inoculated control trees suggesting that mycorrhiza can increase salt tolerance of this sensitive tree species to the level of more tolerant tree species. With *Populus* no effect of inoculation on root colonization and salt tolerance of trees was observed. This fast-growing, fairly salt tolerant tree species, quickly developed abundant mycorrhization after planting, also in the non-inoculated control.

3. Field experiment along the motorway A2

Objective

About 50% of newly planted trees along highways has to be replaced within 5 years. Apart from bad soil conditions after construction works, de-icing salt is an important stress factor for these trees.

The application of mycorrhizal fungi with trees is relatively new in Europe. Pius Floris tree care company since several years uses mycorrhizae when planting trees and restoring big old trees.

The aim of this experiment was to investigate if the addition of a commercial mycorrhizal inoculum or other conventional tree care treatments in the rooting zone will have a (salt)stress alleviating and growth-promoting effect on already established (older) trees. The choice of the location along the motorway was motivated by our interest in improving tree growth in urban saline environments.

Material and methods

The experiment was laid out in summer 2000 along the motorway A2 Vught-Eindhoven on the eastern sidestroke in the vicinity of Vught. The soil was sandy with a pH between 6 and 7. The soil profile was disturbed as a result from the construction works and at places water accumulated at a depth of about 1 meter probably due to compaction and wet lowlands nearby. A relatively homogeneous set of 180 Oak (*Quercus robur*) trees standing in a single lane on a distance of about 2 km (124.3 to 126.5) were selected and labeled with metal plates. These trees had been planted and taken care of by 'Pius Floris Boomverzorging Vught BV' by order of 'Rijkswaterstaat' after the construction of the motorway and were about 6 to 8 years old.

In February 2001, just before the growing season, the different root treatments were applied by use of the Tree Fertilizer Injector (TFI) of the PFB Company:

Control	no root treatment
Perlite	creating air channels and injection of perlite
Manure	creating air channels and injection of perlite plus worm castings
Pisolithus	creating air channels and injection of perlite plus Mycortree® Injectable <i>Pisolithus tinctorius</i> ectomycorrhizal spores

These treatments comprised a total of 120 trees, 30 trees per treatment in groups of 5 trees spread over 3 blocks of 40 trees (8 x 5). The 5 treated trees were separated by 2 or 3 buffer trees resulting in a total of about 180 trees.

In addition to the deicing salt that arrived from the road, half of the trees (15 trees per root treatment) received extra salt (also in summer) to increase stress. Municipal deicing salt (mainly NaCl, small amount of CaCl₂) was scattered around the trees on a surface of about 10 m² at a concentration of 1 kg/m² in May 2001, October 2001, May 2002 and July 2002.

Salt concentration was regularly checked by in situ EC (electrical conductivity) measurements along a vertical soil profile (0 to 100 cm) in the rooting zone of the trees (Table 3.1). During the summer months the added salt stayed in the upper 10 cm of the soil profile due to high evapotranspiration. With addition of extra water in July we tried to flush the salt further down into the rooting zone at least temporarily. In September high salt concentrations were measured in deeper layers (20 to 50 cm) and in October all the added salt was flushed out of the profile (below 100 cm) and was replaced by a new application of salt.

Table 3.1 In situ bulk electrical conductivity (mS/cm) measured along a vertical soil profile in the rooting zone of a salt treated tree at different dates during the experiment. For more details on the EC measurement method see Annex.

Depth (cm)	EC bulk 05/06/01	EC bulk 06/09/01	EC bulk 23/10/01	EC bulk 10/06/02	EC bulk background
5	1.64	0.13	0.25		0.08
10	2.83	0.30	0.16	0.27	0.05
15		0.58	0.16	0.20	
20	0.16	1.75	0.17	0.20	0.06
30		1.59	0.20	0.77	
40	0.01	1.84	0.21	0.51	
50		2.36	0.29	0.26	
100			0.51		

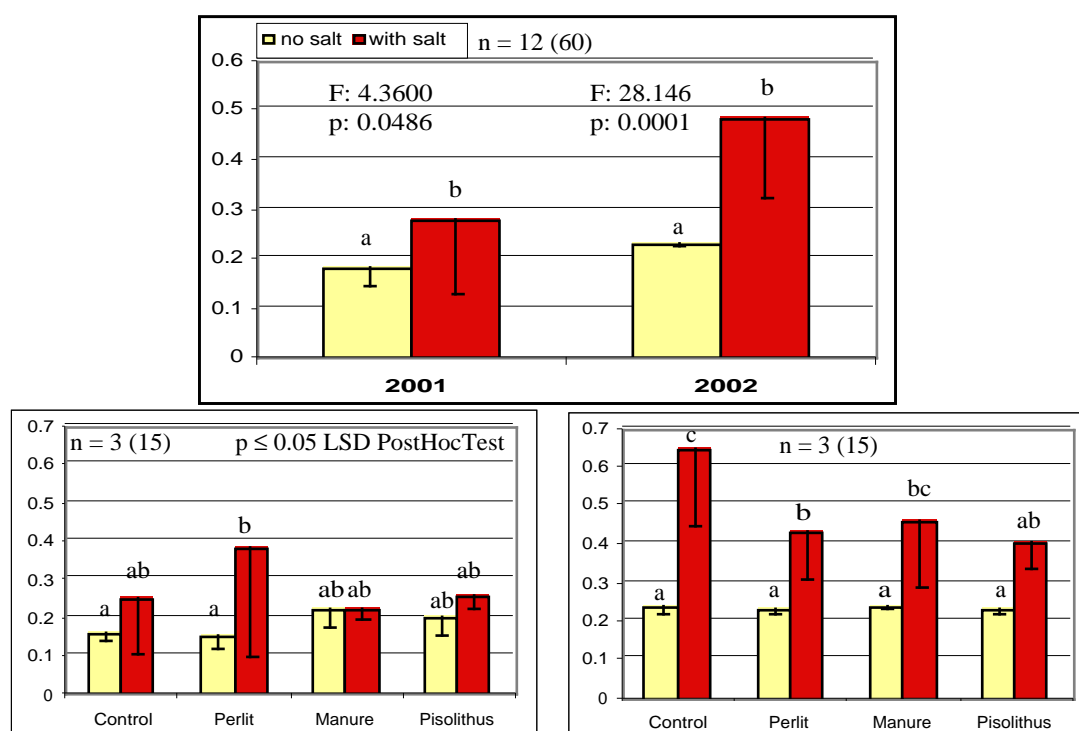
At the end of the growing season (September 2001 and 2002) leaf samples were taken to be analyzed for Cl accumulation (Eurolab, Deventer, NL). Per group of 5 neighboring trees having received the same root treatment an amount of 500g leaflets (fresh weight) was stripped of 5 randomly cut branches per tree. In Total 24 pooled samples from the 120 trees of the experiment were analyzed.

Root samples were taken in August 2000, before the treatments were applied, to check indigenous ectomycorrhizal colonization by assessing mycorrhizal root tips under a dissecting microscope. In December 2001 roots were sampled from Pisolithus treated trees and analyzed morphologically (P. Mleczko, partner nr. 5, Krakow, PL) and by RFLP and DNA sequencing (J. Baar, Applied Plant Research, Horst, NL) to check the presence of Pisolithus and the diversity of indigenous ectomycorrhizal fungi.

Tree growth was measured as stem circumference in mm at a height of 1.30 m in January 2001, in November 2001 and in September 2002.

Results

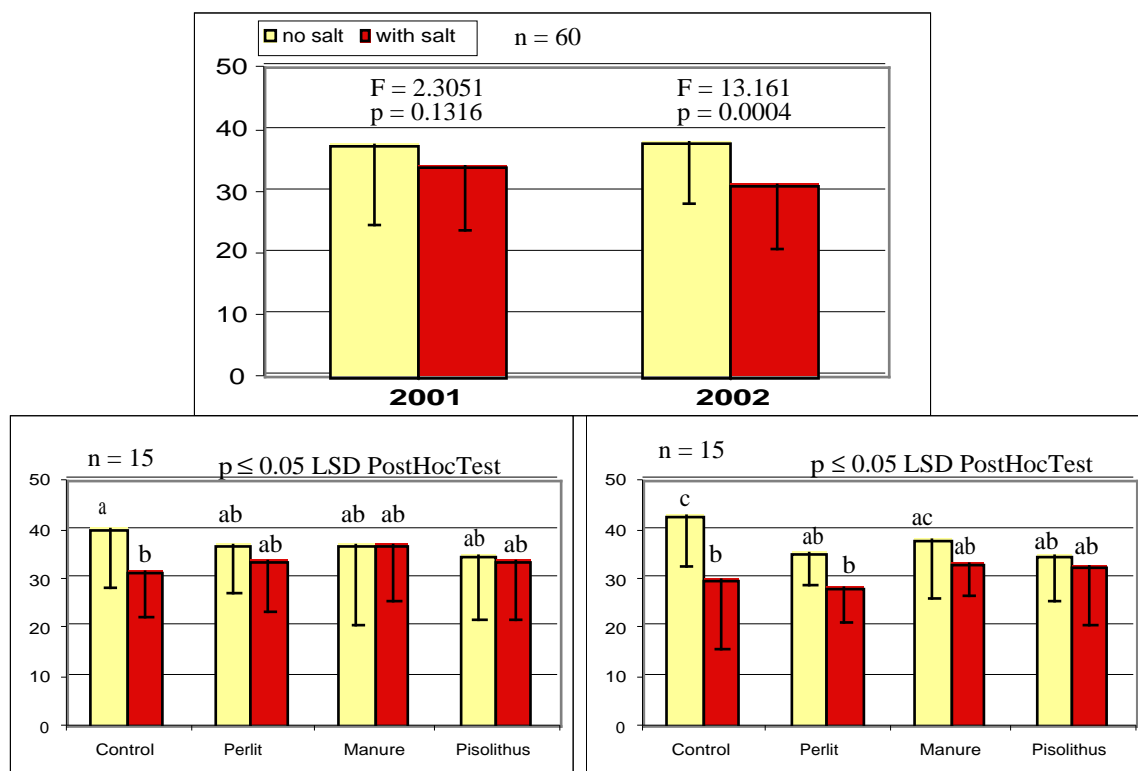
Fig.3.1 Foliar chloride concentrations (% dry matter) of oak trees at the A2 site in September 2001 and 2002; above: average of all root treatments (n = 12): below: root treatments separated (n = 3); means + SD.



The foliar chloride analysis carried out at the end of the growing season (September 2001 and 2002) revealed an increase of 50% in 2001 and 100% in 2002 of leaf chloride in salt treated trees compared to non salt treated trees (Fig. 3.1 above). In 2001 the average chloride concentration in the salt treated oak trees (0.282 % of dry matter) was according to literature still below thresholds for the appearance of leaf salt toxicity symptoms, which was reflected by the only incidental occurrence of salt stress symptoms such as leaf burn. In 2002 with an average chloride concentration of 0.489% the literature threshold for salt toxicity in oak trees was reached, which was reflected by the consistent occurrence of marginal leaf burn, particularly with control trees without TFI root treatment. The increase in foliar chloride was also highest with the untreated control and with perlite (Fig. 3.1 below).

Tree growth of the oak trees was significantly decreased by salt application: in 2001 by 9% and in 2002 by 23% (Fig. 3.2 above). This was consistent with the increased foliar chloride concentrations. Split into the different root treatments (none, perlite, manure, pisolithus) the growth decreasing effect of salt in 2001 was highest with the control (22%) and lowest with manure (1%) and pisolithus (2.5%) treatment (Fig. 3.2 below). Also in 2002 the growth decrease was highest with control (30%) and perlite treated trees (20%) and lowest with manure (13%) and pisolithus (6%) treated trees (Fig. 3.2 below). However, under salt stress there was no significant difference in growth of trees having received different root treatments, whereas without salt stress control trees performed best .

Fig.3.2 Growth of oak trees at the A2 site measured as increase of stem circumference (mm) at 1.30 meter height in 2001 and 2002; above: average of all root treatments (n = 12): below: root treatments separated (n = 3); means + SD.



Root samples taken in August 2000, when the experiment was set up, revealed a low root density, a high proportion of old roots and a low indigenous ectomycorrhizal colonization of the oak trees at the A2 site.

Root samples taken in December 2001 (after TFI-treatments) confirmed that the mycorrhizal condition of the oak trees at this location was poor as was the whole root system development (Table 3.2). There were only little mycorrhizal root tips, many of them not vital.

Ascomycetes like *Cenococcum* sp. were dominant. The addition of extra salt decreased the abundance and diversity of mycorrhizae. On trees injected with *Pisolithus tinctorius* spores development of *Pisolithus* mycorrhizae could not be observed.

Table 3.2 Mycorrhizal status of oak trees at the A2 site in December 2001; RT = root tips.

Tree nr.	Treatment	Mycorrhizal status as analyzed by:	
		RFLP, DNA-sequencing J. Baar	Classical morphotyping P. Mlezco / K. Turnau
A2-2525	Pisolithus	Tomentella sp. / 50-60 RT Thelephoroid / 1-5 RT	3 morphotypes no Pisolithus
A2-2648	Pisolithus	Lactarius sp. / 1 RT	no ectomycorrhizae
A2-2517	Pisolithus + salt	Tomentella sp. / 5-10 RT	-
A2-2643	Pisolithus + salt	Cenococcum sp. / 1-5 RT	no ectomycorrhizae

Conclusion

In the field trial along the motorway A2 high soil salt concentrations significantly increased leaf chloride accumulation and decreased growth of oak trees, mycorrhizal abundance and diversity. Trees injected with manure or pisolithus were less affected by salt than trees injected with perlite only or untreated control trees. However, no *Pisolithus tinctorius* mycorrhizae were found colonizing Pt-inoculated trees.

4. Field experiment with roses

Objective

Roses are generally salt sensitive plants but popular for plantings in urban areas. As arbuscular mycorrhizal fungi are suspected to alleviate plant salt stress, this experiment was laid out to test if AM inoculation at planting can increase salt tolerance of roses.

The originally planned inoculation with *Glomus geosporum* ‘salt ecotype’ inoculum from partner 1/1d could not be carried out because there was not enough inoculum available. *Glomus intraradices* inoculum was used as a reference strain, upon which all concerned project partners agreed, since this strain proved to be stress (heavy metal) tolerant. A commercial inoculum (PlantHealthCare VAM cocktail) was equally used.

Material and methods

The experiment was set up in April 2001 on a 78 m² terrain of Pius Floris Boomverzorging Vught, Baarzenstraat 17, 5260 CA Vught, The Netherlands. The soil was sandy with a neutral pH. 234 roses (Meidiland La Sevillana) were planted in groups of 13 plants in plots of about 4 m². There were 6 treatments with each 3 plots randomly arranged, thus 18 plots in total. The treatments were the following: (1) not inoculated control, (2) inoculated with *Glomus intraradices* inoculum from partner 1/1d, (3) inoculated with the commercial PlantHealthCare inoculum VAM cocktail, (4, 5, 6) same treatments as above but with applied salt stress. Inoculum was added with planting of the roses in the recommended dose. Salt stress was applied one year after planting to allow plants and mycorrhiza to establish. De-icing salt was added in April 2002 at a concentration of about 10 mS/cm electrical conductivity ($EC_{\text{saturation extract}}$) in the rooting zone.

Results

The roses responded strongly to the salt stress with leaf burn and stem dieback. Two months after salt application, in June 2002, up to 30% of plants were lost in the salt treated plots compared to 0.3% in unsalted plots (Table 4.1). Arbuscular mycorrhizal root colonization was considerably reduced in salt treated plots (Table 4.2). However, there was no significant difference between non-inoculated control and inoculation treatments (Table 4.1 and 4.2).

Table 4.1 Percentage lost rose plants in June 2002.

Treatment	plant loss (% ± Stdv)
Control	2.6 ± 3.6
Control + Salt	23.1 ± 6.3
G. intraradices	2.6 ± 3.6
G. intraradices + Salt	28.2 ± 3.6
PHC VAM cocktail	0.0 ± 0.0
PHC VAM cocktail + Salt	33.4 ± 7.3

Table 4.2 Percentage colonized root length (gridline intersect-method after blue staining) of roses in June 2002.

Treatment	CRL (% ± Stdv)
Control	55 ± 7
Control + Salt	15 ± 2
G. intraradices	53 ± 8
G. intraradices + Salt	17 ± 3
PHC VAM cocktail	60 ± 8
PHC VAM cocktail + Salt	21 ± 6

Conclusion

The roses in Vught and their mycorrhiza were sensitive to salt stress. Inoculated plants did not show higher mycorrhization or higher salt tolerance.

5. Conclusions and recommendations

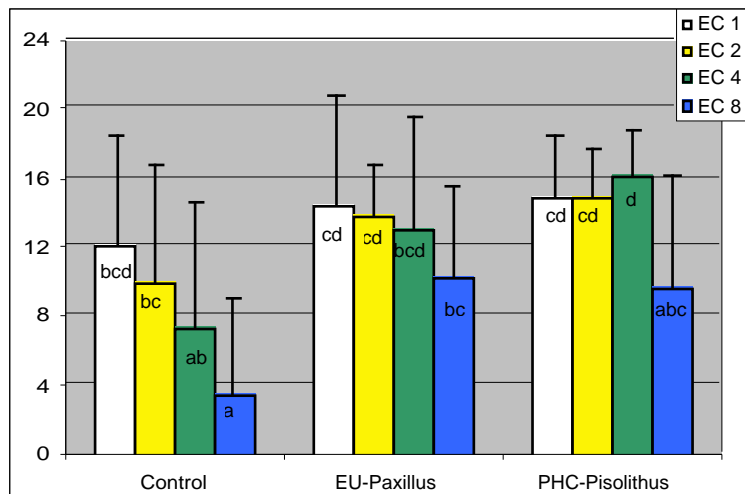
There is not much knowledge available on the impact of mycorrhizal fungi on the salt tolerance of trees.

Three years of nursery and field experiments with trees under practical conditions enlarged our view on the feasibility of using mycorrhizal fungi to improve the performance of trees in saline or salt-polluted soils. We came to the following conclusions and recommendations for application.

Mycorrhiza can considerably improve salt tolerance of trees.

Particularly ECM (ectomycorrhizal) fungi seem to efficiently protect their host tree against salt stress. We could show that improved mycorrhization increased salt tolerance of the sensitive tree species *Tilia cordata* and *Fagus sylvatica* to the level of salt tolerant tree species (Fig.2.5.3 and Fig.5.1).

Fig. 5.1 Growth of *Tilia cordata* trees during two years (2001+2002) with different inoculation treatment and at different salt concentrations, measured as increase in stem diameter (mm), values are means (n = 7) with standard deviation. Means indicated by the same letter are not statistically different ($p < 0.05$).



It seems important that the fungi are well established on the roots before exposure to salt.

In several cases (*Fagus*, *Tilia*, *Aesculus pavia*) inoculation increased mycorrhization and/or salt tolerance of the trees.

However, in most cases the inoculated mycorrhizal fungi were not found colonizing the roots whereas indigenous mycorrhizae were abundantly present. That means that the introduced fungi could not compete with the indigenous fungi under the given environmental conditions. For ectomycorrhizal fungi pH conditions seem to play an important role.

An AM (arbuscular mycorrhizal) fungus isolated from saline soil (*Glomus geosporum*, supplied by partner 1) could not compete with the indigenous AM fungi of *Aesculus pavia* trees even at the highest salt level (EC 16).

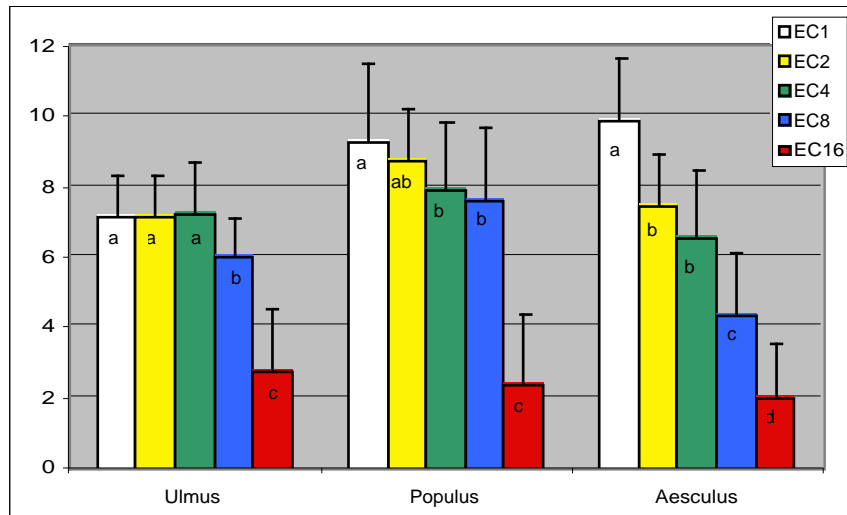
Therefore, mycorrhizal inoculation of non-sterile nursery seedlings or older trees seems rather unlikely to result in the establishment of the inoculated fungi. Stimulation of root growth and indigenous fungi might make more sense to improve mycorrhizal status and tree performance.

Selected fungi (highly efficient, tolerant) should be inoculated on sterile young seedlings under controlled conditions.

Generally, the indigenous fungi (AM and ECM) seemed to be fairly salt tolerant i.e. present at the highest salt levels. A decrease in mycorrhization was mostly correlated with a decrease of root development indicating a plant mediated effect (decreased availability of assimilates for the fungus).

Salt sensitivity of tree species differed greatly (Fig.5.2).

Fig. 5.2 Effect of salinity on tree growth in 2001, as increase in stem diameter (mm), values are means (n = 18) with SD. Means of one tree species indicated by the same letter are not statistically different (p < 0.05).



Tree specific tolerance mechanisms could interfere with mycorrhiza. In the case of *Ulmus x hollandica*, a salt tolerant tree species, AM colonization decreased very quickly with higher salt concentrations, though root development was hardly impaired. This suggests a plant-mediated effect on mycorrhization for example via high salt concentrations accumulated in the root cells.

For (re)vegetation of salt-impacted areas it is recommended to use salt tolerant tree species and/or trees with a good mycorrhizal status, preferable with fungi adapted to the site conditions (pH, salt,...).

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