Effect of waterlogging on boreal forest tree seedlings during dormancy and early growing season

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Academic dissertation

To be presented, with the permission of the Faculty of Science and Forestry of the University of Eastern Finland, for public examination in the auditorium Futura 100 of the University of Eastern Finland, Yliopistonkatu 7, Joensuu, on 16th September 2016, at 12 o'clock noon.

ABSTRACT

More precipitation in the form of rain in winter together with deteriorated ditch networks may expose drained peatland forestry to winter or spring waterlogging in boreal forests. The response of main boreal forest species on soil waterlogging is important for predicting forest productivity and justifying the demand for ditch network maintenance. In this study, the aim was to find out the physiological and growth responses of 1-year-old Norway spruce (*Picea abies* (L.) Karst.), silver birch (*Betula pendula* Roth) and pubescent birch (*Betula pubescens* Ehrh.) seedlings subjected to one-month waterlogging at the end of the dormancy phase, and to find out the morphology, physiology and growth of silver birch and pubescent birch seedlings subjected to one-month waterlogging at the beginning of the growing season.

Two experiments were carried out in growth chambers. In experiment 1, Norway spruce seedlings were subjected to either no waterlogging or waterlogging for one month at 2 ^{o}C, after which a six-week follow-up growing season allowed the seedlings to recover. In experiment 2, silver birch and pubescent birch seedlings went through a four-week dormancy (weeks 1–4), a four-week early growing season (weeks 5–8), and a four-week late growing season (weeks 9–12). The treatments were 1) no waterlogging throughout the experiment (NW); 2) four-week waterlogging during dormancy (Dormancy waterlogging, DW); 3) four-week waterlogging during the early growing season (Growth waterlogging, GW); and 4) four-week dormancy waterlogging followed by four-week growth waterlogging during the early growing season (DWGW).

Dormancy waterlogging reduced root volume and increased root mortality, but did not affect dark-acclimated chlorophyll fluorescence (F_v/F_m) or the biomass of leaves, stems and roots in Norway spruce. Root biomass and root hydraulic conductance (K_r) of silver birch was reduced but aboveground organs were not affected by dormancy waterlogging. On the contrary, in pubescent birch root morphology, biomass and K_r were not affected, but gas exchange was reduced by dormancy waterlogging. However, the biomass of leaves and stems was not negatively affected in pubescent birch. In conclusion, these tree species can tolerate a one-month winter waterlogging well.

Growth waterlogging led to the reduction of stomatal conductance (g_s), and thus the reduction of light-saturated photosynthesis rate (A_{max}) in both birch species, although recovery was seen during the follow-up growing season. It also led to lower leaf area in both birch species. Growth waterlogging led also to decreased the K, Ca, Mg, Mn and B contents of leaves in silver birch, whereas in pubescent birch only Ca and Mg contents were decreased. In pubescent birch, fine cluster roots, the incidence of non-glandular trichomes, and stem lenticels were increased by growth waterlogging. However, silver birch did not show such acclimation to waterlogging. In conclusion, growth waterlogging caused more negative effects to both birch species than dormancy waterlogging. The morphological differences explained why pubescent birch may grow better in wet soil than silver birch.

Keywords: boreal forest, dormancy, waterlogging, hypoxia, root, biomass, photosynthesis, F_v/F_m, starch, nutrients, stomata, trichome

ACKNOWLEDGEMENTS

LIST OF ORIGINAL ARTICLES

The thesis is based on the following articles, which are referred to in the text by the Roman numerals I-III. Articles I-II are reproduced with the kind permission of publishers.

I   Wang A.F., Roitto M., Lehto T., Zwiazek J.J., Calvo-Polanco M., Repo T. (2013). Waterlogging under simulated late-winter conditions had little impact on the physiology and growth of Norway spruce seedlings. Annals of Forest Science 70: 781-790.
http://dx.doi.org/ 10.1007/s13595-013-0325-5

II   Wang A.F., Roitto M., Sutinen S., Lehto T., Heinonen J., Zhang G., Repo T. (2016). Waterlogging during dormancy and early growth affect root and leaf morphology of *Betula pendula* and *Betula pubescens* seedlings. Tree Physiology 36: 86-98.
http://dx.doi.org/ 10.1093/treephys/tpv089

III   Wang A.F., Roitto M., Lehto T., Sutinen S., Heinonen J., Zhang G., Repo T. (2016). Photosynthesis, nutrient accumulation and growth of two birch species exposed to waterlogging in late dormancy and in the early growing season. Manuscript.

The author's contribution

I The experiment was planned and designed by Ai-fang Wang with the guidance of Tapani Repo, Tarja Lehto and Marja Roitto. Ai-fang Wang was responsible for running the experiments, data analysis, and she wrote the manuscript. All co-authors contributed to the article through comments for improvement.

II-III Ai-fang Wang was responsible for running the experiments, data analysis, and wrote the manuscripts. Tapani Repo, Tarja Lehto, Marja Roitto originated the research idea and advised Ai-fang Wang in designing and setting up the experiment. Sirkka Sutinen analysed the anatomical parameters and advised in writing. Jaakko Heinonen advised in statistical analysis. All co-authors have commented on the manuscript.

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LIST OF ABBREVIATIONS

A_{max}	Light-saturated net assimilation rate
CCI	Chlorophyll content index
DW	Waterlogging during dormancy
DWGW	Dormancy waterlogging followed by growth waterlogging
E	Transpiration rate
F_v/F_m	Dark-acclimated chlorophyll fluorescence
GW	Waterlogging during growth
HPFM	High Pressure Flow Meter
g_s	Stomatal conductance
K_r	Root hydraulic conductance
L_p	Root hydraulic conductivity
NW	No waterlogging
W	Waterlogging
WUE	Water use efficiency

1. INTRODUCTION

1.1 Background

In Finland, one third of the land area is composed of peatlands (8.7 million ha), and half of them have been drained to improve forest productivity (Finnish statistical yearbook… 2013). Peatlands are ecosystems with a high level of ground water and are further characterized by a naturally accumulated more than 30 cm thick peat layer on the surface (Joosten and Clarke 2002). Spruce swamp forests are boreal peatlands with a cover of Norway spruce (*Picea abies* (L.) Karst.), and also some pubescent birch (*Betula pubescens* Ehrh.). The water table is normally positioned well below the soil surface through most of the growing season, which allows the spruce to persist even without drainage. Spruce swamp forests have been commonly drained due to their large timber production potential (Päivänen and Hånell 2012). In Finnish peatlands drained for forestry, the water table typically lies at -30 to -40 cm, which is considered deep enough to sustain a well growing tree stand by means of transpiration and canopy interception (Sarkkola et al. 2010; Sarkkola et al. 2012). Lowering of the water table increases the rate of peat mineralization and CO_2 emissions; however, increased tree growth and litter production in conditions with a lower water table can fix more CO_2 (Ojanen et al. 2013).

Peatland forests account for about a quarter of the annual increment of the total growing stock in Finland (Hökkä et al. 2002). The standing volume in peatland forests is still increasing, since most of the forest drainage took place between 1960 and 1980, and these forests are beginning to reach their maximum growth rates (Minkkinen et al. 2008). Peatland forests are therefore an increasingly important resource for the forestry sector in Finland.

Several decades after drainage the ditch network has deteriorated in many cases as a result of vegetation invasion in ditches, collapse of ditch walls and peat subsidence (Paavilainen and Päivänen 1995). The deterioration depends on ditch space, digging technique, peat composition, peat thickness and the texture of the mineral subsoil (Timonen 1983; Isoaho et al. 1993). Ditch network maintenance (cleaning the ditches or digging complementary ditches) aiming to ensure sufficient drainage is required after 20 years in the northern and after 25-30 years in the southern part of Finland (Hökkä et al. 2000). Even though ditch network maintenance improves tree growth (Ahti 2005; Sikström and Hökkä 2016) it may impair water quality in receiving water courses, in addition of being a cost for the land owner, too. Therefore, unnecessary maintenance of ditch network should be avoided if it does not result in higher growth (Hökkä and Laurén 2014). Sikström and Hökkä (2016) pointed out that better understanding is needed of the relation between soil water and tree growth for the justification of ditch network maintenance.

Climate change is predicted to increase the mean annual temperature by 2-5°C during the next century in northern Europe including Finland (IPCC 2001; Kellomäki et al. 2010), the warming being the largest in winter (IPCC 2007). Therefore, increased precipitation especially in winter months (IPCC 2007), and increased snow melt frequency, are expected to increase the groundwater table and soil waterlogging in winter and spring, especially on sites where ditches do not work efficiently.

1.2 Waterlogging and roots

Waterlogging is characterised by the saturation of soil pores with water either in the short-term or permanently (Alaoui-Sossé et al. 2005). The degree of tolerance of plants to soil waterlogging is often estimated according to survival or growth and productivity in forest stands after exposure to waterlogging conditions for different times (Kozlowski 1997). However, there is no standard method for waterlogging tolerance assessment. Tolerance to waterlogging varies greatly with the plant species, the plant's age, the time and duration of waterlogging and the mobility of water (Kozlowski and Pallardy1997). The immediate consequence of soil waterlogging is oxygen deprivation in the root system. Therefore, roots have a key role in the waterlogging tolerance of trees. In waterlogging conditions, in root cells energy production through mitochondrial respiration may be replaced by ethanolic fermentation (Kreuzwieser et al. 2004). The oxygen affinity of the mitochondrial cytochrome c oxidase is very low in these circumstances, in the order of 1-10μM (Krab et al. 2011). Due to the low efficiency of this process to yield energy, the roots must compensate for the low yield by decreasing the demand for energy by a reduction of root growth or nutrient uptake, and by consuming more carbohydrates per unit time for the generation of energy equivalents (Kreuzwieser et al. 2004). In fact, flooding intolerant species tend to accumulate photosynthetic products in leaves and needles, but not transport them to roots (Sudachkova et al. 2009; Repo et al. 2016a). In a study on northern red oak (*Quercus rubra* L.) seedlings, downshifting of translocation of current photosynthates is the first physiological indication of flooding (Sloan et al. 2016).

Growth of fine roots and production have been shown to correlate positively with the depth of the groundwater table (Finér and Laine 1998; Murphy et al. 2009), and thus with soil oxygen content accordingly. Precipitation in July has been found to correlate negatively with the diameter growth in drained peatland Scots pine (*Pinus sylvestris* L.), whereas in mineral soils the growth of trees correlated positively with the mean precipitation in May and June (Hökkä et al. 2012). This explains the different responses of annual growth to precipitation in drained peatlands and in mineral soil.

In sensitive tree species e.g. Sitka spruce (*Picea sitchensis* (Bong.) Carr.), waterlogging results in dieback of fine root tips (Nicoll and Coutts 1998). Furthermore, waterlogging decreases root biomass and the root /shoot ratio in several other tree species (Megonigal and Day 1992; Lopez,and Kursar 1999; Possen et al. 2011). Although waterlogging causes injury to old roots, the tree root systems also have mechanisms of morphological and anatomical acclimation. Adventitious roots are formed commonly near the base of the stem, at the interface between the water saturated soil and atmosphere. They are commonly associated with improved tolerance to flooding (Mergemann and Sauter 2000; Steffens et al. 2006).

Other adaptations of roots include the formation of hypertrophic lenticels and flood-adapted roots with aerenchyma (Bertolde et al. 2010; Tanaka et al. 2011; Alves et al. 2013). Hypertrophic growth is the swelling of tissues at the stem base and is believed to result from radial cell division and expansion. Formation of hypertrophic lenticels is a common anatomical change observed in woody species (Kozlowski 1997). Hypertrophic lenticels are considered as entry points of oxygen from shoot to root (Armstrong 1968; Hook et al. 1971). There is also evidence that hypertrophic lenticels serve as excretory sites to eliminate potentially toxic compounds such as ethanol, acetaldehyde and ethylene which result from anaerobic metabolism in the roots (Pangala et al. 2014).

One of the most important responses to waterlogging is the development of aerenchyma in the root cortex. The enlarged gas spaces in tissues may enhance the venting of phytotoxic compounds produced in the roots toward the shoot and the atmosphere, and enhance the

longitudinal diffusion of gases in the roots, thus increasing their aeration (Evans 2004). The impairments and morphological adaptations in roots have been mostly studied during or after waterlogging in the growing season.

1.3 Water relations and gas exchange

Root water transport is divided into radial and axial transport. The axial transport consists of the water moving along the xylem vessels to the aerial parts. Radial water flow consists of water moving from the soil solution to the root xylem vessels which contribute the main resistance to root water transport (Doussan et al. 1998). Radial water transport involves the apoplastic path (through the cell walls and intercellular spaces) and the cell-to-cell path (through plasmodesmatal connections or across plasma membranes) (Steudle and Peterson 1998). Root hydraulic conductance (water flux versus driving force, K_r) or conductivity (hydraulic conductance expressed on the root surface or volume basis, L_p) has been used widely for the estimation of water uptake capacity (Aroca et al. 2012). The change of L_p in hypoxic plants has also been linked to the radial water transport pathway of roots (Bramley et al. 2010; Calvo-Polanco et al. 2012). The reduction of L_p under flooding stress has been attributed to resistance to transmembrane water flow resulting from cytosolic acidification and inhibition of aquaporin activity (Tournaire-Roux et al. 2003). Flooding did not change L_p in lupins (*Lupinus angustifolius* L., *Lupinus luteus* L.) in which radial water transport is dominated by the apoplastic path, whereas it led to decreased L_p in wheat (*Triticum aestivum* L.) in which the cell-to-cell path dominates radial water transport (Bramley et al. 2010). The root hydraulic conductivity of adventitious roots in tamarack (*Larix laricina* (Du Roi) K. Koch) subjected to flooding stress was higher than non-adventitious roots, in which flooded adventitious roots was found to have a higher ratio of apoplastic to cell-to-cell water flow (Calvo-Polanco et al. 2012). Changes in K_r and L_p are related to root structure and anatomy. Suberization and the formation of Casparian bands in the endo- and exodermis of roots impede transport along the apoplastic pathway, thus contributing to a lower L_p of whole root system (Steudle 2000). Increase in K_r is connected to the growth in the number and diameter of xylem vessels in Lupinus species (Bramley et al. 2009).

As a methodological consideration, heavily damaged roots probably also have high K_r values as measured by the High Pressure Flow Meter (HPFM) because in this apparatus, water is driven into roots in the opposite direction to normal water uptake. The water pressure driven to the root system at the root collar during measurement would be released more easily in damaged than in undamaged root systems. Therefore, a very steep increase in an HPFM result might be used as an indicator of damage.

Stomatal closure is one of the earliest plant responses to soil inundation that affects assimilation rate by restricting CO_2 diffusion into the leaf (Kozlowski 1982; Kozlowski 1997; Folzer et al. 2006). Stomatal closure due to waterlogging has been shown to be mediated by different chemical signals, abscisic acid (ABA) transmitted from roots to shoots (Jackson and Hall 1987; Jackson et al. 2003; Else et al. 2006), reduced K uptake (Kozlowski and Pallardy 1984) and reduced root water uptake (Schmull and Thomas 2000). Stomatal size was reported to affect the rate of response and therefore stomatal conductance, i.e. the leaves built for higher rates of gas exchange have smaller stomata and faster dynamic characteristics (Drake et al. 2013).

In addition to stomatal limitations, the photosynthetic rate can be reduced by non-stomatal limitations (Ashraf and Harris 2013). Photosynthesis is affected by the capacity of

photosynthetic machinery under flooding stress, which can be seen as reduced chlorophyll content (Mielke and Schaffer 2010) and consequently also as reduced dark-acclimated chlorophyll fluorescence (F_v/F_m) (Baker and Rosenqvist 2004). In addition, mesophyll diffusion conductance of CO_2 from sub-stomatal cavities to chloroplasts (g_m) is a key trait affecting photosynthesis (Evans et al. 2009; Niinemets et al. 2009; Terashima et al. 2011). Leaf anatomical traits such as cell wall thickness and chloroplast distribution, which are affected by environmental stress, are determinants of mesophyll conductance (Flexas et al. 2012; Tosens et al. 2012; Flexas et al. 2014).

1.4 Nutrients

Waterlogging decreases the nutrient contents of leaves by affecting the availability of nutrients in the soil and nutrient uptake capacity. Waterlogging often decreases availability of the soluble potassium (K) and nitrate (NO_3^-) because of leaching, whereas it enhances the availability of reduced forms of manganese (Mn^{2+}) and iron (Fe^{2+}). Waterlogging reduces nutrient uptake capacity as a result of the reduction in the absorbing surface of roots, nutrient uptake rate, and expression of transporters (Kreuzwieser and Gessler 2010). The lack of ATP in waterlogged roots is a reason for the reduced nutrient uptake rate (Kreuzwieser et al. 2004).

Nitrogen (N) is an important constituent of photosynthetic proteins and enzymes, so increased N-availability in plants results in increased net photosynthesis and foliage production. In boreal forests, conifers usually take up ammonium and amino acids, while the uptake of nitrate is generally low (Lupi et al. 2013). The concentrations of amino acids, particularly of aspartate, asparagine, glutamate and glutamine, were lower in the roots of flooded European beech (*Fagus sylvatica,* sensitive to flooding), Pedunculate oak (*Quercus robur*, moderately flood tolerant) and *Populus tremula × P. alba* (flood tolerant) trees than in controls (Kreuzwieser et al. 2002). After 36 days of treatment, the nitrogen content of flooded Carrizo citrange seedlings had decreased more than 2.3-fold compared to control seedlings, and reductions in nitrogen concentration ranged from 21 to 55% (in roots and leaves, respectively) (Martínez-Alcántara et al. 2012). Three and 8.5 weeks of soil waterlogging decreased the needle nitrogen content of five-week-old Scots pine seedlings but the N-content recovered after three weeks of stress when the soil oxygen content returned to normal level (Repo et al. 2016b).

Phosphorus (P) exists in soil both in organic and inorganic forms (e.g. orthophosphates HPO_4^{2-}, $H_2PO_4^-$). Phosphate ions generally react by adsorbing to soil particles or by combining with elements such as calcium (Ca), magnesium (Mg), aluminum (Al), and iron (Fe), and forming compounds that are solids in soil. Plants absorb soluble P in the soil solution mainly in the orthophosphate form (Brady and Weil 2007). When soil is flooded, soil pH moves toward neutrality (pH 6.5-7.5) both in acidic and alkaline soil and soil reduction levels increase. As a result, phosphorus can be released from insoluble, adsorbed and bound forms (Ponnamperuma 1972), thereby becoming more available for uptake by roots. However, soluble P is also liable to leaching losses.

The single most consistent nutritional shift reported by plant hypoxia and flooding is a drop in leaf or plant K concentration (Fisher and Stone 1990). The loss of potassium ions from the leaves is thought to impair the function of the potassium ion pump responsible for maintaining the turgor of guard cells that open stomatal pores for gas exchange (Sojka 1992).

In submerged soils, insoluble Fe^{3+} and Mn^{4+} are reduced to the soluble ferrous (Fe^{2+}) and manganous forms (Mn^{2+}) (Ponnamperuma 1972). Reduced forms of iron and manganese are more readily taken up by roots. High concentrations of Fe^{2+} and Mn^{2+} are toxic to plants, however. During waterlogging, toxic concentrations of Mn^{2+}, Fe^{2+} may occur (Setter et al. 2009). Therefore, tolerance to one or more ion toxicities can be an essential trait to improve plant performance in waterlogged soils (Khabaz-Saberi et al. 2006).

1.5 Stage of annual development

Tree responses to waterlogging stress are much dependent on the stage of annual development. The annual cycle of trees includes two main phases, i.e. dormancy and growth. Furthermore, dormancy has conventionally been divided into several consecutive physiological phases (Lang et al. 1987). In autumn, a few weeks after growth cessation, the buds enter endodormancy when growth is constrained even under favourable growing conditions. Endodormancy is triggered by a sufficient exposure to low chilling temperatures (-5 to +10 ^{o}C) during the autumn months and early winter (Nienstaedt 1966, 1967; Sarvas 1974; Leinonen 1996). Under Finnish conditions, the chilling requirement for triggering of endodormancy is fulfilled by the end of the year (Sarvas 1974; Hänninen 1990). The second period of dormancy is ecodormancy (post-dormancy or quiescence) during which the buds are capable of development but they are still suppressed by adverse environmental conditions such as low temperatures. This stage is typical of later winter and early spring. Ecodormancy is terminated by rising temperatures (e.g., T > 5°C), and finally budburst takes place when the accumulated temperature exceeds a specific threshold (Sarvas 1972; Campbell and Sugano 1975; Hannerz 1994). The growth phase starts at bud burst, and ends after growth cessation. The effect of waterlogging during the endodormancy and ecodormancy phase might be different due to the above-mentioned inner physiological variation of plants within and between the phases. Roots typically are never truly dormant but during the winter are in a quiescent state, with a high competence to start growing.

Whether waterlogging occurred during the dormancy phase or the growth phase, the cause of possible injuries is the same, i.e. oxygen deprivation, but the response time is different. There are several studies on tree responses to waterlogging during the growing season (Dreyer et al. 1991; Nicolás et al. 2005; Rengifo et al. 2005; Mielke and Schaffer 2010; Repo et al. 2016a). In that phase, the high rate of metabolic oxygen consumption makes oxygen shortage more acute than in dormancy. In the growing phase, plants have several acclimation mechanisms for alleviation of the dangers of hypoxia or anoxia. On the contrary, growth-dependent escape mechanisms may not operate in winter, but tolerance has to be based on prolonged adaptation to waterlogged conditions (Crawford 2003). However, the responses of trees exposed to waterlogging during dormancy and at the beginning of the growth season are much less studied compared to those in the middle of the growing season (Crawford 2003).

1.6 Study species

Norway spruce (*Picea abies* (L.) Karst.), silver birch (*Betula pendula* Roth) and pubescent birch (*Betula pubescens* Ehrh.) are economically and ecologically important tree species in

boreal forests. Norway spruce has a wide distribution ranging from the Pyrenees, Alps and Balkans northwards through Germany to Scandinavia and eastwards to west Russia. Norway spruce has a shallow root system. It is a relatively adaptive species but grows best on moderately fertile, moist mineral soils and fertile peatlands with shallow peat. It is regarded as a flood-intolerant species according to field observations (Glenz et al. 2006). In Finland, Norway spruce is the second most widely distributed conifer species after Scots pine and it grows in mixed forests with broad leaved species as well, mainly silver birch and pubescent birch.

Silver birch and pubescent birch have a wide distribution range on the Eurasian continent, ranging from the Atlantic Ocean to eastern Siberia. However, birches grow most abundantly in the temperate and boreal forests of Northern Europe (Hynynen et al. 2010). Birch species are light-demanding pioneers, which rapidly occupy open areas after forest fires and clear-cuttings due to fast early growth (Fischer et al. 2002). The root systems of birch trees are often deep and intensive. Most of the birch stock occurs in mixed stands dominated by conifers, where they are very important for the biodiversity of mostly coniferous forests. However, silver birch is also grown in pure even-aged artificially regenerated stands. In the forest management of pure silver birch stands, the goal is typically to produce high-quality sawn timber or plywood. Silvicultural practices aim at the production of large-diameter, straight and defect-free birch stems (Viherä-Aarnio 2007). The timber of pubescent birch is of poor durability when untreated and it never reaches the larger, more financially attractive size classes, often showing poor stem form especially when growing on peatlands and other wet sites (Verkasalo 1997). Therefore, the main aim of growing pubescent birch is to produce pulp wood and fuel wood with low costs. Silver birch is generally associated with well-drained but not dry and relatively fertile sites (Hynynen et al. 2010). Pubescent birch can survive also in compact soils and in wet peatlands (Niemistö et al. 2008). The physiological and morphological adaptation mechanisms of pubescent birch to wet peatland in comparison to silver birch have not been addressed before.

There is large variation in the waterlogging tolerance between tree species (Jones and Sharitz 1998; Johnson 2000; Küssner 2003). Those differences are explained by different acclimation and adaptation mechanisms to avoid or to tolerate hypoxic conditions (Kozlowski 2002; Glenz et al. 2006). Survival may vary even among closely related species of the same genus (Kozlowski 1997; 2002).

1.7 Study objectives and hypothesis

In this study, three boreal forest species were selected to study their tolerance to waterlogging during different seasons. Boreal trees stay dormant or in an inactive phase at most of the time during their annual cycle but their responses to waterlogging in that phase are not well known. Waterlogging causes deprivation of soil oxygen content, which disturbs root function and consequently affects shoot growth. Knowledge of the effects of waterlogging in winter and spring on Norway spruce and both birch species in boreal areas is important also for the prediction of the future forest growth and production.

The aim of this study was to find out the responses of boreal tree species to simulated waterlogging in late winter and early spring. The specific objectives were as follows:
i. To explore the effects of soil waterlogging in late winter on the physiology and growth of 1-year-old Norway spruce, silver birch and pubescent birch seedlings;

ii. To find out the effects of soil waterlogging in early spring on the morphology, physiology and growth of silver birch and pubescent birch seedlings;

iii. To find out the differences in waterlogging responses between dormancy and the growth period and between two birch species.

The hypotheses were as follows:

i. Waterlogging in late winter has a negative effect on the physiology and growth of all of the three species during the follow-up growing season;

ii. Waterlogging in early spring has more harmful effects than in late winter on the two birch species;

iii. Pubescent birch has more morphological and physiological acclimation and adaptation mechanisms to waterlogging stress compared to silver birch.

2. MATERIALS AND METHODS

2.1 Materials

There were two experiments (Exp. 1, Article I, and Exp. 2, Articles II and III) that were included in this study. In Exp. 1, one-year-old cold-stored dormant Norway spruce seedlings (Plantek-81F; pot volume 85 cm^3; height 18-24 cm; origin Suonenjoki, Eastern Finland, 62º39' N, 27º03' E, 130 m a.s.l.) were transported to the Natural Resources Institute Finland (Luke), Joensuu unit (62°36' N, 29°45' E, 80 m a.s.l.) and stored in darkness at 2°C before the start of the experiment. The growing medium was peat. At the start of the experiment, the seedlings were in the second phase of dormancy, i.e. quiescence, where growth can resume whenever conditions become favourable. The seedlings were replanted, with the peat plugs intact, into black plastic pots (7 × 7 × 6 cm) with quartz sand (particle size 0.5-1.5 mm, Nilsiä Quartz, SP. Mineral, Finland) as the culture medium. Immediately after replanting, 128 seedlings were moved into a growth chamber (PGW36, Conviron, Winnipeg, Canada). They were distributed into eight containers (dimensions 33 × 33 × 13 cm, 16 seedlings/container) and four replicate containers (n = 4) for the designed two treatments. The containers were placed in the growth chamber in a randomised order.

In Exp. 2, one-year-old silver birch (PL25, peat plug volume of 380 cm^3, seedling height 40–72 cm) and pubescent birch (Ek28, peat plug volume of 280 cm^3, seedling height 40–66 cm) seedlings in containers were cultured at a commercial tree nursery in Saarijärvi, Central Finland (62º 46' N, 25º 37' E), with seeds from local seed orchards (Sv422 and Sv421, for silver and pubescent birch, respectively). The seedlings overwintered under snow cover. They were transported to Joensuu, Eastern Finland (62º 36' N, 29º 45' E, 80 m a.s.l.), at the end of March 2011 and stored for one day in a growth chamber at 2°C. Within the next two days, they were replanted in pots (11 × 11 × 12 cm) in mineral soil with the peat plugs intact. All told, 288 pots (144 per species) were distributed across 48 basins (35 × 23 × 12 cm), with each basin containing six seedlings of the same species. The basins were placed in two growth chambers (PGW36, Conviron, Winnipeg, Canada). Each chamber contained 24 basins (12 per species), and they were allocated into three replicate blocks per chamber. In each block, there was one basin for each of the four treatments (see below) and each species. Thus, each treatment had six replicate basins with six seedlings for each species.

2.2 Waterlogging treatments

Exp. 1 included a four-week dormancy period (weeks 1–4) and a six-week follow-up growing season (weeks 5–10). There were two treatments: (1) No waterlogging in the dormancy phase (NW) and (2) waterlogging in the dormancy phase (W).

Exp. 2 included a four-week dormancy period (weeks 1–4), a four-week early growing season (weeks 5–8), and a four-week late growing season (weeks 9–12). The treatments were: 1) no waterlogging (NW; weeks 1–12), 2) waterlogging during dormancy (Dormancy waterlogging DW; weeks 1–4), 3) waterlogging during the early growing season (Growth waterlogging GW; weeks 5–8), and 4) dormancy waterlogging followed by growth waterlogging (DWGW; weeks 1–8).

2.3 Growth conditions

The water level was maintained at the soil surface of the pots in the waterlogging treatments. The seedlings that were not subjected to waterlogging treatment were irrigated as needed during the dormancy and the growing season. The chemical composition of the waterlogging and irrigation water was adjusted to match the precipitation in southern Finland (Sallantaus 1992). During dormancy, the air temperature was 2°C, air relative humidity (RH) 90%, photoperiod 6/18 h (day/night), and photosynthetically active radiation (PAR) 200μmol m^{-2} s^{-1}. During the growing season, the air temperature was 22/15°C (day/night), air relative humidity 70/80% (day/night), photoperiod 18/6 h (day/night), and PAR 400μmol m^{-2} s^{-1}. Incandescent lamps (Airam, 60 W, Airam, Finland) and fluorescent tubes (Sylvania Cool White, 215 W; Sylvania, Canada) were used for illumination. The changes in photoperiod, temperature, and RH from dormancy to growing season were made gradually over a one-week span and the changes in photon flux density over five days. The daily changes between day and night conditions took place gradually within two hours.

2.4 Harvests and measurements

Soil oxygen content was monitored during waterlogging and during the follow-up growing season. In Exp. 1 (Article I), harvests were carried out at the end of dormancy waterlogging (H0), after 14 days of follow-up growth (H14), after 28 days of follow-up growth (H28), and after 42 days of follow-up growth (H42) to assess the physiology, biomass and nutrients (leaf: F_v/F_m, soluble sugar and starch content, biomass, nutrients; stem: biomass; root: hydraulic conductance and conductivity, length, volume, biomass).

In Exp. 2 (Article II, III), harvests were carried out at the end of dormancy waterlogging (week 4), at the end of growth waterlogging (week 8), two weeks after growth waterlogging (week 10) and four weeks after growth waterlogging (week 12). Physiological measurements were taken during growth waterlogging and the follow-up growing season by using the same seedlings at different sampling times for leaf photosynthesis, leaf chlorophyll content index (CCI), leaf dark-acclimated chlorophyll fluorescence, individual leaf area, and stem diameter. From harvested seedlings leaf carbohydrate content, leaf biomass; stem lenticel density, stem biomass, root hydraulic conductance/conductivity, root length, number of root tips, root surface area, root diameter, black root proportion and root

biomass were determined. Leaf stomata and trichome density was measured at the late follow-up growing season at week 12. Leaf nutrient contents were measured at two weeks after the end of growth waterlogging at week 10.

2.4.1 Soil oxygen content (Exp. 1, 2)

The oxygen content was measured by means of a 4-Channel Fiber-Optic Oxygen Meter (OXY-4, PreSens, Germany). An optical sensor (Oxygen Dipping Probe DP-PSt3-L2.5-St10-Yop, PreSens, Regensburg, Germany) was inserted into the soil at the middle depth of the pot, and the air-saturated value, as a percentage, was noted after the reading levelled off.

2.4.2 Gas exchange (Exp. 2)

Starting before the end of growth waterlogging (at week 8), one seedling per basin was randomly taken to measure the gas exchange in saturated-light conditions at one-week intervals. The measurements were made for one leaf of each seedling with a photosynthesis measurement device (ADC-LCpro+, Portable Photosynthesis System, ADC-BioScientific Ltd. Hoddesdon, Herts, England) in a growth chamber with constant conditions. A leaf chamber (chamber area 6.25 cm^2) was used for the measurements with the following conditions: leaf temperature of 24.0°C, PAR on leaf surface of 696 µmol m^{-2} s^{-1}, ambient CO_2 concentration of 394 µmol mol^{-1}, and water vapour pressure into leaf chamber of 15.6 mBar. For small leaves with a leaf area less than 6.25 cm^2, the actual leaf area was estimated in order to correct for light-saturated net assimilation rate (A_{max}), transpiration rate (E) and stomatal conductance (g_s) and water use efficiency (WUE=A_{max}/E).

2.4.3 Dark-acclimated chlorophyll fluorescence (Exp. 1, 2)

In Exp. 1, five previous-year needles were picked from each seedling (40 needles per treatment) for the F_v/F_m measurements at each harvest time. Five needles were attached on a tape side by side and measured at room temperature with a portable chlorophyll fluorescence meter (MINI-PAM, Heinz WalzGmbh, Effeltrich, Germany). In Exp. 2, after two weeks of growth waterlogging (week 6), two leaves were randomly selected from the upper part of the shoots from each seedling for F_v/F_m measurements at one-week intervals until the end of the experiment.

2.4.4 Soluble sugar and starch content (Exp. 1, 2)

In Exp. 1, eight Norway spruce seedlings (one per container) were used for soluble sugar and starch content measurements. The needles were dried at 40°C to constant weight and then ground into a powder. In Exp. 2, six leaves were sampled during the growing season at two weeks intervals from the long shoots in the middle height of one seedling per basin for carbohydrate analyses. The leaves were weighed for their fresh mass, packed in tinfoil, inserted briefly in liquid nitrogen and stored in -80˚C. The leaves were dried and milled to powder before the analyses.

The analyses followed the protocol of Hansen and Møller (1975). The soluble sugars were extracted using 80% aqueous ethanol and the concentration was determined spectrophotometrically at 630 nm after reaction with anthrone using D-glucose as a standard. The starch was extracted from the residue using 30% perchloric acid. The starch

content was determined spectrophotometrically at 625 nm with anthrone using starch in 30% perchloric acid as a standard.

2.4.5 Leaf area (Exp. 2)

After bud burst, two leaves were randomly selected at the same height in one seedling from each basin for measurement of leaf expansion. The leaves were photographed twice at week 6 and from then on at one-week intervals. Coordinate paper was used as a background for calibration. The image analysis for leaf area was performed with Adobe Photoshop CS6 (Adobe Systems Nordic AB, Kista, Sweden). In the analysis, the leaf area was measured as the number of pixels (n1), translated to metric units (A_{leaf} in unit mm^2) on the basis of the number of pixels in the calibration area (100 mm^2) (n2) on the coordinate paper by means of the equation $A_{leaf} = $ (n1/n2)*100. In addition, the leaf area used in the calculation of the number of stomata and trichomes was measured in a procedure similar to that outlined above.

2.4.6 Stomata, glandular trichome, and non-glandular trichome density in leaves (Exp. 2)

When leaf expansion was completed, one leaf was sampled midway along a long side shoot in the middle of the main stem of one seedling per basin, for calculation of the density of stomata, glandular trichomes, and non-glandular trichomes (at week 12). The area from the leaf edge up to the central vein between the two side veins in the middle of each leaf was copied to transparent tape from both sides of the leaf (II, Figure 1a). The replicas were placed on an objective glass, and the area from the edge up to the central vein was evenly divided into three areas. Photographs were taken from each set of three areas with a digital camera (Leica Microsystems CD Camera, Heerbrugg, Switzerland) under a light microscope (Leica DM2500, Germany) with 20x objective magnification for counting the stomata and with 2.5x objective magnification for counting the glandular and non-glandular trichomes (II, Figure 1b, c, d). The number of stomata was counted from the entire image area of 2.3 mm^2 for calculation of stomatal density (number of stomata per unit surface area) in each of the three photographed sections. The densities of glandular and non-glandular trichomes were calculated from a 4 mm^2 area of each the three photographed sections. In addition, the total numbers for the whole leaf area were approximately evaluated.

2.4.7 Stem lenticel density (Exp. 2)

The number of stem lenticels was calculated at the three last harvests from the plants that were used for scanning of root systems. One centimetre of stem was cut from above the uppermost root at the root collar. Photographs were taken from opposite sides of the stem, always in horizontal orientation in relation to the picture frame to assure the same length of the stem in the picture (Leica Microsystems CD Camera, Heerbrugg, Switzerland) under a stereo microscope (Wild, Heerbrugg, Switzerland) at 6x objective magnification and analysed with Adobe Photoshop CS6 (Adobe Systems Nordic AB, Kista, Sweden). The number of lenticels was counted per unit surface area of each photograph. The mean value between the two opposite sides from three samplings was used for statistical analysis.

2.4.8 Root hydraulic conductance/ conductivity (Exp. 1, 2)

One seedling per basin was harvested at each sampling time for the measurement of root hydraulic conductance (K_r, expressed in mg MPa^{-1} s^{-1}) using a High Pressure Flow Meter (HPFM, Dynamax, Inc., Houston, Texas, USA). The shoot was cut at about 15 mm above the root collar while the root system remained intact. The cut surface was attached to the HPFM. The measurement is based on monitoring the flow of water by gradually increasing pressure (ranging from 0 to 0.5 MPa) (Tyree et al. 1995). The root hydraulic conductivity (L_p) was obtained by dividing of the root conductance by the root surface area as determined by scanning (see below).

2.4.9 Root morphology and biomass (Exp. 1, 2)

In both experiments, the roots that were used for root hydraulic conductance measurements were separated from soil by washing with tap water. In Exp.1, dead roots were separated from live ones by visual and microscopical assessment of cross sections. The live roots, including brown and black roots, were scanned (STD4800 scanner, Régent Instruments Inc., Sainte-Foy, Canada), and the root volume, root length, root surface area, and number of root tips were assessed by means of a WinRHIZO program (WinRhizo, Régent Instruments Inc., Sainte-Foy, Canada). The black roots' proportions were not calculated in this experiment. Both dead and live roots were dried at 40°C, and weighed.

In Exp. 2, the dead roots were not collected, but the live roots were scanned (Epson Expression 1640Xl scanner, Quebec, Canada), and the length, surface area, volume, number of tips, mean root diameter, and proportion of black roots relative to total length were analysed (WinRHIZO Pro (2012b), Régent Instruments, Inc., Quebec, Canada). The viability of black roots was checked in a sample by means of a stereo microscope and they were characterised in terms of the white colour of the xylem tissue under the cortex. In addition, the black roots in our material were firm instead of very fragile typical for dead and decomposing roots. For biomass measurements, the roots were dried at 40 °C to constant weight. In both experiments, the leaves and stems were collected at each harvest time for assessing dry weight.

2.4.10 Nutrients (Exp. 1, 2)

In Exp. 1, previous-year and current-year needles of all seedlings from each harvest (12 seedlings per treatment) were used for N and other nutrients analyses. In Exp. 2, the dried leaves used for biomass measurements at the late growing phase (week 10), were used for N and other nutrients analyses. For N analyses, a LECO CHN-1000 elemental analyser was used (LECO Corporation, St. Joseph, MI, USA). Powder samples were digested with HNO_3–H_2O_2 and analysed with an inductively coupled plasma atomic emission spectrophotometer (ICP/AES) (TJA Iris Advantage, Thermo Jarrell Ash Corporation, Franklin, MA, USA) for phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), boron (B), copper (Cu), and zinc (Zn) measurements. All the nutrients were expressed based on the dry mass.

2.5 Statistical analyses

The effects of waterlogging on all of the variables in both experiments were analysed by means of a mixed linear model (procedure MIXED in SPSS 15.0.1, SPSS, Inc., Chicago,

Illinois, USA). In Exp. 1, the significance of the difference between the treatments at different sampling times was tested by the Bonferroni method at a 95% confidence level. In Exp. 2, the average effect (main effect) of dormancy waterlogging (DW) which contrasts the average for NW and GW plots against the average for DW and DWGW plots and the average effect of growth waterlogging (GW) which contrasts the mean for NW and DW plots against the mean for the GW and DWGW was estimated by the Bonferroni method. The significance of the difference between the treatments (DW, GW, and DWGW) and the control (NW) at various sampling times was tested via the least significant difference (LSD) method. For each variable, the mean value for the basin was used in the statistical analyses. The number of replicate containers was four in Exp.1 and the number of replicate containers was six in Exp. 2.

3. RESULTS

3.1 Effects of dormancy waterlogging on roots of spruce and birch seedlings

DW reduced the root volume of Norway spruce at the end of waterlogging and during the following growing season (Article I, Figure 3). However, there were no differences between the treatments in root length, root surface area, number of root tips, biomass (I, Figure 2) or root hydraulic conductance (I, Figure 1d).

Similar to Norway spruce, DW had no significant effect on root length, root surface area, number of root tips, root biomass or root hydraulic conductance in pubescent birch either. On the contrary, in silver birch seedlings, DW led to decreased root length, number of root tips, root surface area and biomass during the follow-up growing season at 4 weeks and 6 weeks after completion of DW treatment (Article II, Figure 2), and decreased K_r throughout the follow-up growing season (II, Figure 4). DW led to increased black root proportion during the follow-up growing season in silver birch but the effect on pubescent birch was not significant (II, Figure 3).

3.2 Effects of growth waterlogging on roots of birch seedlings

Root length, surface area and number of tips were not affected by GW in either birch species. A significant negative effect of GW on root biomass was found in both species during the growing season (II, Figure 2i, j, Table 3). Furthermore, root length, number of root tips and root surface area were higher for GW than NW seedlings at the end of GW in pubescent birch seedlings (II, Figure 2b, d, f). At the same time, temporary enhanced formation of fine cluster roots was observed in GW seedlings in pubescent birch. GW led to increased black root proportions in both species during the growing season (II, Figure 3). During the growing season, the K_r and Lp values were significantly higher in pubescent than in silver birch and increased in both species with the progress of the growing season (II, Table 3 and Figure 4). GW caused decreased K_r only in silver birch.

3.3 Effects of dormancy waterlogging on foliage

DW did not affect F_v/F_m in Norway spruce (I, Figure 1a) and silver birch seedlings (Article III, Figure 2c). In pubescent birch, DW-treated seedlings had lower F_v/F_m than the control seedlings, however (III, Figure 2d). DW led into slightly increased sugar and starch content in the needles of Norway spruce (I, Figure 1e, f), however, it did not have any effect on starch content in either birch species. On the contrary, sugar content decreased in pubescent birch seedlings after DW during the follow-up growing season (III, Figure 3b, Table 2). DW did not affect leaf or stem biomass in Norway spruce (I, Figure 2), moreover, DW led to decreased stem biomass temporarily in silver birch (III, Table 3). The only effect of DW on pubescent birch was a temporary increase in leaf biomass (III, Table 2 and 3).

DW did not have any effect on leaf area, stomatal density, or glandular trichome density in either birch species (II, Table 3 and 5). CCI was reduced during the follow-up growing season by DW treatment in pubescent birch seedlings (III, Figure 2b, Table 1). Water use efficiency (WUE) was also reduced by DW in pubescent birch seedlings (III, Figure 1f, Table 1). Non-glandular trichomes in leaves were slightly increased by DW in pubescent birch seedlings (II, Table 4, 5). DW led to decreased the Ca content of needles in Norway spruce (I, Figure 5d) and the Mg and K content of leaves in silver birch during the follow-up growing season (III, Table 4).

3.4 Effects of growth waterlogging on leaves of birch seedlings

In silver birch, GW had effects on A_{max} and g_s (III, Table 1) that were significantly lower in GW and DWGW compared to NW seedlings at weeks 8 and 11 (III, Figure 1 a, c). In pubescent birch, lower values were found in GW and DWGW compared to NW in A_{max} and g_s at week 8 (III, Figure 1 b, d).

In silver birch, WUE was lower in GW and DWGW seedlings, significantly so in GW than in NW seedlings at week 8 and were then increased being higher in GW and DWGW seedlings compared to NW at weeks 9 and 10 (III, Figure 1e). In pubescent birch, WUE was lower in DW, GW and DWGW seedlings than in NW seedlings at week 8. Moreover, it was lower in DW than NW seedlings at weeks 9 and 11(III, Figure 1f).

The CCI of the leaves was significantly affected by GW in both birch species (III, Table 1). In silver birch, GW and DWGW seedlings had significantly higher CCI compared to NW seedlings during the whole growing season (III, Figure 2a). In pubescent birch, the CCI of the leaves in GW and DWGW did not differ from that in NW seedlings at certain sampling times (III, Figure 2b).

F_v/F_m of the leaves was significantly affected by GW in both species (III, Table 1). In silver birch, F_v/F_m was significantly higher in GW and DWGW seedlings than in NW seedlings at weeks 10 and 11 (III, Figure 2c). Furthermore, it was higher in GW than in NW seedlings at week 9 and higher in DWGW than in NW seedlings at week 7 (III, Figure 2c). In pubescent birch, F_v/F_m was somewhat lower in GW than in NW seedlings at week 9 (III, Figure 2d).

The sugar content of leaves was not affected by GW in either birch species (III, Table 2, Fig. 3a, b). GW significantly affected the starch content in silver birch (III, Table 2). Significant treatment effects were seen in silver birch as lower starch contents in GW seedlings at week 8 and in DWGW seedlings at weeks 8, 10 and 12 compared to NW seedlings (III, Figure 3c). In pubescent birch, significantly lower starch content compared to NW seedlings was found in DWGW treatment at week 12 (III, Figure 3d).

GW decreased individual leaf area similarly in the two birch species (II, Table 3 and Figure 5). The leaf area was about 1.4 times larger in NW than in GW and DWGW seedlings in both species during the growing season. Stomatal density on the lower side of the leaves was significantly higher in silver than in pubescent birch (II, Tables 4 and 5). GW increased the stomatal density in silver birch only (II, Table 5). However, no differences were found between the treatments in the mean total number of stomata per individual leaf area.

In silver birch, the density of glandular trichomes increased during GW (II, Table 5). The density of glandular trichomes was 1.6 and 2.2 times higher in GW and DWGW than in NW seedlings on both sides of the leaves respectively (II, Table 4). In addition, the mean total number of glandular trichomes per individual leaf area was significantly higher in DWGW than in NW seedlings on both sides of the leaves. The density of non-glandular trichomes was not affected by GW.

In pubescent birch, DWGW seedlings showed twice as high a density of non-glandular trichomes on the upper side of the leaves in comparison to NW seedlings (II, Table 4). The mean total number of non-glandular trichomes ($\pm$SE) per individual leaf area in DWGW seedlings was higher than in NW treatment.

3.5 Effects of waterlogging on stem lenticel density of birch seedlings

Based on the average value across the last three sampling times, the density of stem lenticels was significantly higher in pubescent than in silver birch (II, Tables 4 and 5). The effects of dormancy and GW were not significant for stem lenticel density in either species (II, Table 5). Instead, an interaction effect was found for silver birch (II, Table 5). The GW silver birch seedlings had significantly lower stem lenticel density than NW seedlings (II, Table 4).

4. DISCUSSION

The first hypothesis that waterlogging in late winter has negative effects on the physiology and growth of the three species' seedlings during the follow-up growing season is supported by the present data (Table 1). The physiological and growth responses varied between species. However, all three species showed recovery by the end of the experiments. The roots of silver birch were most sensitive to DW, whereas Norway spruce and pubescent birch were less affected. According to the earlier field observations, silver birch prefers the well-drained sites (Hynynen et al. 2010) but the root response of silver birch to waterlogging at late winter had not been studied before. Norway spruce is classified as a flood-intolerant tree species (Glenz et al. 2006), however, the results showed that Norway spruce seedlings could tolerate one month winter waterlogging well.

The second hypothesis that waterlogging in the early growing season has more harmful effects than in late winter in two birch species is supported with the findings in this thesis (Table 1, Table 2). However, low oxygen use in winter may mitigate the harmful effects of DW (Crawford 2003).

Proof for the third hypothesis that pubescent birch has higher potential for acclimation and adaptation to waterlogging stress compared to silver birch was seen especially in

Table 1. Comparison between the three tree species studied on the effects of DW on physiological, morphological and growth parameters at the end of DW and during the follow-up growing season. Stomata density and trichomes were measured at week 12 only. The other parameters were measured several times at the end of DW (roots and stems) and during the follow-up growing season (roots, stems and leaves).

Organ	Attribute	Dormancy waterlogging effect		
		Norway spruce	Silver birch	Pubescent birch
Roots	Length	No	Decrease	No
	Surface area	No	Decrease	No
	Number of tips	No	Decrease	No
	Volume	Decrease	Decrease	No
	Biomass	No	Decrease	No
	Black root proportion	_	Increase	Increase (week 4)
	Hydraulic conductance (K_r)	No	Decrease	No
	Hydraulic conductivity (L_p)	No	No	No
Leaves /needles	Light-saturated net assimilation rate (A_{max})	_	No	Decrease (DW-week 8)
	Stomatal conductance (g_s)	_	No	Decrease (DW-week 8)
	Water use efficiency (WUE)	_	No	Decrease
	Chlorophyll content index (CCI)	_	No	Decrease
	Dark-acclimated chlorophyll fluorescence (F_v/F_m)	No	No	No
	Soluble sugar	Increase (slightly)	No	Decrease
	Starch	Increase (slightly)	No	No
	Leaf area	_	No	No
	Biomass	No	No	Increase
	Stomata density	_	No	No
	Glandular trichome	_	No	No
	Non-glandular trichome	_	No	Increase (slightly)
Stems	Biomass	No	Decrease (week 10)	No
	Lenticel density	_	No	No

morphological characteristics (Table 1, Table 2). Pubescent birch may grow in compact soils and in wet peatlands (Niemistö et al. 2008). The results partly explained the mechanism of the better tolerance of pubescent birch compared to silver birch to waterlogging. They will be explained specifically in the following chapters.

Table 2. Comparison between the two birch species studied on the effects of GW on physiological, morphological and growth parameters during the follow-up growing season. Stomata density and trichomes were measured at week 12 only. The other parameters were measured several times at the end of GW and during the follow-up growing season (roots, stems, leaves). Leaf area, CCI and F_v/F_m was measured during the GW.

Organ	Attribute	Growth waterlogging effect	
		Silver birch	Pubescent birch
Roots	Length	No	Increase (week 8)
	Surface area	No	Increase (week 8)
	Number of tips	No	Increase (week 8)
	Volume	No	Increase (week 8)
	Biomass	Decrease	Decrease
	Black root proportion	Increase	Increase
	Hydraulic conductance (K_r)	Decrease	No
	Hydraulic conductivity (L_p)	No	Decrease (week 8)
Leaves	Light-saturated net assimilation rate (A_{max})	Decrease	Decrease (week 8)
	Stomatal conductance (g_s)	Decrease	Decrease (week 8)
	Water use efficiency (WUE)	Decrease (week 8)	Decrease (week 8)
	Chlorophyll content index (CCI)	Increase	No
	Dark-acclimated chlorophyll fluorescence (F_v/F_m)	Increase	No
	Soluble sugar	No	No
	Starch	Decrease	No
	Leaf area	Decrease	Decrease
	Biomass	Decrease	Decrease (week 12)
	Stomata density	Increase	No
	Glandular trichome	Increase	No
	Non-glandular trichome	No	Increase (DWGW treatment)
Stems	Biomass	Decrease	Decrease (week 12)
	Lenticel density	Decrease	No

4.1 Root morphology and biomass

DW affected the roots of Norway spruce and silver birch, but not pubescent birch seedlings. In silver birch, root length, root surface area, number of root tips and root biomass in dormancy waterlogged seedlings was lower during the early follow-up growing season. This indicated some root damage that did not fully recover in the early growing season. It seems that the damaged roots disintegrated during the follow-up growing season in a manner similar to that noted earlier in Scots pine (Sutinen et al. 2014). In Norway spruce, waterlogging led to decreased root volume during the next growing season, but biomass did not change. Moreover, the proportion of dead roots which was increased at the beginning of the growth phase, reduced later. Therefore, it is suggested that the roots were damaged slightly in waterlogging treatment, but they could recover. The result is in accordance with post-drainage recovery of several conifer species after waterlogging during dormant phase

(Coutts and Philipson 1978; Pelkonen 1979; Coutts and Nicoll 1990). When Sitka spruce and lodgepole pine (Pinus contorta Douglas ex Louden) rooted cuttings were grown in Perspex tubes with peat as growing substrate and the lower part of root system was flooded for 28 days at 6˚C, both species survived and growth took place after drainage (Coutts and Philipson 1978). Scots pine and Norway spruce seedlings that were kept in ditches with a slow stream of water for their root systems before the onset of root growth, showed slight decrease in growth but the seedlings survived well after planting (Pelkonen 1979). When clones of Sitka spruce root cuttings were grown in 2 m tall transparent acrylic tubes of peat and exposed to waterlogging of the lower part of the roots after completion of growing season, the roots had little increase in dieback but the seedlings survived in the following spring (Coutts and Nicoll 1990).

GW decreased root biomass in both birch species which is in accordance with the results for other broad-leaved tree species (Tsukahara and Kozlowski 1986; Mielke et al. 2003; Alaoui-sossé et al. 2005). Previously, waterlogging during the growing season caused more damage to roots than during the dormant phase (Coutts and Philipson 1978; Pelkonen 1979; Coutts and Nicoll 1990). This is probably related to the high root respiration rate and thus high oxygen demand.

Enhanced fine cluster root formation was temporarily observed in GW seedlings of pubescent birch at the end of GW. A similar increase and later rapid disappearance of adventitious and fine cluster roots has been reported for *Hakea* species exposed to waterlogging (Poot and Lambers 2003). Root clusters are well known for their role in mineral acquisition in nutrient-poor soils (Dinkelaker et al. 1995). GW caused a reduction in Ca and Mg content in the leaves of pubescent birch which may have triggered cluster root formation to compensate for nutrient deficiency. Their rapid disappearance indicates a short life span for the cluster roots, however. Either DW or GW did not stimulate the formation of these kinds of roots in silver birch seedlings. Therefore, this phenomenon seems to be an adaptation of pubescent birch to wet sites. According to a previous study, pubescent birch has been found to form hypertrophic tissue and adventitious roots in waterlogged roots (Rinne 1990), moreover, aerenchyma normally forms in the cortex of young adventitious roots (Armstrong et al. 1994) and is also found in the secondary tissue of some woody plants (Kozlowski 1997).

4.2 Water relations, photosynthesis and carbohydrates

DW did not affect the root hydraulic conductance (K_r) or conductivity ($L_p = K_r$/root surface area) in Norway spruce during the following growing season. Both DW and GW led to decreased K_r, (but not L_p) in silver birch but not in pubescent birch, however. The change in K_r is related to root morphology and anatomy. Increase in K_r is connected to the increased number and diameter of xylem vessels in Lupinus species (Bramley et al. 2009). On the other hand, the decreased L_p is suggested to be due to increased resistance of transmembrane water flow resulting from cytosolic acidification and inhibition of aquaporin activity (Tournaire-Roux et al. 2003). The decreased K_r took place simultaneously with the decreased root surface area both in dormancy and growth waterlogged silver birch seedlings. Therefore, it is suggested that the decreased rate of water transport in silver birch roots was partly due to root impairment and consequently the reduced size of the root system. The decreased L_p of roots of pubescent birch seedlings caused by GW at week 8 may be explained by a temporary increase in fine root surface

area. Some flood-tolerant species like tamarack are able to develop new adventitious roots with high L_p values when exposed to flooded conditions (Islam et al. 2003; Calvo-Polanco et al. 2012). It seems that the L_p of temporary fine roots is not very high, however, and their role may be greater for nutrient uptake.

DW did not affect A_{max}, g_s and WUE in silver birch but it decreased WUE in pubescent birch. The decreased WUE in pubescent birch may imply that even though the roots were not visibly damaged the leaves got a signal from the roots affected by waterlogging and adjusted their photosynthesis machinery accordingly. GW led to decreased A_{max}, g_s, and WUE at the end of GW in both birch species but this effect did not last after drainage. Waterlogging has been found to lead to decreased gas exchange in other tree species but the rate and strength of response depends on the species and the phase of annual development (Kozlowski 1997; Repo et al. 2016a). It has been suggested that the reduction of root hydraulic conductance may contribute to the decrease of stomatal conductance (Schmull and Thomas 2000). However, it seems that the stomatal closure of leaves in silver birch and pubescent birch seedlings during GW is mediated by chemical signals (Araki 2006; Else et al. 2006).

DW did not affect F_v/F_m in Norway spruce, and F_v/F_m and CCI in silver birch seedlings during the following growing season. F_v/F_m and CCI was expected to decrease by the waterlogging treatment (Baker and Rosenqvist 2004; Mielke and Schaffer 2010). However, the results of this study indicated that the photosynthetic machinery of these two species was not affected by one month DW. By contrast, DW led to decreased CCI compared to the control at most sampling times in pubescent birch. This reduction took place simultaneously with reduced sugar content in DW. The explanation for the temporary increase in leaf biomass in that phase remained unclear, however. GW did not reduce the F_v/F_m or CCI in either birch species. In silver birch, instead of reduction, a higher level of CCI following GW than NW and DW was observed in the recovery period. This might be connected with the higher nitrogen content of the leaves, as reported previously (Evans 1989).

Starch content was slightly increased in needles of dormancy waterlogged Norway spruce seedlings during the early follow-up growing season. This might be associated with declined phloem transport and the inhibited sink effect of roots for carbohydrates in stressed plants (Palomäki et al. 1994; Sudachkova et al. 2009) and low N availability at the end of DW (Utriainen and Holopainen 2001). Limited N supply is reported to promote starch accumulation in chloroplasts in Scots pine needles (Palomäki and Holopainen 1995). In contrast, the starch content in the leaves of both birch species was not affected by DW, but was decreased during GW in silver birch, unlike the increase seen in conifers (Norway spruce, black spruce (*Picea mariana* (Mill.) BSP), Scots pine) (cf. Islam and Macdonald 2004; Sudachkova et al. 2009; Repo et al. 2016a) and broad-leaved species (White oak (*Quercus alba* L.), river red gum (*Eucalyptus camaldulensis*)) (Gravatt and Kirby 1998; Kogawara et al. 2006). In addition, the starch content in the leaves of pubescent birch was not affected by GW. The conclusion is that the hypoxia stress in the birch seedlings was not severe, therefore allowing the allocation of carbohydrates to roots as well.

4.3 Leaf and stem morphology

The leaf areas were significantly smaller in GW and DWGW than in NW in both birch species. Similar results have been reported previously for other tree species as well (Lopez

and Kursar 1999; Mielke et al. 2003; Mielke et al. 2005). This is explained by water deficiency in the above-ground parts with low transpiration rate under waterlogging (Parent et al. 2008). Compared to NW the leaf biomass in the GW and DWGW conditions were reduced in silver birch whereas those in pubescent birch were mainly similar to NW during the whole follow-up season. Thus it is possible that the leaves of growth waterlogged pubescent birch seedlings were thicker compared to the controls but this kind of thickening did not occur in silver birch. The thicker and smaller leaves have less intercellular areas and increased amount of palisade or spongy mesophyll cells that tolerate stressful conditions like high irradiation, cold weather, drought and flooding (Guerfel et al. 2009; Tosens et al. 2012; Zhang et al. 2012). Contrary to pubescent birch, a smaller leaf area in silver birch was accompanied by smaller biomass, which is probably connected to root damage in DW and DWGW.

The increase in the density of trichomes in growth waterlogged birch seedlings is partly related to the reduced leaf area. However, in light of the significantly higher total number in the leaves after DWGW treatment, it is probable that DWGW treatment induced an increase in glandular trichomes in silver birch seedlings and an increase in non-glandular trichomes in pubescent birch seedlings. It has been suggested that both types of trichomes are involved in protection against water loss by reducing air movement at the leaf surface and by increasing the thickness of the boundary layer of leaves (Tattini et al. 2000). Moreover, both types of trichomes were mainly on the veins (II, Figure 1b, c) so they can also be physically bound to the water movement between the inner and outer parts of the leaves as suggested by Fernández et al. (2014). The increase in density of stomata in silver birch was accompanied by the reduction in leaf area by GW. Moreover, the estimated total number of stomata per leaf in different treatments was the same which suggests that apparently the increase is related to the reduction in leaf area.

GW treatment showed a significantly lower lenticel density than NW in silver birch. Formation of new lenticels has been reported to occur simultaneously with stem diameter growth in flood conditions (Pangala et al. 2014). In this study, the stem diameter of seedlings in both species increased but without treatment difference (II). Accordingly, the lower density indicates that fewer or no new lenticels were formed in silver birch during the diameter growth in GW treatment. In contrast, the density of stem lenticels in pubescent birch remained the same irrespective of the treatment, indicating that new lenticels were formed during stem diameter growth.

4.4 Nutrients

The contents of N, K, Ca, Mg, Mn and Zn of previous-year needles were lower in waterlogged than in control seedlings of Norway spruce at the end of DW. This is either caused by the reallocation of nutrients to new needles or the inhibition of nutrient uptake after waterlogging (Kreuzwieser and Gessler 2010). Boreal conifers rely much on the stored N in stem, roots and older needles and its internal translocation to sustain the development of new tissues, especially at the beginning of the growing season (Gezelius 1986; Näsholm and Ericsson 1990; Millard and Proe 1992). However, all the nutrient concentrations of previous-year needles in waterlogged seedlings recovered to the same level with non-waterlogged seedlings quickly during the follow-up growing season.

In silver birch, the K and Mg content of leaves of dormancy waterlogged seedlings was reduced at week 10, i.e. 6 weeks after completion of the DW. This is likely due to the

reduction of the absorbing surface of roots as a result of the reduction of root length, surface area, number of root tips and root biomass in those seedlings at weeks 8 and 10.

GW led to reduced the content of K, Ca, Mg, Mn, and B in silver birch leaves as well as of Ca and Mg in pubescent birch leaves at week 10 (two weeks after completion of GW treatment). This might be connected to the impaired function of the roots, which had not yet fully recovered from GW. P content did not change but Fe content increased in the leaves of waterlogged silver birch seedlings which might indicate higher availability of Fe and P due to waterlogging (Ponnamperuma 1984; Rubio et al. 1997). The uptake of Mn under waterlogged conditions can also be reduced by high Fe concentrations (Khabaz-Saberi and Rengel 2010). The manganese content was high especially in DW treated pubescent birch seedlings but it did not exceed toxic levels (Rikala 2012). The increase in the N content of growth waterlogged silver birch seedlings was accompanied with a smaller leaf area and lower leaf biomass, leading also to a concentration effect.

5. CONCLUSIONS

The aim of this study was to find out the response of the seedlings of Norway spruce, silver birch and pubescent birch to waterlogging at the end of the dormancy phase (simulated late winter) and to explore the differences of two birch species in their responses to waterlogging in the early growing phase (simulated early spring). Waterlogging during the end of the dormancy phase (DW) induced root impairment in Norway spruce and silver birch seedlings which became evident during the following growing season. In addition, the roots of silver birch were more strongly affected than those of Norway spruce. In contrast, DW had little negative effect on aboveground physiology, growth and nutrient status on Norway spruce and silver birch seedlings. This indicates that both species are potentially tolerant to short-term winter waterlogging. It is known that pubescent birch may tolerate wet soils. Therefore, it is understandable that the morphology of the roots of pubescent birch were not as strongly affected as those of silver birch. However, physiological attributes, i.e. gas exchange, CCI and sugar content in pubescent birch was decreased by DW without the decrease of leaf, stem and root biomass. The explanation for this remains unknown. The results indicated that these three species have different reactions to late winter waterlogging but that biomass production would not be reduced significantly.

Waterlogging during the early growth phase affected gas exchange, starch content, the accumulation of biomass and nutrient concentrations more in silver birch than in pubescent birch. Morphological traits, such as trichomes in leaves, stem lenticels and fine root growth, were related to the better waterlogging resistance of pubescent birch compared to silver birch. If the waterlogging events in late winter and early spring increase in future, the growth of silver birch may be affected on some sites that are suitable for growth in the current climate. More studies are needed with adult trees to get a more comprehensive view of overall responses.

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