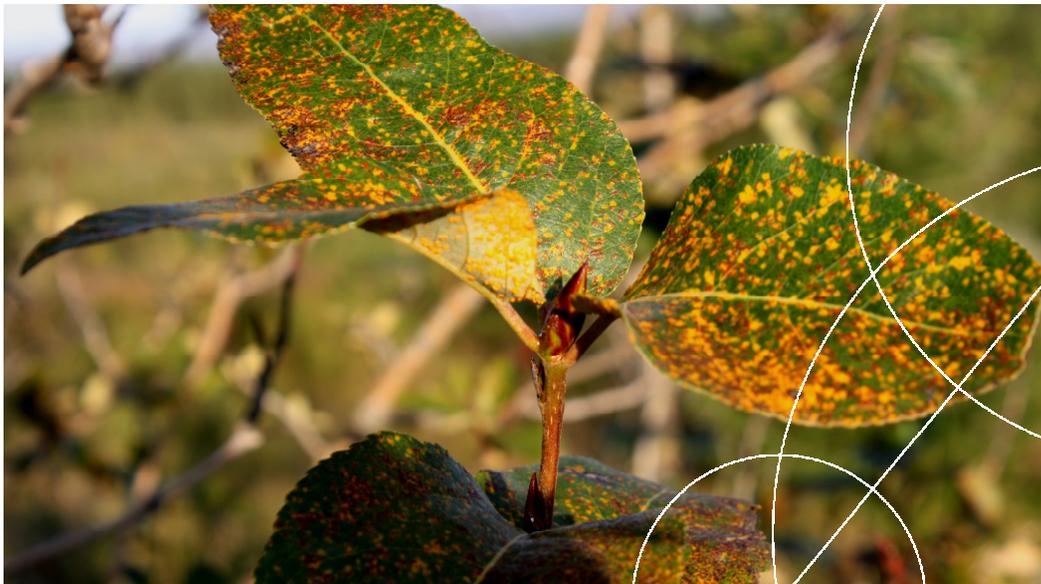**Master's thesis**

Helga Ösp Jónsdóttir

Effects of poplar leaf rust *Melampsora larici-populina* on frost resistance in poplars

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Abstract

Melampsora larici-populina the causal agent of poplar leaf rust was first found in Iceland in the year 1999 causing substantial damage on poplars. Rust infection may lead to decreased photosynthesis, early defoliation, increased susceptibility to other pests and diseases, reduced growth, affect the wood quality and increase the susceptibility of frost damage. To determine the effects of poplar leaf rust on frost resistance in poplars in the autumn, plants were infected with rust at two different times during the growing season and the frost resistance was examined by freezing tests at several times during the autumn. Based on the results from this study it may be concluded that leaf rust has an impact on the frost resistance in poplars in the autumn. However, no correlation was observed between the rust severity and level of frost damage which may indicate that the amount of rust does not give any implications for the level of frost damage, rather that only the presence of the rust is enough for interfering with the frost resistance development of plants. The different infection times did not seem to have impacts on the level of frost damage. No clear connection was observed between the origins of the clones i.e. inland or coastal clones, or between different susceptibility towards the rust. The results implied that some plants were increasing its frost resistance in spite of rust infection. Nevertheless, in order to conclude that the frost resistance development is only delayed further research would be needed.

Key words: Poplar leaf rust *Melampsora larici-populina*, black cottonwood *Populus balsamifera* ssp. *trichocarpa* T. & G., frost resistance, frost damage.

Contents

1. Introduction

Melampsora rust fungi are one of the most important pathogens causing epidemics in poplar plantations worldwide, especially in North America and Europe (Major *et al.* 2010). *Melampsora larici-populina* Kleb. is one of the poplar leaf rust species causing the most economic losses in poplar cultivation in Europe (Frey *et al.* 2005). Infection by poplar leaf rust on poplars can lead to decreased photosynthesis, early defoliation, increased susceptibility to other pest and diseases, delay of flushing time of the tree in next growing season, reduced growth of the tree and possible influence on wood quality of the tree (Gérard *et al.* 2006, Steenackers *et al.* 1996). Also, *Melampsora* species may increase the susceptibility of early frost damage (Thielges & Adams 1975; after Schreiner 1959). *Melampsora medusae,* causing leaf rust on eastern cottonwood (*Populus deltoides*), is thought to increase the risk of winter injuries as a result when damaged leaves fail to respond to decreasing day light and do not induce hardiness in the stems (Tabor *et al.* 2000). Verwijst 1990 studied *Salix viminalis* and response to *Melampsora epitea* Thüm. and frost. Certain susceptible clones showed larger proportion of frost damage following infection by *Melampsora epitea* than the uninfected clones (Verwijst 1990).

M. larici-populina was first found in south Iceland in the year 1999 causing substantial damage on poplars (Halldórsson *et al.* 2001) and is now the main pathogen causing damage on poplars. Another problem in forestry in Iceland is frost damage (Halldórsson & Sverrisson 2006). Most of the poplar clones used in forestry in Iceland are susceptible to leaf rust and many of them are also susceptible to frost damage, either spring or autumn frost (Sverrisson *et al.* 2006). In the year 2010 rust severity was unusually severe and in spring and summer 2011 many poplars had top dieback which was considered to be due the combination of rust and frost damage (Halldór Sverrisson, personal communications). Breeding program of poplars has been established in order to ensure plant material for Icelandic forestry, where one of the objectives are to find poplar material that grows without incident, e.g. frost tolerant material and with tolerance against diseases such as poplar leaf rust (Sverrisson *et al.* 2006). However, it is important to know if, and how much leaf rust is affecting frost resistance when seeking for new material in forestry and in breeding programs for regions where both rust and frost damage can be a significant problem.

The aims of this study were to a) examine the effects of poplar leaf rust *Melampsora larici-populina* on frost resistance of black cottonwood (*Populus balsamifera* ssp. *trichocarpa* T. & G.) in the autumn, b) examine if there is a correlation between the rust severity and frost damage c) to test whether different infection times of the rust affects frost resistance in poplars, and d) to test whether

different freezing dates in the autumn are affecting frost resistance development of infected poplars. The questions that will be addressed are: Is poplar leaf rust influencing frost resistance of poplars and leading to increased frost damage in the autumn? Is the level of frost damage related to the amount of rust on plants? Can different infection time of poplar leaf rust lead to different levels of frost damage? How do different freezing dates affect frost damage in infected poplars? Is the cold hardiness developing in the plants in spite of infection by leaf rust and the hardiness process therefore only delayed?

1.1 Poplars in Icelandic forestry

Black cottonwood has become the most important introduced deciduous tree species in forestry in Iceland. The origin of black cottonwood is in western North-America, from California to Alaska (Snorrason & Sigurgeirsson 2006). It was first introduced to Iceland in the year 1944 from Alaska and since then several collections have been established, from both inland and coastal areas in Alaska. A great variability can be seen between different clones of poplars originating from inland or coastal areas e.g. in leaf flushing, defoliation, frost resistance and tolerance against pest and diseases. Thus, the clone selection is important for successful poplar cultivation (Ottesen *et al.* 2006). The use of black cottonwood increased in 1990 with more use in experimental plantations and since then have the regional afforestation projects increased the use as well (Blöndal & Gunnarsson 1999). In the year 2009, black cottonwood counted for 5.7% of planted trees in Iceland (Gunnarsson 2011). Black cottonwood is the fastest growing cultivated tree species in Iceland with annual wood production of 6-20 m^3/ha (Snorrason & Sigurgeirsson 2006).

1.2 Leaf rust *Melampsora larici-populina*

Melampsora species causing leaf rust are one of the most important leaf diseases of poplars (Pei & Shang 2005). Poplar leaf rust *Melampsora larici-populina* is one of the several *Melampsora* species that is the most frequent and widespread, occurring on poplars that belong to three different sections of the *Populus* genus; *Tacamahaca, Aegiros* and *Leucoides* (Pei *et al.* 2005a), as well as on hybrids with *Aegiros* and/or *Tacamahaca* as their parents (Feau *et al.* 2007).

The rust fungi are biotrophic parasites, depending on the living tissues of plants for their development, proliferation and reproduction (Feau *et al.* 2007). Most of the rust fungi can produce up to five spore stages during their life cycle; basidiospores, spermatia, aeciospores, urediniospores

and teliospores, therefore considered as macrocyclic (Pei *et al.* 2005, Pei & Shang 2005). *M. larici-populina* is a heterocecious macrocyclic basidiomycete, producing the five spore stages during its life cycle (Fig. 1) (Barrés *et al.* 2008). In late summer and autumn, the rust forms teliospores and overwinters on fallen poplar leaves. Teliospores germinate in the spring to produce haploid basidiospores that infect larch. Dikaryotic aeciospores are generated by fertilization between spermagonia on larch needles and the aeciospores are capable of infecting poplars (Pei *et al.* 2007). When aeciospores land on poplar leaves, dikaryotic urediniospores are produced repeatedly (Yu *et al.* 2009). It is the repeatable production of asexual urediniospores or the vegetative cycle, which under favourable condition can lead to rust epidemics (Duplessis *et al.* 2009, Gérard *et al.* 2006, Hacquard *et al.* 2011, Pei *et al.* 2007). The urediniospores germinate and in order to colonize the plant tissue they produce germ tubes and penetrate the stomata, forming various infecting structures in the intercellular space, penetrating the host cell wall and differentiating into haustoria (Laurans & Pilate 1999). The haustoria is essential for nutrient uptake, furthermore it is thought to be involved in the suppression of host defence responses and in redirection or reprogramming the host´s metabolic flow (Voegele *et al.* 2009).

The outcome of rust infection is determined with an early recognition step. During an incompatible interaction, a local defence is activated and fungal growth is restricted by necrosis of the cells containing haustorium as well as the surrounding cells and by lignin accumulation. In compatible interactions, the host does not recognise the pathogen and the late expression of defense-releated genes is not sufficient to restrict host colonization and the rust proliferates in the leaf (Duplessis *et al.* 2009). Lignin deposition is thought to be one of the major defence reactions and a massive production of monolignols is induced upon rust infection (Major *et al.* 2010, Rinaldi *et al.* 2007). Also strong production of secondary metabolites has been detected in colonized leaves which likely lead to the synthesis of phytoalexins (Rinaldi *et al.* 2007). With transcriptome analysis the *Populus-Melampsora* interactions has been studied and revealed that several genes encoding for different types of PR proteins are up-regulated after *Melampsora* infection, suggesting their important roles in pathogen defence. The genes encode for PR-1, PR-2, PR-3, PR-5 and PR-9. The function of PR-1 is unknown, PR-2 and PR-3 likely hydrolyses fungal cell walls, PR-5 includes osmotins and osmotin-like proteins (antifungal cytotoxin) and PR-9 are peroxidases associated with lignin formation that increase lignin deposition at infection sites (Rinaldi *et al.* 2007, Duplessis *et al.* 2009).
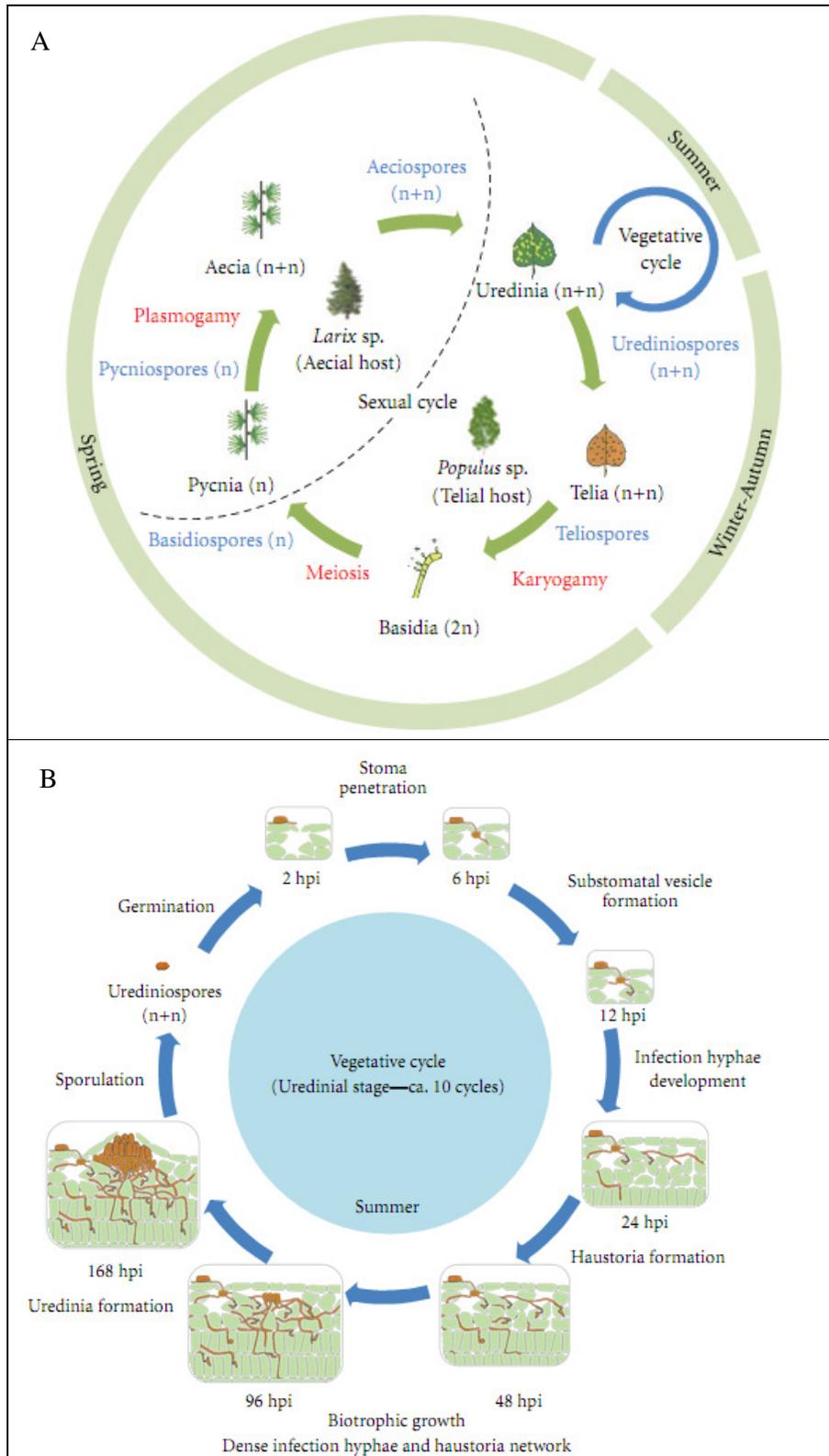
Fig. 1. Life cycle of *Melampsora larici-populina* (A) and vegetative cycle on poplars (B) (Hacquard *et al.* 2011).

M. larici-populina is native to Eurasia and its distribution is thought to cover the distribution of its natural host *Populus nigra* e.g. in Western Europe where *Populus nigra* is native. During the past century, distribution of the rust has expanded and its distribution range is now covering the most poplar-growing region (Barrés *et al.* 2008).

The poplar leaf rust was found for first time in Hveragerði in South Iceland in August 1999. It was obvious that in all spots with severe rust larch trees stood close to the poplars. A sample of rust affected leaves were sent to Jean Pinon in INRA in Nancy in France and it was confirmed that the rust fungus was *Melampsora larici-populina.* Soon after the rust was also found in a nearby town, Selfoss. Because of the distribution and the severity of the rust attack, it seems likely that the rust had arrived in the summer 1998, but there is no way to confirm that. In an attempt to eradicate the rust all larch trees in Hveragerði and most trees in Selfoss were sprayed with fungicide in the spring 2000. This may have slowed down the disease, but did not stop it. The spread of the rust was slow in the next two years, but reached the Reykjavík area in southwest 2001 although it was insignificant there. In 2003 the rust had reached inland areas in South-Iceland and also southeastern parts of the country (Halldór Sverrisson, personal communication). In 2004 the rust was found in the east and in NW-Iceland. The same year a little rust was found in the inland areas of the western part. The total distribution and severity of the disease is shown on fig. 2 (Sverrisson *et al.* 2005).
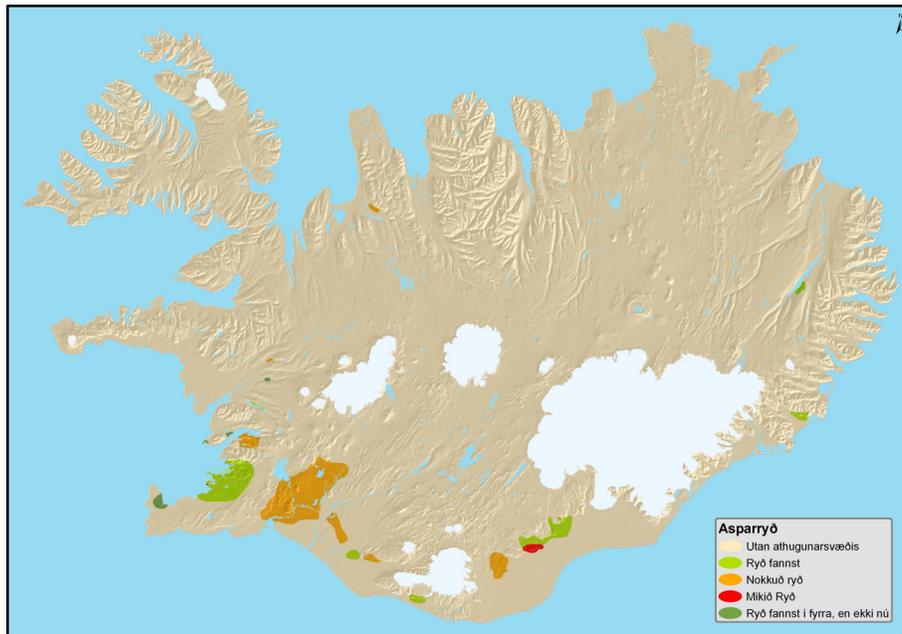

Fig. 2. Distribution of poplar rust in Iceland 2004 (Sverrisson *et al.* 2005), white = rust not checked, light green = low rust severity, yellow = medium rust severity, red = high rust severity, dark green = rust found 2003 but not 2004.

In 2005 the distribution was more or less unchanged, but the rust was more severe in some localities in the south. In the period 2006-2011 the distribution is mainly unchanged. The rust seem to have disappeared at one of the places in the east and in some localities in the west. In 2009 rust was found on some nursery plants in Akureyri, N-Iceland. Those plants were brought to Akureyri from a nursery in the south. No rust has been found there since. In NW-Iceland the rust has been found in one additional locality. The severity of the rust has been similar most of the years except in the summer 2010, when it was unusually bad in inland areas in the south. This is probably the result of an unusually long and warm summer. Surprisingly the rust has never been serious in the capital area in the southwest. This is difficult to explain, but some differences in climatic conditions must be the reason. Firstly it is possible that the basidiospores are produced too early in the spring when the larch needles have not appeared. The mild coastal winter climate could trigger the teliospores to germinate too early. The second reason could also be connected to the coastal climate in the summer. The daily temperature difference between day and night is little. This means that the conditions for dew formation in the night are unfavourable (Halldór Sverrisson, personal communication). In dry periods dew is very important for germination of urediniospores (Jean Pinon, personal communication). Dew is very common in inland areas where difference between day and night temperatures is great.

For the absence or sparse distribution of rust in West-, North- and East-Iceland we do not have good explanations. Probably the overall cooler and shorter summers do have some effect. For example in the long and warm summer in 2010 there was very severe attack of rust in the northwestern part of the country. The rust had been there since 2004, but never doing much damage until 2010. In this year the result is severe dieback of shoots and branches. Another reason for unsuccessful performance of the life cycle of the rust is spring frost damage on infected larch needles. In Iceland it is common to have warm periods in late winter, which can lead to both basidiospore production and bud break of the larch. The spores will infect the needles normally but a sudden frost period can kill off the infected needles (Halldór Sverrisson, personal communication).

The genetic variation of the rust in Iceland is being studied, and the first results suggest that there are three populations of rust present in Iceland. One population is in the east, one covering the south and south-west, and one population in the north. No gene-flow seems to be present between the eastern population and the other two, as small gene-flow was present between the north and south populations. The results do give indications for that the rust may have arrived to Iceland probably at different times, and that the genetic material may be dispersed between areas (Elefsen *et al.* 2011).

1.3 Frost resistance in trees

Plants in the temperate and boreal regions are forced to cope with large seasonal variation in temperature (Howe *et al.* 1999). Many plants sense the changes in the environment when winter approaches and exhibit an increase in freezing tolerance (Thomashow 1999). In order to withstand cold temperatures, trees undergo annual periods of dormancy (Howe *et al.* 2003, Frewen *et al.* 2000). Dormancy is in general, defined as the temporary suspension of visible growth of any plant structure containing a meristem. Endodormancy, which develops in fall, is characterized by a requirement for sustained exposure to low, near-freezing temperatures before active shoot growth can resume in the spring (Howe *et al*. 1999; after Lang 1987). Cold acclimation is a term used for describing the change from frost susceptible stage to a frost tolerant stage (Weiser 1970). The annual growth cycle of trees consists of many traits that are correlated with cold hardiness, such as: growth cessation and bud set, initiation of cold acclimation, development of endodormancy, development of maximum cold hardiness, endormancy release via chilling, and initiation of primary growth and vegetative bud flush (Howe *et al.* 2003). The timing of bud set is critical, as if the buds do not set soon enough and an adequate level of frost hardiness does not develop before the first frost of autumn, the tree may get damaged (Frewen *et al.* 2000). Several factors are assumed to be involved in endodormancy related process as important regulators of the endodormancy such as, light, temperature, abscisic acid (ABA) and gibberellins (GAs) (Rohde *et al.* 2000).

According to Weiser 1970, woody species acclimate in two distinct stages. In the first stage, the acclimation is induced by short days and low temperatures. Decreased photoperiods cause growth cessation which is a prerequisite to cold acclimation in woody plants. The short-day induced leaves are a source of a translocatable factor that promotes acclimation and moves from the leaves to the overwintering stems through the bark (Weiser 1970). Exposure to frost temperature is triggering the second acclimation and there is no translocatable factor involved (Weiser 1970).

Frost damage may occur as result of intracellular freezing. In the first stage of freezing, water in the extracellular spaces becomes frozen and ice formation begins. The extracellular ice may result in mechanical damage which can heal rapidly when favourable conditions return. In the second stage of freezing, ice is forming intracellularly (Levitt 1980). When all readily available extracellular water is frozen, protoplasmic water moves out from the cell to the extracellular ice nuclei (Weiser 1970). The rate of the water movement is limited by the permeability of the lipid plasma membrane

that surrounds the cells. Even though the membrane is highly permeable, it slows down the water movement (Levitt 1980). After continued slow movement of cellular water out to the extracellular ice, the water movement may become restricted if the temperature decreases rapidly enough so that the water movement does not occur with sufficient speed and eventually stops. The water is trapped and intracellular ice formation occurs spontaneously which results in the disruption of the protoplasm, thus death (Weiser 1970, Levitt 1980). Also, it has been studied that freezing induced damage may result indirectly as severe dehydration associated with freezing (Thomashow 1999; after Steponkus 1984, Steponkus *et al.* 1993). During freezing when all readily available extracellular water has frozen, vital water is moved from the protoplasm to the extracellular ice i.e. movement of unfrozen water from the inside of the cell to the extracellular spaces, resulting in dehydration and death (Weiser 1970, Thomashow 1999). Additionally, extracellular ice can also adhere to cell walls and membranes and cause cell rupture (Olien & Smith 1977), and protein denaturation may also occur at low temperature resulting in cellular damage in some plants (Guy *et al*. 1998).

Frost resistance that allows plants to survive freezing temperature has been categorized into two classes: frost tolerance and freezing avoidance (Levitt 1980). Frost tolerance is accomplished if the water movement from the inside of the cell to the extracellular space is kept at an equilibrium i.e. movement of water is rapid enough for the ice formation outside the cell. Even so, plants also need to be tolerant to dehydration if frost tolerance should be ensured. Dehydration stress tolerance may therefore act as a secondary factor in frost tolerance (Levitt 1980). Freezing avoidance involves prevention of freezing of sensitive tissues in plants e.g. with supercooling of the water or with accumulation of cryoprotectant substances (Janská *et al.* 2010).

When plants acclimate in the autumn, their metabolic activities undergo changes and the synthesis of cryoprotectant substances is established, such as soluble sugars, sugar alcohols and nitrogenous compounds. Those substances together with dehydrin proteins and cold-regulated proteins stabilise the plasma membrane phospholipids and proteins, cytoplasmic proteins and maintain hydrophobic interactions while other substances protect the plasma membrane from ice adhesion and cell disruption (Janská *et al.* 2010). Antifreeze proteins may also be synthesized during cold acclimation which inhibit the activity of ice formation (Janská *et al.* 2010, Mofatt 2006). Plants may get less hardened or develop less frost resistance if plants are diseased, deficient in mineral nutrients or if

they have not been able to accumulate enough carbohydrate reserves because of loss of foliage or shortened growing period (reviewed in Sakai & Larcher 1987).

Exposure to short-days prior to low temperature induces the cold acclimation for both eastern and black cottonwood. The timing of growth cessation and bud set that is induced by short-days varies between latitudinal ecotypes of poplars (Howe *et al.* 1999). Northern ecotypes have longer critical photoperiod i.e. longest photoperiod that elicited a short-day response, and a greater photoperiodic sensitivity, i.e. the change in response per unit in photoperiod, compared to the southern ecotypes. Thus, the southern ecotypes display a delay in the timing of bud set compared with the northern ecotypes (Howe *et al.* 1995). Photoperiod is very important for the induction of bud set in poplars and it has been demonstrated that the short-day induced bud set is controlled by phytochromes (Frewen *et al.* 2000, Howe *et al.* 1996). Phytochromes are photoreceptors that are involved in many light-mediated responses, including photoperiodic responses in poplars (Howe *et al.* 1999, Howe *et al.* 1996). As a result, the phytochrome genes are considered as candidate genes for playing important roles in endodormancy related traits (Howe *et al.* 1999) and three phytochrome genes have been detected in black cottonwood, *PHYA*, *PHYB1* and *PHYB2* (Howe *et al.* 1998). Abscisic acid (ABA) is known as a water stress inducible plant hormone and a growth inhibitor and is thought to be involved in short-day induced growth cessation and dormancy induction in buds (Arora *et al.* 2003). ABA is thought to act as a key regulator in enhancing cold hardening, e.g. ABA deficient mutants of *Arabidopsis* are not able to achieve normal cold hardiness, and ABA is also thought to be involved in the cold hardening of woody plants (Junttila *et al* 2002). Genes involved in ABA signalling have been detected in poplars, as the expression of *ABI3* and *ABI1* have been detected in autumn buds at the time when growth ends (Rohde *et al.* 2000). Also, the level of ABA has been shown to increase when exposed to low temperature, irrespective of photoperiod as the genetic manipulation of phytochromes in hybrid aspen did not prevent the effect of low temperature on ABA levels (Junttila *et al.* 2002). Gibberellins (GAs) are plant hormones that regulate many aspects of plant growth, such as photoperiodic control of growth cessation in woody plants (Howe *et al.* 1999; Olsen *et al.* 1995, Olsen *et al.* 1997). Certain steps in the biosynthesis of GAs seem to be inhibited by short photoperiods in hybrid aspens and this inhibition can be mediated through the phytochrome (*PHYA*) (Junttila *et al.* 2002, Olsen *et al*. 1997). This inhibition step of GA synthesis is important for the induction of growth cessation, frost hardening and dormancy development (Junttila *et al.* 2002, Olsen *et al.* 1995, Olsen *et al.* 1997).

2. Materials & methods

2.1 Plant material

Four clones of black cottonwood (*Populus balsamifera* ssp. *trichocarpa* T. & G.), 'Pinni', 'Súla', 'Halla', 'Sæland', were cultivated from cuttings in Sólskógar, a plant nursery in north Iceland, during the summer. In order to grow the plants under rust free conditions, a plant nursery in north Iceland was chosen, where poplar leaf rust has not established yet. Cuttings were collected from black cottonwoods in a forest in eastern part of Iceland. The cuttings were put in Hiko trays (35 cavities per tray) filled with Finnish peat on the 9th and 10th of June and grown inside a greenhouse for three weeks. After the three weeks, plants were taken out from the greenhouse and placed outside in the plant nursery for further growth during the summer. On the 26th of August all plants were moved again into a greenhouse to prevent possible damage from night frost. All plants were kept under natural light and well watered condition. In total 648 plants were used for the experiment with 6 replications per clone and trays with plants of similar size were chosen. The clones 'Sæland' and 'Halla' are inland clones whereas 'Pinni' and 'Súla' are considered as coastal clones. All four clones are considered to be frost tolerant based on an Icelandic study by Ævarsson 2007 where frost tolerance of various clones of black cottonwood in the autumn was determined. The tolerance against poplar leaf rust has also been studied for many clones of black cottonwood by Halldórsson *et al.* 2001. The clones 'Sæland' and 'Súla' were considered as rust tolerant and 'Pinni' and 'Halla' as susceptible towards the rust.

2.2 Inoculation

Plants were transported from the plant nursery to the Iceland Forest Service in south Iceland on the 25th of July and 11th of August for inoculation. Plants were kept in trays, 28 plants per tray. Control plants were kept at the plant nursery. Rust infected poplar leaves were collected from a poplar plantation at the forest service. Urediniospores were suspended in 400 mL distilled water and concentration of the spore suspension was determined by counting spores using an improved Neubauer hemocytometer (~14.000 urediniospores/mL). Concentration of spore suspension made for inoculation in August was adjusted to be the same as in July, however the spores were suspended in 800 mL distilled water, because the plants were bigger in August. The spore suspension was sprayed on the plants in the trays using a spray bottle, onto the lower surface of the leaves and then until runoff. 50 mL of spore suspension was sprayed on each tray in July and 80 mL

per tray in August. After inoculation, each tray was enclosed with clear plastic bag for 48 h to maintain high humidity for spore germination (Fig. 3), and the trays placed outside.

Fig. 3. Trays enclosed with plastic bag after inoculations. Pics: HÖJ.

2.3 Rust assessment

Before each freezing test, pictures were taken of three leaves from each plant with digital camera for rust assessment. The pictures were colour analysed with image analyse software (WinSeedle, Regent Inc., Canada) in order to assess the leaf rust severity as area of diseased tissue in percentage, see example in fig.4. The software is able to distinguish the infected leaf area from the healthy leaf area based on the colour of the leaf, see example in fig. 5. The healthy colour class was set to be green and disease colour class was set to be yellow, orange, brownish orange or brown in case of necrotic areas due to rust. The overall rust severity of each plant was based on the mean of three leaves from each plant.
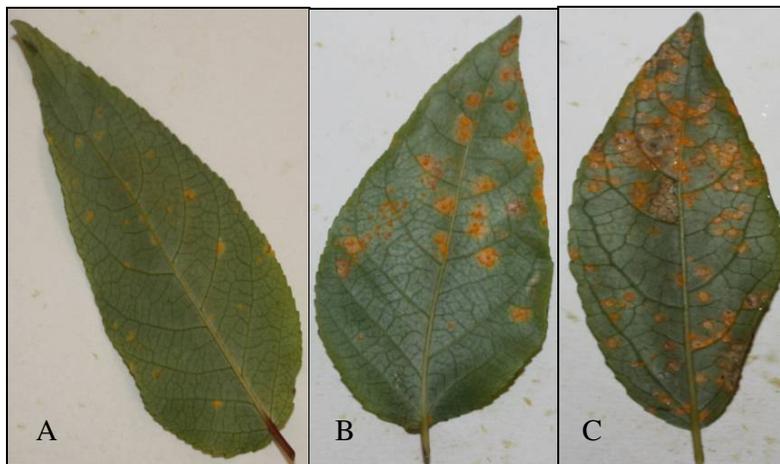

Fig. 4. Different levels of rust. (A) 4% rust severity, (B) 16% rust severity, (C) 40% rust severity. Pics: HÖJ.
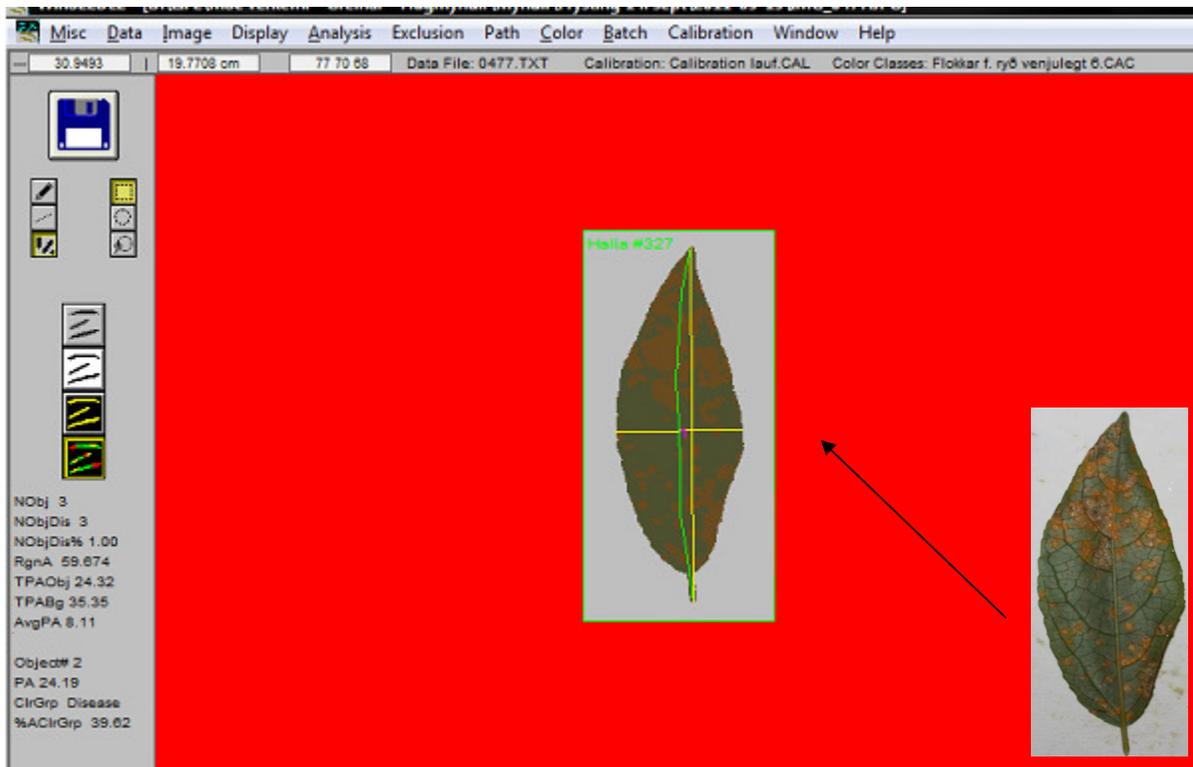
Fig. 5. An example of a leaf analysed by the computer software based on the colour of the leaf. The leaf represents 40% rust severity.

2.4 Freeze testing

Frost tolerance of the plants was tested by using computer-controlled freezing chambers, the temperature can be controlled quite accurately by a computer with approximately 1°C accuracy. Freezing process was based on methods from previous frost tolerance studies (Skúlason *et al.* 2001, Ævarsson 2007). For the first hour the temperature was decreased to +4°C. For the next five hours, the temperature was decreased from +4°C to -4°C. Then temperature was decreased by 2°C each hour until the minimum temperature was reached. The minimum test temperature was kept for two hours. Thawing rate was 2°C per hour until temperature reached +4°C. An example of freezing profile can be seen in figure 6, from 27th of September where test freezing temperature was -12°C.

Fig. 6. Freezing profile from 27th of September where test freezing temperature was -12°C, the green line represents the aimed temperature and red line represents actual temperature.
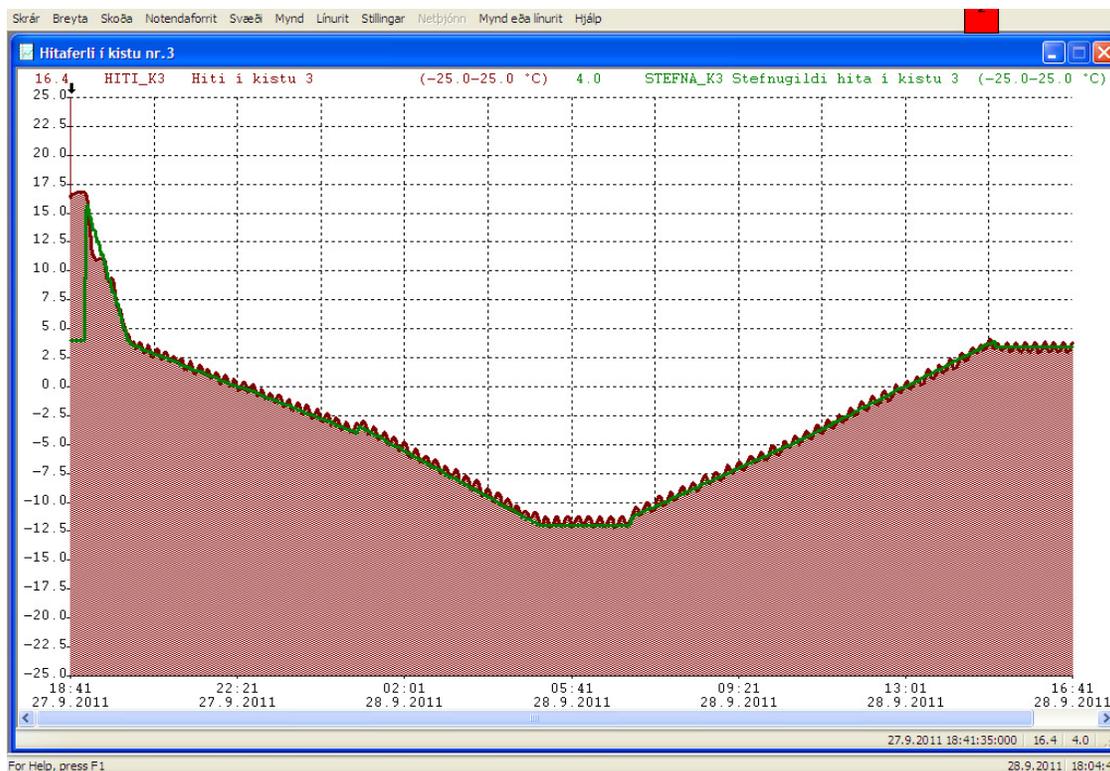
Infected and non-infected (control) plants were frozen at three different dates in the autumn, 1st, 14th and 27th of September. Plants were also kept at +4°C in one freezing chamber as unfrozen control, in order to see if any other damage was present on the plants beside the frost damage. Freezing dates, temperature and number of plants can be seen in table 1.

Table 1. Freezing dates, freezing temperature and number of plants.

	+4°C	-4°C	-6°C	-12°C	-16°C
1.09.2011	72	72		72	
14.09.2011	72		72	72	
27.09.2011	72			72	72

10 cm long shoots were cut from the top shoot of each plant containing the terminal bud, put in a moistened plastic bag and placed in a freezing chamber (Fig. 7). On 1st of September plants were frozen at -4°C and -12°C. On September 14th plants were frozen at -6°C and -12°C. The change from -4°C to -6°C was made because -4°C was not sufficient temperature for resulting in any frost

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damage, i.e. all the plants did not show any damage. On September 27th plants were also frozen at -16°C instead of -6°C because of small visible frost damage.

Fig. 7. The facilities of the freezing chambers (left) and samples ready in one chamber for freezing (right). Pics: HÖJ.

2.5 Frost damage assessment

After freezing tests, the shoots were taken out from the freezing chambers and put upstanding in soil filled trays and placed in a moisturizing chamber for ~2 weeks before the assessment of frost damage. In the moisturizing chamber the shoots were sprayed with very fine mist for 20 seconds every 15 minutes. For assessment, the uppermost 5 cm of each shoot was assessed, each shoot was cut longitudinally and the cambium layer assessed visually for any injuries, e.g. discoloration of the tissue or necrosis (Fig. 8). Frost damage was assessed as damaged area per stem in percentage, thus the average frost damage represents the average damaged area of the entire stems assessed.
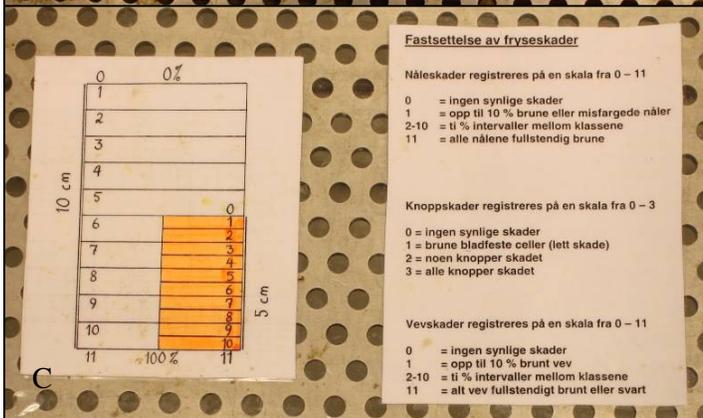
Fig. 8. (A) Samples in moisturizing chamber, (B) and (C) equipments used for frost damage assessment, (D) healthy shoot and (E) totally damaged shoot. Pics: HÖJ.

2.6 Temperature data

In order to see how likely freezing temperatures occurs in late summer and autumn in Iceland i.e. August and September, temperature data was obtained from the Icelandic Meteorological Office and analysed. By looking at data from three weather stations in south Iceland, where rust is known to be prevalent in the area in most years, data from the last ten years revealed that freezing temperatures in August does not occur very often, but is more frequent in September. The lowest temperature of the month and the date of the lowest temperature can be seen in table 2. Even though the data from the weather station revealed rather mild temperature, it may be pointed out that the weather stations are located about 2 meters above ground, thus the temperature may be much lower on and above the surface and a chilling factor by the wind is also not incorporated.

Table 2. Lowest temperature (°C) from three weather stations in August and September 2001-2011 (date of freezing temperature).

Weather station:	2011	2010	2009	2008	2007	2006	2005	2004	2003	2002	2001
Kirkjubæjarklaustur											
August	4	4	4	5	4	6	5	4	7	5	6
September	2	3	0	3	1	3	-1(24/9)	3	-2(23/9)	3	2
Hæll											
August	3	3	3	2	3	3	-1(26/8)	4	7	2	4
September	-1(14/9)	1	-5(29/9)	1	-2(17/9)	1	-5(24/9)	0	-5(23/9)	1	2
Hjarðarland											
August	0	1	0	1	-1(28/9)	2	0	1	5	0	2
September	-4(14/9)	-1(22/9)	-7(29/9)	0	-5(17/9)	-1(22/9)	-7(24/9)	-2(28/9)	-7(23/9)	-1(8/9)	0

2.7 Statistical analysis

Prior to data analysis, the normality of the data was checked with Shapiro-Wilk normality test. Both rust severity and frost damage failed the normality test. Due to the non-normality of the data, non-parametric methods were used for analysis. Kruskal-Wallis one-way analysis of variance (ANOVA), the non-parametric alternative to one-way ANOVA (Sokal & Rohlf 1995), was used in order to test for difference in sample medians between non-infected and infected plants in July and August. Tukey´s test was used for all pairwise multiple comparison with a significance level of $p \leq 0.05$. When comparing frost damage between the two different infection times, Mann-Whitney U-test was used to test for difference in frost damage with a significance level of $p \leq 0.05$. Spearman´s rank correlation analysis was tested for correlation between rust severity and frost damage, with a significance level of $p \leq 0.05$. Average data are presented as medians. All statistical analysis was carried out using Sigmaplot 11.0 (Systat Software Inc., San Jose, CA, U.S.A.).

3. Results

3.1 Effects of rust on frost resistance in autumn

Rust inoculations were successful as rust pustules from both July and August inoculations established on the leaves two weeks after inoculations. Furthermore, the rust spread gradually on the leaves throughout the experiment (Table 3), and an example of the disease development for each clone can be seen in fig. 9 when rust was assessed before each freezing test at -12°C. Unfrozen control plants, kept at +4°C at each freezing test showed no visible damage in the cambium layer, thus it could be concluded that all plant material was useable for the frost damage assessment. The first freezing test was performed on the 1st of September at two different freezing temperatures, -4°C and -12°C. No visible frost damage was seen on shoots frozen at -4°C, whereas shoots frozen at -12°C showed 100% frost damage (Table 3).

Table 3. Medians of rust severity (%) and frost damage (%) on different freezing dates, at three different temperatures and under three treatments (control, plants infected in July and plants infected in August).

| Freezing date	Treatment	Freezing temperature	Rust severity %	Frost damage %
1. September	Control	+4°C	-	0
1. September	Infected in July	+4°C	5	0
1. September	Infected in August	+4°C	2	0
1. September	Control	-4°C	-	0
1. September	Infected in July	-4°C	4	0
1. September	Infected in August	-4°C	3	0
1. September	Control	-12°C	-	100
1. September	Infected in July	-12°C	3	100
1. September	Infected in August	-12°C	2	100
14. September	Control	+4°C	-	0
14. September	Infected in July	+4°C	18	0
14. September	Infected in August	+4°C	5	0
14. September	Control	-6°C	-	0
14. September	Infected in July	-6°C	16	0
14. September	Infected in August	-6°C	6	0
14. September	Control	-12°C	-	0
14. September	Infected in July	-12°C	19	74
14. September	Infected in August	-12°C	5	79
27. September	Control	+4°C	-	0
27. September	Infected in July	+4°C	47	0
27. September	Infected in August	+4°C	47	0
27. September	Control	-12°C	-	0
27. September	Infected in July	-12°C	45	18
27. September	Infected in August	-12°C	28	80
27. September	Control	-16°C	-	70
27. September	Infected in July	-16°C	39	100
27. September	Infected in August	-16°C	33	90
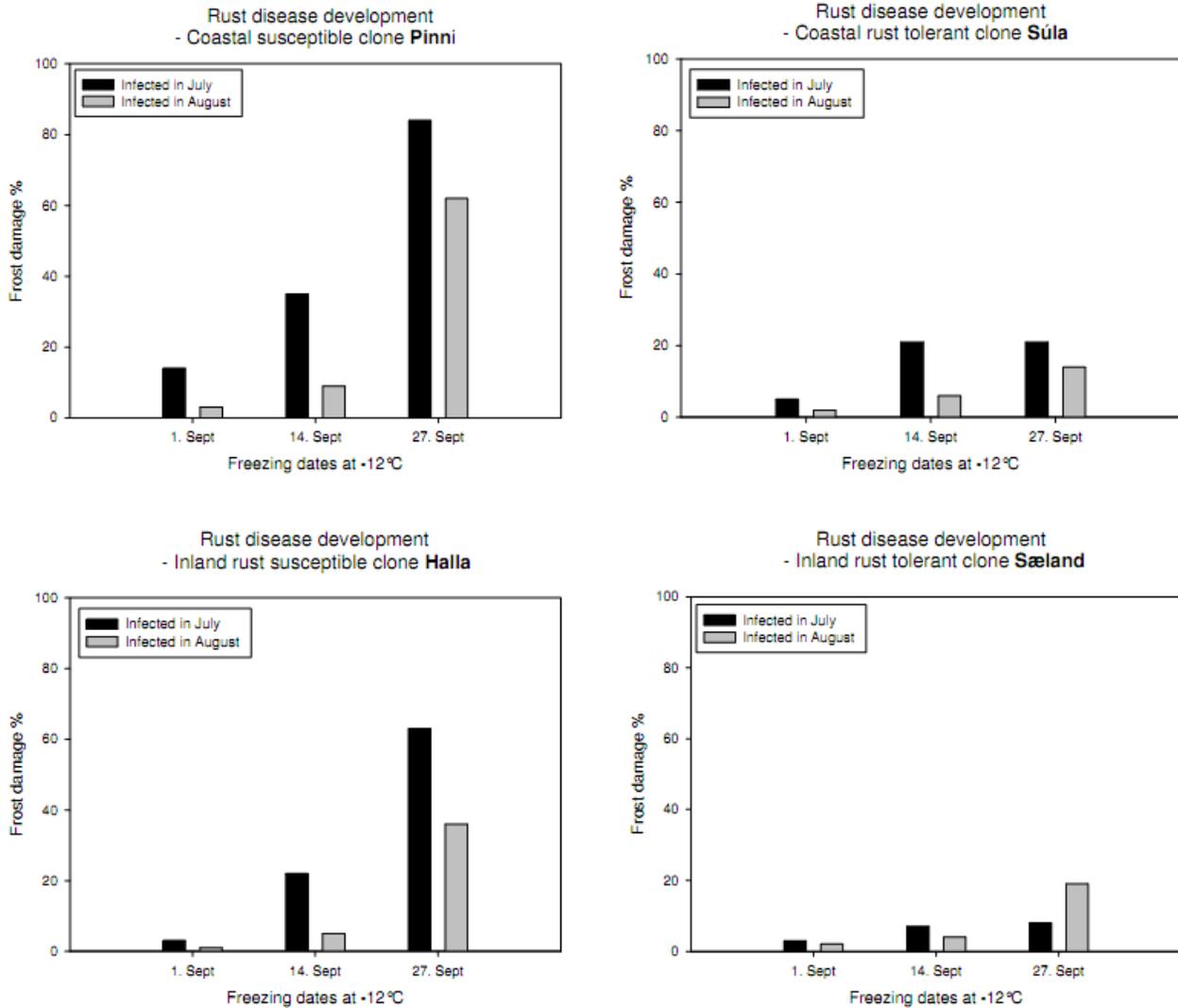
Fig. 9. An example of the development of rust severity when rust was assessed before each freezing date at -12°C for each clone.

Second freezing test was performed on the 14th of September at -6°C and -12°C. Very little frost damage was seen on few shoots frozen at -6°C but the overall average median for all the shoots was 0% (Fig. 10). At -12°C, control plants did not show any visible frost damage 0%, but plants infected by rust in July and August had frost damage with medians 73% and 79%, respectively. Significant difference was observed between the control plants and infected plants (Kruskal-Wallis: H=48.4, n=24, p<0.001). Plants infected in July and August had significantly more frost damage compared with the control plants (Tukey test: p<0.05).
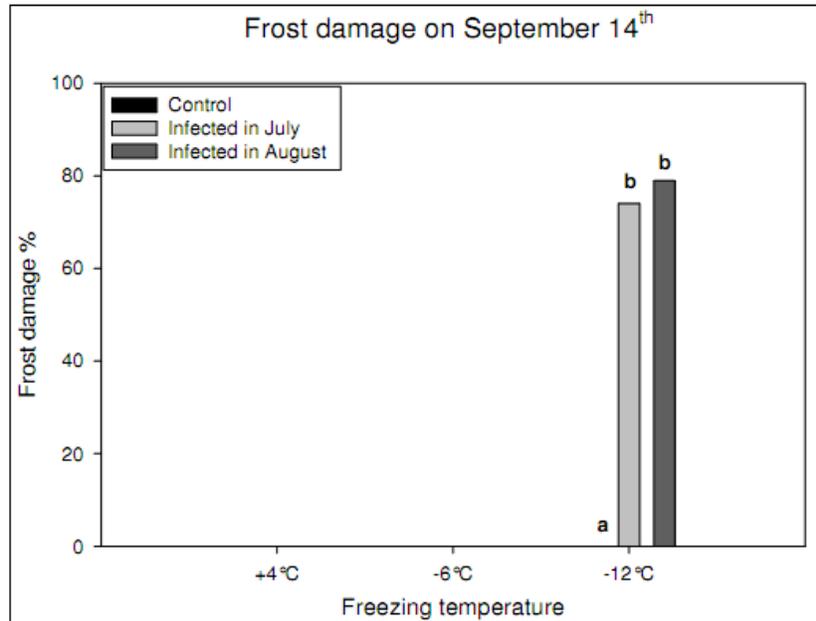
Fig. 10. Median of frost damage (%) on September 14ᵗʰ at -6°C and -12°C, no frost damage on average at -6°C, control plants showed no frost damage at -12°C but plants infected by rust in July and August had frost damage. Different letters indicate significant difference (*P*<0.05).

Third freezing test was performed on 27ᵗʰ of September at -12°C and -16°C. At -12°C, control plants did not show any frost damage 0%, but plants infected in July had 18% frost damage on average and plants infected in August had 80% frost damage on average (Fig. 11). Significant difference was observed between the control plants and rust infected plants (Kruskal-Wallis: H=38.7, n=24, *p*<0.001). Plants infected by rust in July and August had significant more frost damage compared with control plants (Tukey test: *p*<0.05). At -16°C, control plants had 70% frost damage on average as plants infected in July and August had 100% and 90% frost damage on average, respectively. Significant difference was observed between control plants and rust infected plants (Kruskal-Wallis: H=23.0, n=24, *p*<0.001). Plants infected in July and August had significant more frost damage compared with the control plants (Tukey test: *p*<0.05).
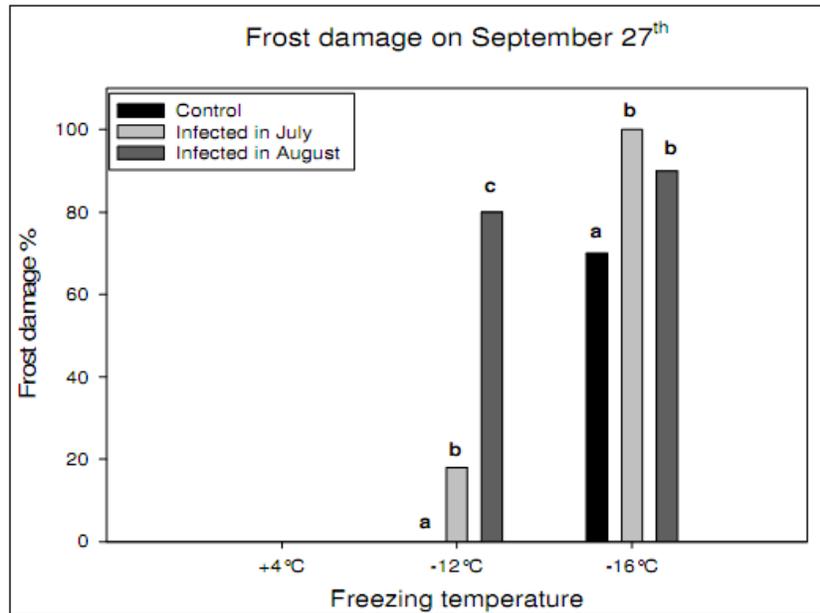
Fig. 11. Median of frost damage (%) on 27th September at -12°C and -16°C. Control plants did not show any frost damage at -12°C but rust infected plants did have frost damage (%). At -16°C, control plants and rust infected plants had frost damage. Different letters indicate significant difference ($p<0.05$).

3.2 Clone origin and frost damage

When looking at frost damage of the four different clones used in this study, no control plants showed any frost damage when frozen on 14th of September at -12°C. By looking at the frost damage on the 14th of September at -12°C (Fig. 12, Table 4) when infected in July, the results indicate that the coastal rust susceptible clone 'Pinni' and inland rust tolerant clone 'Sæland' had less frost damage than coastal rust tolerant clone 'Súla', whilst there is no difference between the other clones (Kruskal-Wallis, H=15.14, n=6, $p=0.002$; Tukey test, $p<0.05$). When infected in August, the results indicate that the coastal rust susceptible clone 'Pinni' had less frost damage than the inland rust susceptible clone 'Halla' and coastal rust tolerant clone 'Súla', whilst no difference was found between the other clones (Kruskal-Wallis, H=16.8, n=6, $p<0.001$; Tukey test, $p<0.05$).
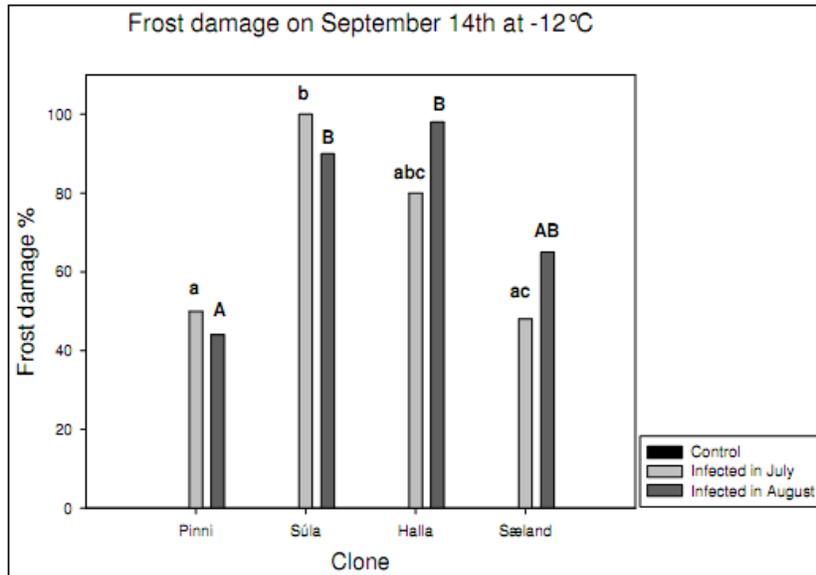
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Fig. 12. Median of frost damage (%) of different clones on 14th September at -12°C. Control plants did not show any frost damage for all clones, but rust infected plants of all clones did have frost damage. Different letters indicate significant difference (*p<0.05*).

The freezing test on 27th of September at -12°C (Fig. 13, Table 4) revealed that the coastal rust susceptible clone 'Pinni' had increased frost damage compared to the 14th of September at same temperature. The coastal rust tolerant clone 'Súla' had little less frost damage than earlier, but the decrease was not great. But interestingly, both the inland clones did not show any frost damage if infected in July, but when infected in August the frost damage had not decreased greatly. The results indicate that when infected in July, both inland clones 'Halla' and 'Sæland' had less frost damage than the coastal rust tolerant clone 'Súla' but not compared with the other clone (Kruskal-Wallis, H=10.7, n=6, *p*=0.013, Tukey test, *p*<0.05). When infected in August, no significant difference in frost damage was found between the four clones (Kruskal-Wallis; H=1.5, n=6, *p*=0.67).
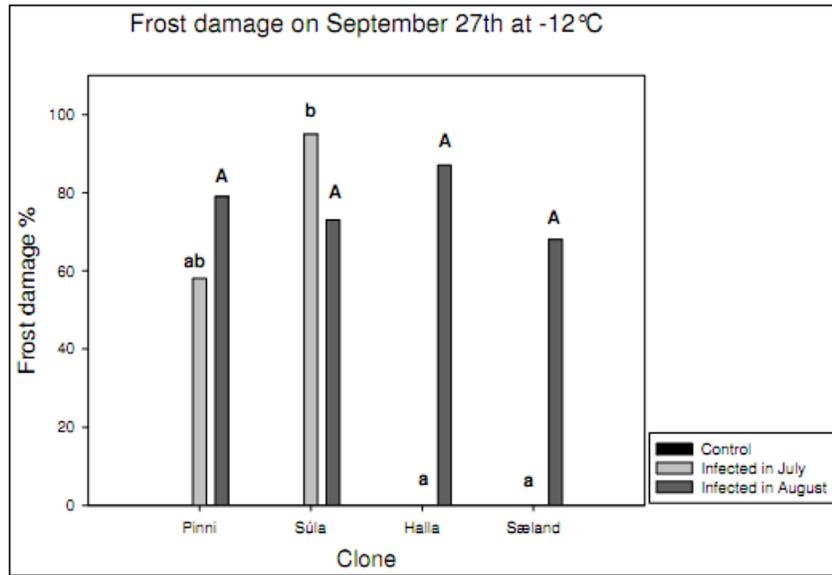
Fig. 13. Median of frost damage (%) of different clones on 27th September at -12°C. The inland clones infected in July did not show any frost damage, whereas the coastal clones infected in July have frost damage. All clones infected August have frost damage. Different letters indicate significant difference (*p*<0.05).

The freezing test on 27th of September at -16°C revealed that control plants had frost damage, except the inland rust susceptible clone 'Halla' (Fig. 14, Table 4). In general all plants showed rather high proportion of frost damage, or over 60%. Since control plants had frost damage, it might be a sign of that the plants were not fully hardened on that time to tolerate the -16°C. When looking at the coastal rust susceptible clone 'Pinni', no significant difference in frost damage was found between control plants, and plants infected in July or August (Kruskal-Wallis, H=1.27, n=6, *p*=0.53, Tukey test, *p*<0.05). That might indicate that the frost damage could be due to the reason that Pinni did not have enough frost tolerance against -16°C, rather than due to the rust infection. The control plants of the coastal rust tolerant clone 'Súla' had significantly lower frost damage than the plants infected in July and August (Kruskal-Wallis, H=12.7, n=6, *p*=0.002, Tukey test, *p*<0.05). The control plants of inland rust susceptible clone 'Halla' had significantly less frost damage that plants infected in July and August (Kruskal-Wallis, H=9.8, n=6, *p*=0.007, Tukey test, *p*<0.05) and control plants of the inland rust tolerant clone 'Sæland' also had significantly less frost damage then plants infected in July (Kruskal-Wallis, H=10.5, n=6, *p*=0.005, Tukey test, *p*<0.05).

By comparing frost damage of the plants infected in July, no significant difference was found between the different clones (Kruskal-Wallis, H=5.7, n=6, *p*=0.125). But when plants were infected

in August, the results indicate that the inland rust tolerant clone 'Sæland' had less frost damage than the coastal rust tolerant clone 'Súla', but not when compared with the other clones.
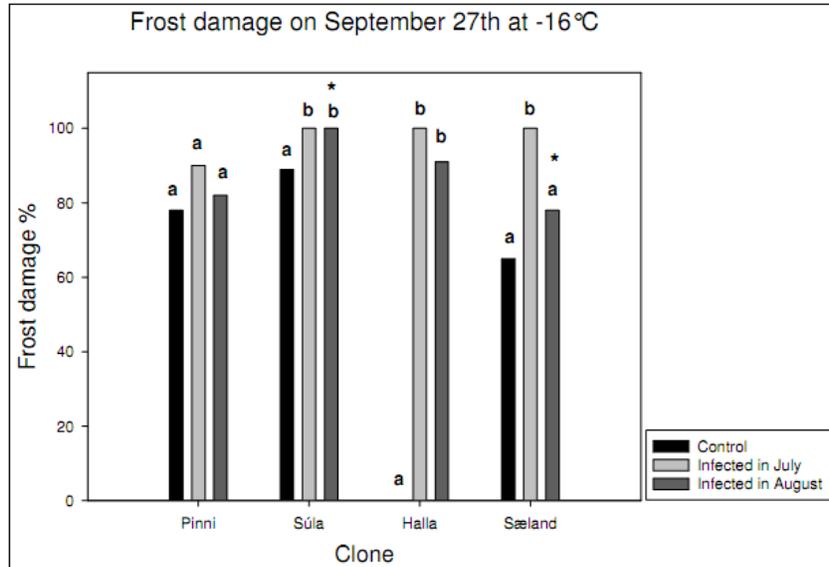

Fig. 14. Median of frost damage (%) of different clones on 27th September at -16°C. The inland susceptible clone 'Halla' is the only one not showing any frost damage as for control plants. All clones infected in July and August have more frost damage than control plants. Different letters indicate significant difference and asterisks indicate significant difference when plants infected in August ($p<0.05$).

Based on these results, there seems to be no obvious relation between clone origin or rust susceptibility and the frost damage. Except that both the inland clones do seem to increase their frost resistance if infected early in the growing season at least when frozen at -12°C.

Table 4. Frost damage of the different inland and coastal clones, tolerant and susceptible refers to the rust.

| | | Frost damage % | | | | | |
| | | 14th Sept. -6°C | | | 14th Sept. -12°C | | |
	Clone name	Control	July	August	Control	July	August
Coastal susceptible clone	Pinni	0	0	0	0	50	44
Coastal tolerant clone	Súla	0	0	0	0	100	90
Inland susceptible clone	Halla	0	0	0	0	80	97
Inland tolerant clone	Sæland	0	0	0	0	48	65
		Frost damage %					
		27th Sept. -12°C			27th Sept. -16°C		
	Clone name	Control	July	August	Control	July	August
Coastal susceptible clone	Pinni	0	58	79	78	90	82
Coastal tolerant clone	Súla	0	95	72	89	100	100
Inland susceptible clone	Halla	0	0	86	0	100	91
Inland tolerant clone	Sæland	0	0	68	65	100	78

3.3 Correlation between rust severity and frost damage

The correlation between the rust severity and frost damage was tested with Spearman Rank correlation for the three freezing tests where frost damage was observed, 14th September at -12°C, 27th September at -12°C and 27th September at -16°C (Fig. 15). No significant correlations were observed on the 14th September at -12°C (Spearman R, R=-0.13, p=0.39), 27th September at -12°C (Spearman R, R=-0.09, p=0.54) or on the 27th September at -16°C (Spearman R, R=0.04, p=0.78). The results indicate that no correlation is found between the rust severity and frost damage on poplars.

14. September at -12°C

27. September at -12°C
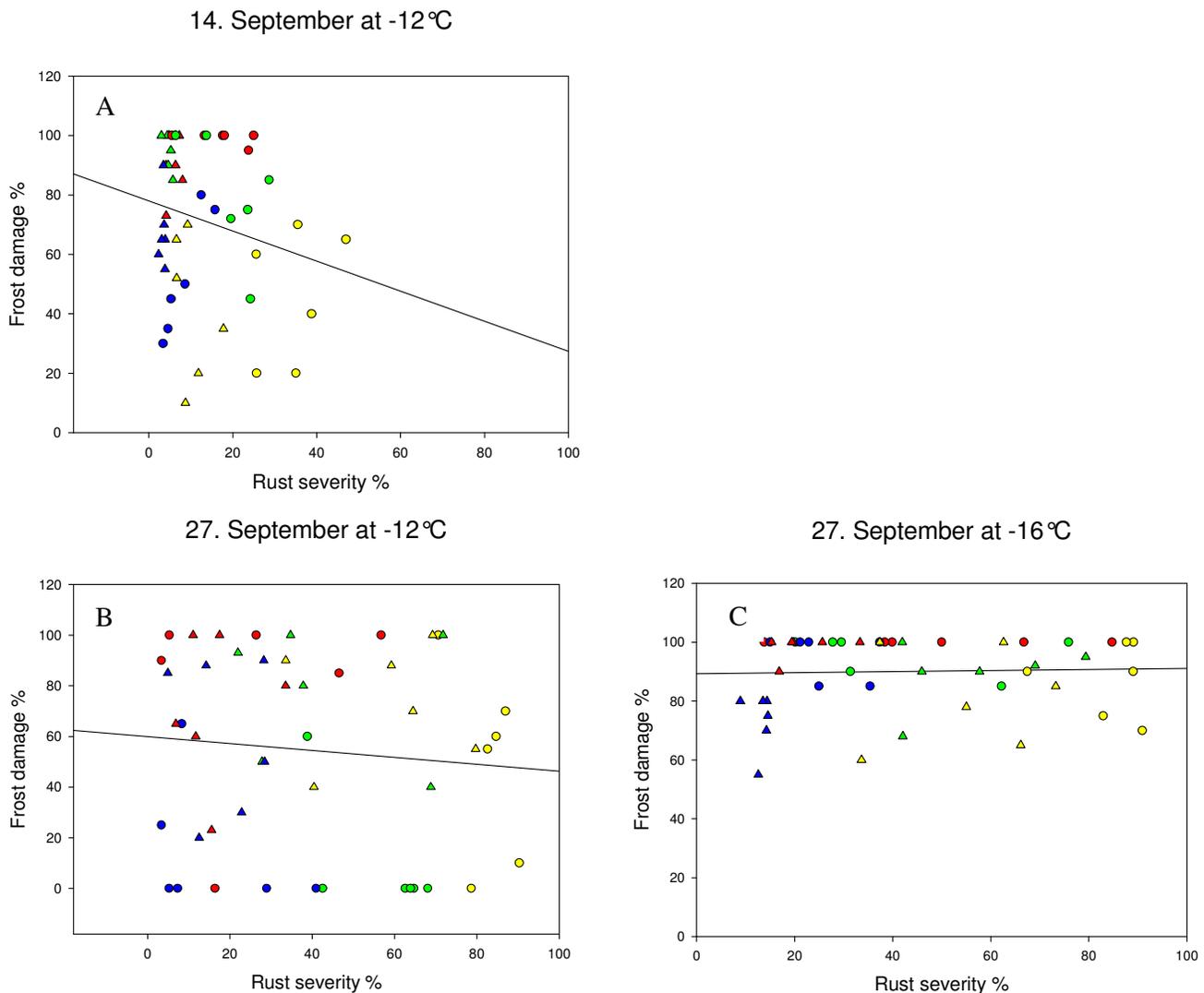
27. September at -16°C

Fig . 15. The correlation between rust severity (%) and frost damage (%) on, A) 14th September at -12°C, B) 27th September at -12°C and C) 27th September at -16°C. No significant correlations were found on the three different freezing dates (Spearman R, p>0.05). o = Infected in July, Δ = Infected in August, ▪ = Coastal rust susceptible clone Pinni, ▪ = Coastal rust tolerant clone Súla, ▪ = Inland rust susceptible clone Halla, ▪ = Inland rust tolerant clone Sæland.

3.4 Effects of different infection time on frost resistance

The different infection times, July and August, did not seem to lead to different frost damage on the 14th of September at -12°C (Table 5) as no significant difference in frost damage was found between the two infection times (Mann-Whitney, $p=0.74$). On the 27th of September, significant difference in frost damage between the two infection times was observed at -12°C (Mann-Whitney, $p=0.01$), whereas no significant difference was observed at -16°C (Mann-Whitney, $p>0.05$). These results do not give any reason for the assumption that late infection lead to less frost damage. In fact, as on the 27th of September at -12°C, the infection from August results in more frost damage than the infection from July.

Table 5. Difference in frost damage (%) between the two infection times. Different letters indicate significant difference ($p<0.05$).

Freezing date	Infection time	Freezing temperature	Frost damage %	
14. September	Infected in July	-12°C	73.5	a
14. September	Infected in August	-12°C	79	a
27. September	Infected in July	-12°C	17.5	a
27. September	Infected in August	-12°C	80	b
27. September	Infected in July	-16°C	100	a
27. September	Infected in August	-16°C	90	a

3.5 Effects of different freezing dates on frost resistance

Plants infected in July seem to be developing frost resistance in spite of being infected with rust (Fig. 16). When comparing difference in frost damage between the three different freezing dates at the same temperature -12°C, a significant difference was observed between the three dates (Kruskal-Wallis, H=31.6, n=24, $p=0.001$, Tukey test, $p<0.05$). Thus there was significantly less frost damage on the 14th compared to the 1st of September as well as on the 27th compared to the 14th of September. Plants infected in August did not show the same pattern for frost tolerance development. A significant difference in frost damage was observed between the 1st and the 14th of September (Kruskal-Wallis, H=21.4, n=24, $p=<0.001$, Tukey test, $p<0.05$), but no significant difference was found in frost damage between the 14th and 27th of September (Tukey test, $p>0.05$). Thus, there was less frost damage on the 14th compared to the 1st of September, but the frost damage was similar between the 14th and 27th of September.
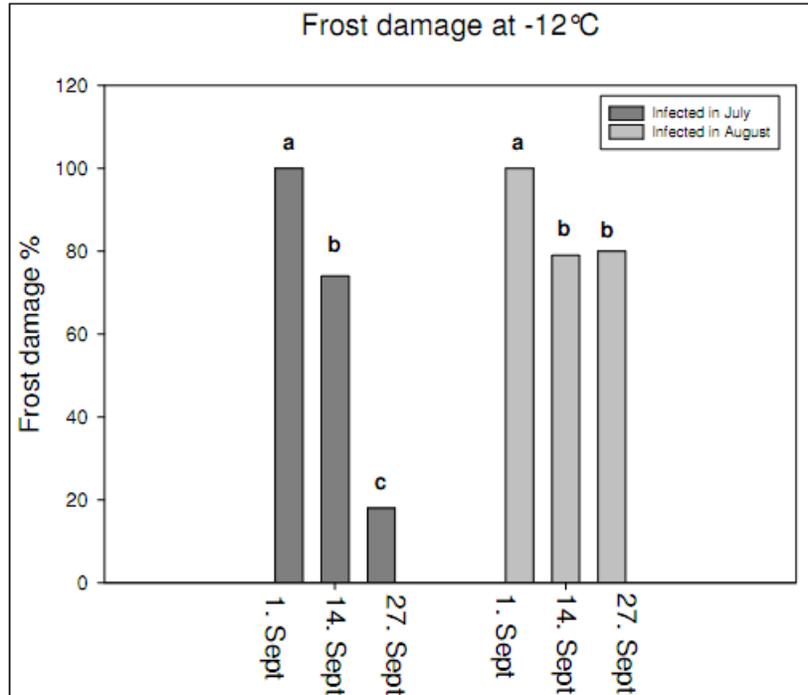
Fig. 16. Frost damage (%) on the three different freezing dates at -12°C, infected in July (right) and August (left). Plants infected in July seem to be developing frost tolerance in spite of infection. Plants infected in August do not seem to be increasing their frost tolerance up to this time point. Different letters indicate significant difference ($p<0.05$).

4. Discussion

Not many studies regarding poplar leaf rust and frost damage can be found in the literature. It is often mentioned in general text books or in general context in papers, often without any proper citations or citations not referring to an experimental source of a study concerning the possible impact of leaf rust on frost damage in poplars. Still, it has been mentioned that rust may increase the susceptibility of early frost damage (Thielges & Adams, 1975; after Schreiner 1959) and that a relatively harmless attack of rust may hinder the normal development of dormancy, leading to early frost damage (Mitchell 1992).

McCamant & Black 2000 studied the freezing tolerance of black cottonwood from coastal, montane and inland regions in the Pacific Northwest in laboratory and field freezing tests. In the field test, inland clones grew poorly in a coastal common garden and had twice the frost damage compared to the inland clones grown in an inland common garden. That was considered to be due to an infection by leaf rust as the inland clones seemed to be more susceptible to the rust compared with the coastal clones. Hence, defoliation due to diseases appeared to decrease the frost tolerance of inland clones

grown in a coastal common garden (McCamant & Black 2000). Verwijst 1990 did study the response of *Salix viminalis* to *Melampsora epitea* Thüm. and frost. His results revealed that certain susceptible clones showed larger proportion of frost damage following infection by *Melampsora epitea* than the uninfected clones (Verwijst 1990).

4.1 Evaluation of the project methods

The inoculations with the artificial rust suspension were successful and rust pustules were visible on the leaves two weeks after inoculations which correspond to previous study by Bergsdóttir & Sverrisson 2004 where the first rust pustules were observed two weeks after inoculation when plants were kept outside (Bergsdóttir & Sverrisson 2004). The concentration of the rust suspension was ~14.000 urediniospores/mL which may not be considered as very high concentration as it is often seen in other studies that both whole poplar plants and leaf disks are inoculated with spore suspension of e.g. 100.000 spores/ml (Beare *et al.* 1999, Zhang *et al.* 2009, Rinaldi *et al.* 2007).

Rust assessment was done by taking pictures with digital camera and then they were colour analysed with image analyse software. Rust was assessed as area of diseased tissue on each leaf in percentage. Because the assessments were done by an image analysing software, the assessment is considered as objective not subjective and is both reliable and accurate. Still, some drawbacks have been noted with the use of digital imaging and colour image analysing such as dealing with multiple diseases, damage or physiologic conditions on the leaves (Bock *et al.* 2010).

Artificial freezing tests have been used for many different plants and have usually been found to be in agreement with plant survival in the field (Levitt 1980). However, it has been argued that if the freezing rate is too slow in the freezing tests intracellular ice may not occur, only extracellular ice (Aronsson & Eliasson 1970). Young poplar seedlings were used in this study and it has been argued that they may differ in frost resistance from adult trees, thus when comparing the results to older, perhaps more stable trees, care should be taken (Sakai & Larcher 1987). The freezing tests were conducted on three different dates. 1st of September in order to test if any effects could be seen this early in the autumn, and on the 14th and 27th of September which corresponds to freezing dates from other frost resistance studies (Ævarsson 2007) if need were for comparing the frost resistance to previous studies. Also, based on the temperature data (Table 2) it is not unlikely that frost may occur on those dates. Four freezing temperatures were used for the freezing tests, -4°C was chosen

first because from a general monthly overview from all weather stations from the last five years (The Icelandic Meteorological Office 2011) revealed that frost temperatures of -4°C occurred in south Iceland in late August in some years. -12°C was chosen as it was found to be suitable frost temperature for predicting the frost resistance in poplars from a previous study (Ævarsson 2007). As -4°C did not cause any frost damage, the frost temperature was lowered to -6°C on second freezing date as could occur in nature (Table 2), and to -16°C on the third freezing date to see if the same effects would be seen as for -12°C, but -12°C was kept at all freezing dates. However it may always be hard to find the exact dates and freezing temperatures that provide the best frost resistance predictions.

Frost damage was assessed visually as percentage damaged area per stem. Since the assessments were visual, it may be considered as subjective (Levitt 1980) and subjective assessment may always be critical i.e. different between times of assessment or between observers. A schematic drawing of a 5 cm segment with a percentage scale (10% intervals) was used as an aid for the assessment in order to assess the damage more precisely.

4.2 Effects of rust on poplars frost resistance in autumn

The results from this study indicate that leaf rust may have impact on poplar frost resistance in the autumn and increase the frost damage. Plants infected with rust had more frost damage, thus by implicating lower frost resistance, than non-infected plants. Other studies may support these findings as the same effects of rust on frost resistance has been detected with poplars in field testing, grown in a common garden study (McCamant & Black 2000), with *Salix* and the leaf rust *Melampsora epitea* (Verwijst 1990), as well as with wheat infected with common bunt *Tilletia foetida* or *Tilletia caries* (Veisz *et al.* 2000). The rust infected poplars displayed considerable more frost damage in the stems than the non-infected poplars. However, it seems that the increased frost damage impact of rust does not apply when relatively mild frosts occur. The first freezing test from the 1st of September revealed that when plants were frozen at -4°C, they did not suffer from any frost damage (Table 3). That could be due to sufficient frost resistance of the plants at that temperature. It also implies that rust does not have any impact on the frost resistance, hence frost damage, at this relatively mild frost this early in the autumn. On the other hand, when plants were frozen at -12°C this early, they displayed large proportions of frost damage or 100% (Table 3), both non-infected plants as well as infected plants. The amount of frost damage was very similar

between the non-infected and infected plants, and no significant difference was found between them. Therefore, the frost damage at -12°C on the 1st of September is likely to be a result of general lack of frost resistance rather than from impacts of the rust infection.

The second freezing test, performed on the 14th of September, also revealed that at rather mild frost temperatures, -6°C in this case, damage amount is not affected by the rust. Both non-infected and infected plants frozen at -6°C showed very little frost damage, with the overall average damage of 0% (Fig. 10). Thus, the plants might have developed sufficient frost resistance to tolerate the -6°C at that time, and the rust did not have any impact on the frost damage at that temperature. But when plants were frozen at -12°C on the same date, non-infected plants did not display any frost damage whereas the infected plants showed significantly more frost damage (Fig. 10). Since none of the non-infected plants had any frost damage, it might be concluded that those plants had developed sufficient frost resistance. However, the plants infected in both July and August had considerable amount of frost damage, or 74 and 79% respectively. Those results imply that the rust does have a great impact on frost resistance and thus the subsequent damage caused at this freezing temperature.

The same pattern could be seen on the 27th of September when plants were frozen at -12°C, that is, a significant difference in frost damage between the non-infected plants and the infected plants (Fig. 11). The non-infected plants did not appear to have any frost damage as the infected plants did. Interestingly, the plants infected in July seemed to have less frost damage compared to the damage on the 14th of September while the amount of frost damage was similar for the plants infected in August between the two freezing dates (discussed below in 4.5). Plants were also tested at -16°C on the 27th of September. Then non-infected plants also displayed frost damage along with the infected ones. This might suggest that not all of the plants have developed enough frost resistance in the end of September to tolerate -16°C. But still, a significant difference was found in frost damage between the non-infected plants and infected plants.

When plants were frozen at -4°C and -6°C, none or very little frost damage was observed. It may have been expected that the plants would have shown some frost damage because after the relatively mild winter 2010-2011, top dieback was present in poplars in Iceland where rust severity had been substantial in the summer before (Fig. 17 & 18). This damage was thought to be a result of decreased frost resistance due to the rust. Based on temperature data from a weather station in the

vicinity of the rusted area (Table 2, Hjarðarland), relatively mild frost temperature did occur in September and the average temperature of the winter months was only 1.4°C. However, by looking closer at the temperature data, the average temperature during the winter does not give any ideas about severe frosts that might occur, and occasional minimum frost temperatures observed during the winter ranged from -7°C to -18°C, with -11°C as most frequent freezing temperature. This might suggest that even though the average winter temperature is relatively high, still, occasional severe frost temperatures may lead to frost damage in poplars if they are not fully frost resistant.

Fig. 17. Poplars at Laugarvatn in Iceland, infected with leaf rust and defoliated by leaf rust while some poplars remain green. Picture taken in early September 2010. Pic: HÖJ.

Fig. 18. Poplars at Laugarvatn in Iceland, picture taken in the same area as figure 17. Poplars with top dieback due to frost damage. Picture taken in early June 2011. Pic: HÖJ.

4.3 Clone origin and frost damage

The level of frost damage among the four clones did not seem to follow either the origin of the clones i.e. inland or coastal, or whether they were susceptible or tolerant against rust (Table 4). For instance on the 14th of September at -12°C, a significant difference was found between the coastal rust susceptible clone 'Pinni' and coastal rust tolerant clone 'Súla', and the inland rust tolerant clone 'Sæland' and coastal rust tolerant clone 'Súla' (Fig. 12). It would have been expected that the inland clones would have less frost damage compared to coastal clones, as it has been studied that inland clones are generally more frost resistant compared to coastal clones (McCamant & Black 2000). Also that more susceptible clones towards rust might have less frost resistance as is indicated with poplars (McCamant & Black 2000) and also in susceptible clones of *Salix* and *Melampsora* rust (Verwijst 1990).

Even though no clear pattern was seen between the level of frost damage and the different clones, two events remained of interest. First, the coastal rust susceptible clone 'Pinni' is known to be highly susceptible to rust and one might consider that if plants are heavily infected by rust, the more

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impact on frost damage might occur. However, on the 14th of September at -12°C, the coastal rust susceptible clone 'Pinni' did not have the most frost damage, and in fact, 'Pinni' had less or similar frost damage than the more rust tolerant inland clone (Fig. 12). But on the 27th at -12°C, the frost damage had increased, and the coastal rust susceptible clone 'Pinni' was the only clone that had increased frost damage on the latter freezing date (Fig. 13). The reason for why the coastal rust susceptible clone 'Pinni' has less frost damage, might be that even though being a coastal clone, 'Pinni' had good frost resistance, which has been seen previously in a freezing test studies (Ævarsson 2007). Also, despite the fact that coastal clones are generally considered to have less frost resistance than the inland clones, variation in frost resistance between coastal clones can exist as McCarmant & Black 2000 observed. They observed that one coastal clone population did display more frost resistance than the other coastal clones and many of the inland clones as well, and discussed that this pattern of different freezing resistance supported the suggestion that clone populations that are separated by more than 100 km may be distinct (McCarmant & Black 2000). Secondly, the inland clones, 'Halla' and 'Sæland' which had frost damage on the 14th at -12°C when infected in July, did not show any frost damage on the 27th at same temperature (Fig. 13). Therefore it might be wondered if the inland clones are developing more frost resistance even though they were infected with rust. However, the same pattern was not seen if the inland clones were infected in August (discussed below in 4.5).

4.4 Correlation between rust severity and frost damage

A significant correlation between the rust severity and frost damage was not observed in this study (Fig. 15). One might consider that a greater amount of rust per plant could lead to more frost damage, or wonder where the threshold lies i.e. how much leaf rust is needed to result in increased frost damage? As Verwijst 1990 mentioned in his discussion that an extensive rust infection may prevent the shoot of *Salix* from entering dormant stage in time (Verwijst 1990). However, here as the results suggest no correlation between the amount of rust and level of frost damage; it might be considered that the amount of rust per plant is not of great importance in relation to increased frost damage. Rather that only the presence of the rust is enough to lead to increased frost damage, since in this study rather low rust severity led to quite severe frost damage and vice versa. Hence, the amount of rust on plants might not be of a great importance in explaining the interactions of rust and frost damage.

4.5 Different timing of rust infection and consequences on frost resistance

The different timing of infection time during the growing season did not seem to matter concerning the frost damage. Whether plants were infected in July or August, no significant difference in frost damage was found between the two infections times, except on the 27th of September at -12°C (Table 5) but that could be explained with increased frost resistance of the inland clones. In general, it might be assumed that if plants are infected in July and environmental conditions are favourable with subsequent disease progression that the rust could lead to more frost damage than if plants were infected in August later in the growing season and therefore not as long time for the rust to establish. Still, the results do imply that the infection times during the growing season do not matter in relation to the level of frost damage. In fact, that might coincide with the results about the correlation between the rust severity and frost damage. Since no correlation was observed between the amount of rust and level of frost damage, it was suggested that the amount of rust is not of a great importance in relation to the level of frost damage, rather that only the presence of the rust is enough to increase the frost damage. Therefore it might not give any difference if plants are infected early in the growing season or late, only the fact that they are infected with rust no matter what time they got infected.

4.6 Effects of different freezing dates on frost resistance of rust infected poplars

Interestingly, infected plants seemed to be developing some sort of frost resistance even though infected by the rust fungi. A significant difference was found between the frost damage between the three different freezing dates when frozen at -12°C, as the frost damage in plants decreased as with subsequent freezing dates (Fig. 16). At least that was the case when plants were infected in July. The reason behind that could be the fact that the clones with inland origin showed this remarkable ability to increase the frost resistance before the last day of freezing, and therefore they did not show any frost damage (Fig. 13). The clones with coastal origin on the other hand, did not increase their frost resistance as they exhibited the similar level of frost damage on the last day of freezing or even little more damage than earlier.

This increase in frost resistance was however not seen within the inland clones when infected by rust in August, a significant difference was observed between the first day of freezing and the second one, but not between the two last freezing dates. The decrease between the first day of freezing and the second is probably due to a general increase in frost resistance as the both non-

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infected plants and infected plants did not seem to have fully developed the resistance on the first freezing date. There was no clear distinction in the frost resistance development between the inland and coastal clones if infected in August as was seen when plants were infected in July.

The different infection time did not seem to have different impacts on the level of frost damage; perhaps the different infection time may instead have some effects on the development of frost resistance of rust infected plants. Since the inland clones exhibited an increase in frost resistance on the last day of freezing tests when infected in July, it could be an indication of a delayed frost resistance. No increase in frost resistance was seen if plants were infected in August, maybe due to the late infection and therefore the plants had not evolved enough frost resistance in the end of September. However, it is not possible to conclude that the plants were developing frost resistance because only two clones exhibited that response and also it was only seen on the last day of freezing. Further investigation with subsequent freezing test later in the autumn would have been needed in order to conclude the suggestion of delayed frost resistance.

It would have been of great interest to see whether the plants infected in August would also have increased their frost resistance, at least the inland clones, if frozen even later than on the 27th of September. Perhaps they could also have developed increased frost resistance as the July infected plants, but was not possible to observe now at the last freezing date because they were infected later in the growing season, and the development of the frost resistance may even be more delayed than with the July infected plants. Also it would have been of interest to see if the coastal clones would have as well increased their frost resistance if frozen later in the autumn. But due to time limits and limitation of plant material it was not possible to proceed with the study with further freezing tests.

4.7 Possible interaction mechanism between rust infection and frost resistance

Verwijst 1990 discussed in his article that the physiological processes that lead to increased susceptibility to frost in *Salix* after infection by *Melampsora* were not yet fully understood but he mentioned a suggestion of that an extensive *Melampsora* infection could prevent the shoots from entering the dormant stage in time, thus causing subsequent frost damage (Verwijst 1990). The findings in present study does not give any information about the mechanism responsible for the interaction of how the rust is interfering with the development of frost resistance, only that the rust

does have some effects. Nevertheless, the reasons may be speculated. One of the consequences of rust infection on poplars is decreased photosynthesis and early defoliation (Gérard *et al.* 2006).

Changes in plant primary metabolism due to pathogens can occur because the induction of plant defences is an energy demanding process. Also, pathogens are withdrawing nutrients from the plant which can lead to even more demand of assimilates from the plant. Thus, the infected area on plants often develops into chlorotic and necrotic areas and a decrease in photosynthetic assimilate production (Berger *et al.* 2007). Therefore it might be reasonable to consider that decreased photosynthesis and early defoliation may interfere with the cold hardening process as plants are considered to get less hardened or develop less frost resistance if they e.g. have not accumulated enough of carbohydrate assimilates and are depleted in photosynthetic assimilates (Sakai & Larcher 1987, Weiser 1970). With transcriptome analysis, it has been observed that during compatible poplar-rust interaction, photosynthetic genes are down-regulated after infection (Major *et al.* 2010, Miranda *et al.* 2007). But results are contradictory between studies and between different pathosystems. Even though the decrease in photosynthesis is considered to be a general respond to pathogen infection, the effect on sugar levels can vary significantly between different pathosystems (Berger *et al.* 2007). For example, wheat infected with stem rust *Puccinia graminis* resulted in increased levels of soluble sugars whereas tomato plants infected with *Botrytis cinerea* resulted in decreased levels of sugars (Berger *et al.* 2007). Many studies have focused on the sugar level from a region of large area of and close to the infection site. However, when analyzing sugar levels in infected region versus uninfected region of an inoculated leaf, strong effects were only observed in the infected region (Berger *et al.* 2007, Chou *et al.* 2000). That could indicate that many measurements missed or underestimated changes and reveals the importance of spatial resolution as the changes could be heterogeneous across a leaf (Berger *et al.* 2007). Chlorophyll fluorescence imaging has revealed that the changes in photosynthesis after infection are local when *Arabidopsis* leaf was infected with *P. syringae* and *B. cinerea* (Berger *et al.* 2007). Major *et al.* 2010 discussed that if the changes in photosynthesis are localized in poplars, they could likely be underestimated by a transcriptome analysis (Major *et al.* 2010). Furthermore, for the wheat-leaf rust *Puccinia triticina* pathosystem, it has been hypothesized that the wheat leaf rust has no effects on net photosynthesis of the remaining green areas of an infected leaf (Robert *et al.* 2005). Same results have been observed for bean rust *Uromyces appendiculoatus*, where it was concluded that the rust did cause a

reduction of photosynthesis, but only on a limited area close to the rust pustules (Robert *et al.* 2005, Bassanezi *et al.* 2001).

These considerations about localized changes in photosynthesis may be an important aspect of the disease effects on photosynthesis in infected plants, as if a plant or leaf is not fully diseased, photosynthesis and production of photosynthetic assimilates may still be active. In present study, the overall rust severity in the end of September was found to range from 28% to 47% (Table 3), and also, defoliation had not yet occurred due to the rust or necrosis. Because the results here did not demonstrate correlation between rust severity and frost damage, and due to the relatively low rust severity on the plants, it might be speculated that the green remaining parts of the leaves might still be photosynthetically active and producing assimilates but with limitations due to the leaf rust, compared to fully diseased leaves that may have impaired photosynthesis and assimilate production and also may exhibit early defoliation. Thus, the plants may not be completely depleted in photosynthetic substances. These suggestions may favour the proposed explanation reviewed in Tabor *et al.* 2000 that the increased frost damage may occur if rust damaged leaves fail to respond to decreased daylength and do not induce cold hardiness in the stems (Tabor *et al.* 2000).

Another aspect of conflicts between biotic and abiotic factors worth mentioning is concerning plant hormones, which may be important factors involved in abiotic and biotic stress processes. For instance, the plant hormone abscisic acid (ABA) is considered to be involved in the cold acclimation process as well as in plant defence mechanism against pathogens, however the of ABA role in plant defence is not well defined (Gusta *et al.* 2005, Maksimov 2009, Mauch-Mani & Mauch 2005). Additionally, even though ABA is considered to be involved in the plant defence mechanism, high concentration of ABA maintained for a while may actually reduce the efficiency of defence that is controlled by salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Maksimov 2009). Research of the abiotic and biotic stress signalling have mostly been in separate fields, despite that it is well known that plants may have to cope with both situations where exposed to biotic and abiotic stresses simultaneously, and recent researches suggest the existence of a overlap between the signalling networks that control abiotic and biotic stress tolerance (Mauch-Mani & Mauch 2005). For instance, Xiong & Lang 2003 investigated a rice protein kinase that was activated by ABA, but if the protein kinase was not activated by ABA and suppressed it resulted in constitutive expression of PR-protein, increase in fungal *Magnaporthe grisea* resistance but

impaired the drought, salt and cold resistance (Xiong & Lang 2003). Even though it has been suggested that the abiotic signalling network controlled by ABA and the biotic signalling network controlled by SA, JA and ET may be interconnected at various levels, it is a very complex network and the analysis of this network is a difficult task (Mauch-Mani & Mauch 2005). Whether the rust-poplar interaction may involve interference of the plant hormone signalling network is not clear and will not be discussed further.

5. Conclusions

Based on the findings in this study, it may be concluded that leaf rust has an impact on frost resistance in poplars in the autumn. However, the rust does not seem to have any effects on frost resistance when exposed to relatively mild frost. But when exposed to -12°C, a significant difference was observed in frost damage between the non-infected plants and infected plants, the infected plants displaying more frost damage. No clear connection seemed to be between the origins of the clones used in this study i.e. inland or coastal clone, or between different susceptibility towards the rust. No significant correlation was observed between the rust severity and level of frost damage, indicating that the amount of rust per plant does not give any implications for the level of frost damage, rather that only the presence of the rust is enough for interfering with the frost resistance development of plants. The infection times did not seem to have impacts on the level of frost damage, i.e. early infection did not lead to more frost damage or late infection to less frost damage. That could however coincide with the result that no correlation is between the rust severity and frost damage, thus, it may be wondered that the infection time is not important in relation to increased frost damage, only the fact that the plants are infected. The results did imply that some plants were increasing its frost resistance in spite of the rust infection, e.g. the inland clones exhibited an increase in frost resistance in the end of September, hence less frost damage, if infected in July. Nevertheless, in order to conclude that the frost resistance development is only delayed when plants are infected with leaf rust, further investigations would be needed. If freezing tests could have proceeded, it might have been possible to see whether all the plants would have exhibited the same pattern, i.e. if coastal clones would have increased their frost resistance as well as the plants infected in August. It should also be noted that young seedlings were used in this study, and often it is considered that seedlings and young plants may differ in frost resistance from adult trees, therefore care should be taken when results from artificial laboratory freezing tests with young plants are compared to older trees out, perhaps more stable trees, in the field (Sakai &

Larcher 1987). However, the present study gives supporting evidence that poplars infected with leaf rust may become more susceptible to frost damage in the autumn.

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