Eurostars E!4247 QFORS

Quality management for vital forest tree seedlings

Final report

Project no: P33854-1

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INTRODUCTION

The research project “Quality management for vital forest tree seedlings” was divided into three phases, a definition phase, a research phase and a development phase. In the definition phase an inventory was made to identify the important control points in nursery practice. In the research phase several biological trials were conducted in order to establish the correlation between gene expression and physiological characteristics. The third and concluding phase was aimed at developing prototype molecular tests that can aid in the decisions nursery managers have to take.

Definition phase

In this phase a questionnaire was made and sent to over 100 nurseries that are growing coniferous species such as *Pinus sylvestris*, *Picea abies* and *Pseudotsuga menziesii* in the Nordic countries, The Netherlands, USA, and Canada. The inventory was aimed at defining the decision criteria that are most commonly used in forestry nurseries. The response was 23%. According to the questionnaire 84,62% use the frozen storage method to store seedlings during winter. Therefore development of a vitality test is desirable. Almost 70% perform a Long Night (LN) treatment. Since the effectiveness of a LN treatment is difficult to determine, would a molecular test be valuable. From all pathogen infections that can occur in the nursery, *Botrytis cinerea* turned out to be the largest infector (34,29%).

Research phase

In the research phase the project focused on three areas of interest for nursery managers i.e. 1) freezing tolerance and storability 2) longnight (LN) treatment and 3) fungal infections. All seedlings used in the physiological experiments were produced at Svenska Skogsplantors nursery Lugnet in Bålsta, Sweden. In addition to the experiments performed in Garpenberg and Vassbo, seedlings were treated at Lugnets nursery to be able to evaluate the treatment effects under practical circumstances. The staff at Lugnet has also been deeply involved in the research phase e.g. with sampling, planning and organizing meetings.

Freezing tolerance and storability

A great portion of forest tree seedlings are put into frozen storage in late autumn to prevent seedlings from damage caused by low and fluctuating winter temperatures, fungae or feeding by rodents. Another advantage with the artificial storage environment is that seedlings can be ready for dispatch at any chosen time. For the seedlings to be able to survive in storage it is crucial that they are in such a state that they can cope with the dark and cold environment in the storage for as much as 6 months. The storability of the seedlings can be estimated from autumn until late December by measuring the freezing tolerance and also by measuring the gene expression of frost tolerance genes. The research phase of this Eurostar project aimed at investigating if seedlings are storable in late winter/early spring

and if there is a possibility to evaluate the vitality of stored seedlings with gene tests. Validation of existing gene test for establishing storability was also performed.

Material and methods

Two trials concerning freezing tolerance and storability were conducted, one in 2008-09 and one in 2010-11. In the first trial both 1-year-old pine and spruce seedlings were included whereas only 1.5-year-old spruce seedlings were used in the latter.

In the first trial, the freezing tolerance of seedlings stored outdoors was tested biweekly, from week 39 until the end of the year using the $SEL_{diff-25}$ method and the gene test ColdNSure. Thereafter they were tested every fourth week. In the second trial, outdoor stored seedlings were tested almost every week from week 37 until week 49. Next to collecting samples for gene expression analysis, seedlings were put into frozen storage (-4 °C). The freezing tolerance of the seedlings put into frozen storage in the first trial was tested in February, March and April and samples for gene expression analysis were collected in January (week 3) and April (week 18). Since the second trial ended in the middle of a "normal" storage period, samples for gene expression analysis from frozen stored seedlings were taken at the end of the project (January, week 3). At the end of the storage period (May/June in the first trial and January in the second trial) the vitality of the seedlings was evaluated in a 3-week root growth capacity test.

Pine results

Storability measurements

The freezing tolerance of the pine seedlings, measured as $SEL_{diff-25}$, increased from September until November when it stabilized (from week 45) at a level at which seedlings are considered to be storable (Figure 1). According to the gene-test ColdNSure and the freezing test ($SEL_{diff-25}$), seedlings were clearly storable at week 45. Unfortunately it was found that gene samples collected on Whatman cards could not be analyzed after long time storage. Therefore ColdNsure results are not available for some of the samples (week 3,7,10,14). In February the pine seedlings, irrespective of storage environment, started to lose freezing tolerance (Figure 1).

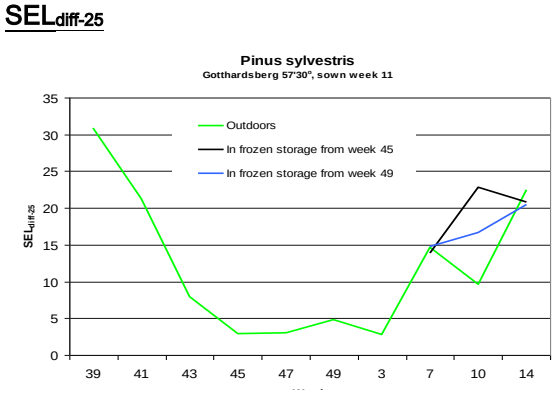

Figure 1. Freezing tolerance, measured as $SEL_{diff-25}$, for 1-year-old pine seedlings from week 39 until week 14.

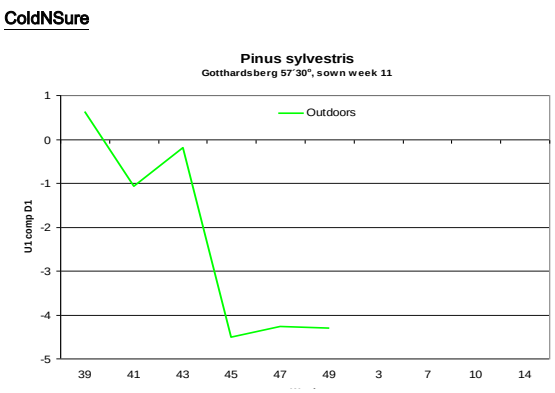

Figure 2. Freezing tolerance, measured as gene expression, for 1-year-old pine seedlings from week 39 until week 49.

Frost tolerance indicators

In order to identify more frost tolerance indicators that could reinforce the existing ColdNsure test NSure performed microarray analysis. Five genes were identified that were upregulated when seedlings become frost tolerant (Figure 3). As expected gene expression declined in March (week 14) when the seedlings deharden. In addition nineteen genes were identified that are downregulated when seedlings develop frost tolerance (Figure 3). In the near future these genes will be validated.

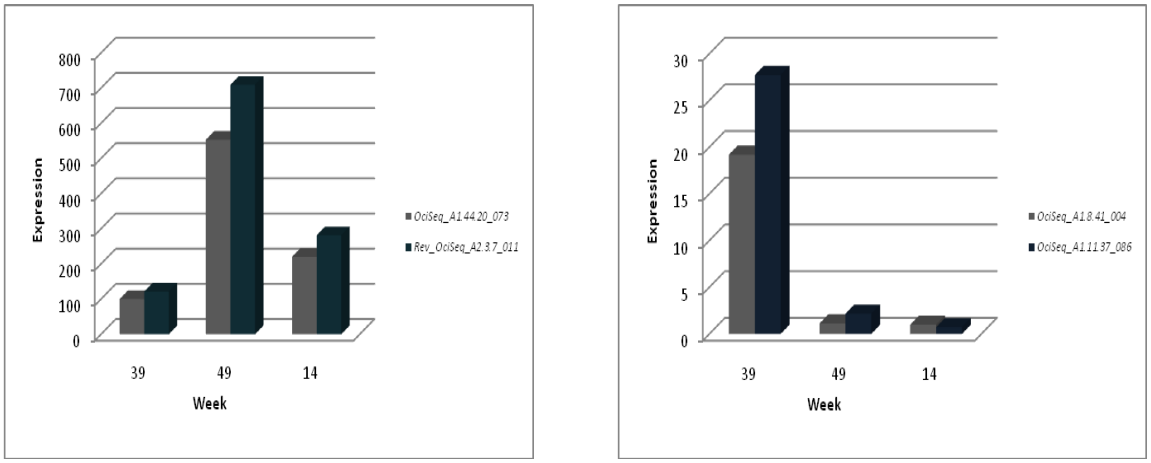

Figure 3. Freezing tolerance, measured as gene expression from buds from 1-year-old pine seedlings grown outdoors. Left, two genes that are upregulated when seedlings become frost tolerant. Right, two genes that are downregulated.

Post storage results

The regrowth tests showed that pine seedlings put into frozen storage in week 39 and 41 did not survive the storage period (Figure 4). Some of the seedlings that were put into storage in week 43 were also damaged. Storage in week 45 to 49 did not cause any damages to the seedlings whereas seedlings stored in Jan/Feb showed minor damages and reduced root growth capacity.

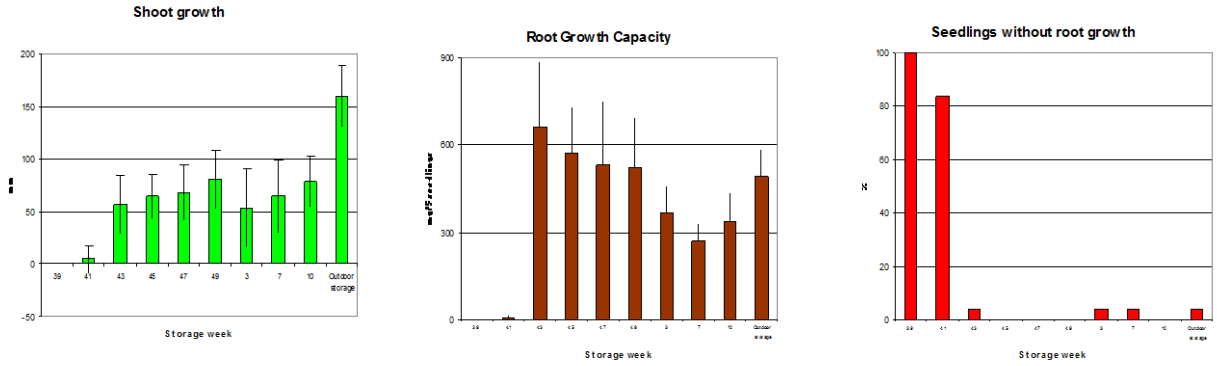

Figure 4. Shoot growth, mm, (left), root growth, mg/5 seedlings, (mid) and seedlings without root growth, %, (right) for 1-year-old pine seedlings in a RGC-test May 18 – June 8 2009. N=25 (left and right) N=5 (mid)

Vitality indicators

Microarray analysis was performed to identify indicators that can say something about the vitality of the seedlings during storage. In total eight genes (4 up / 4 down) were found that can be used to determine the vitality of pine seedlings during the whole storage period (Figure 5). Unfortunately, the difference in level of expression between not fully frost tolerant (week 39,41) and frost tolerant (week 45, 49) seedlings is not huge when compared to the results described below for spruce. However the combination of down- and upregulating genes would help to distinguish the status of the seedlings. More vitality indicators could be revealed with help of Next Generation Sequencing (which turned out to be successful for spruce), but due to time and manpower this was not possible. In the near future a validation experiment should be performed to confirm the found indicators.

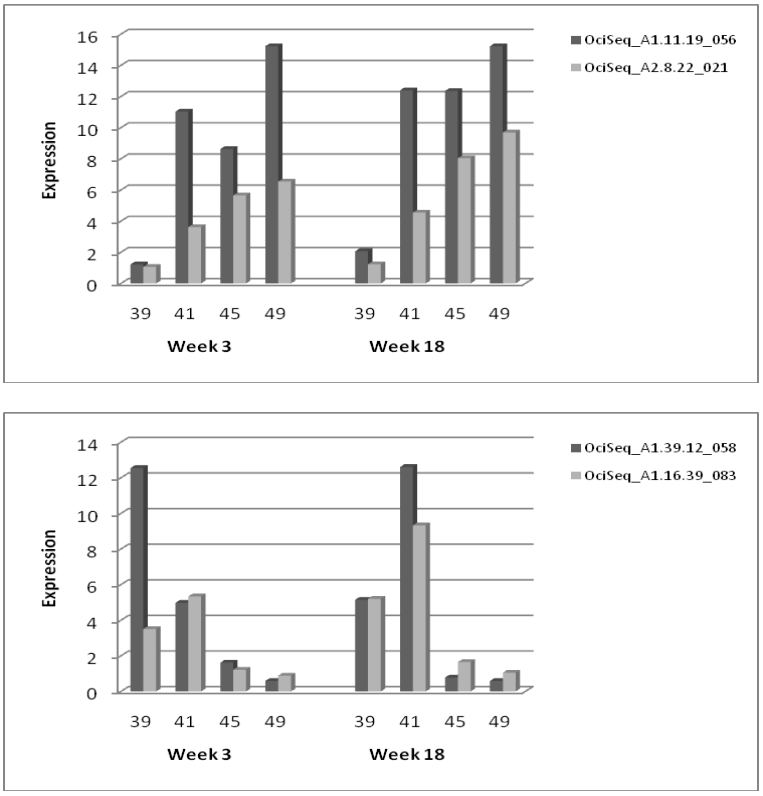

Figure 5. Vitality indicators, measured as gene expression from buds from 1-year-old pine seed-lings during frozen storage. Top, two upregulated genes. Bottom, two down regulated genes.

Spruce results

Storability measurements

In the first trial (2008-09) the seedlings were storable, according both to $SEL_{diff-25}$ (Figure 6) and ColdNSure (Figure 7) already in week 41. Freezing tolerance of the spruce seedlings, measured as $SEL_{diff-25}$, stayed high throughout the observation period (Figure 6).

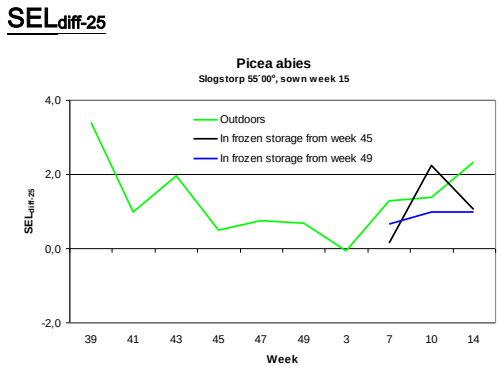

Figure 6. Freezing tolerance, measured as $SEL_{diff-25}$, for 1-year-old spruce seedlings from week 39 until week 14. From week 41 seedlings were storable

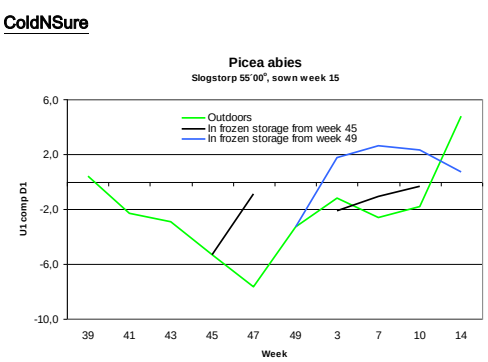

Figure 7. Freezing tolerance, measured as gene expression, for 1-year-old spruce seedlings from week 39 until week 14. From week 41 seedlings were storable.

Both the ColdNsure test as the $SEL_{diff-25}$ method showed dehardening in late winter/early spring for seedlings stored outdoors as well as in frozen storage. The gene test seems to give a more detailed picture of this process.

In the second trial the freezing tolerance of the spruce seedlings, measured as $SEL_{diff-25}$, decreased from week 37 to week 43 and stayed at this level throughout the observation period (Figure 8). According to this measurement the seedlings were storable from week 43. The gene-test ColdNSure was analysed by NSure. According to this measurement the spruce seedlings were storable from week 42 (Figure 9).

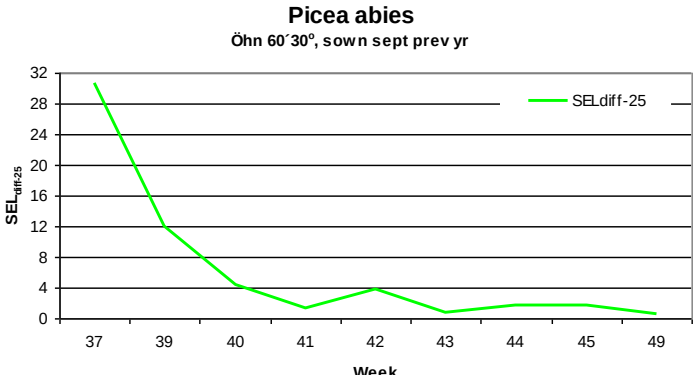

Figure 8. Freezing tolerance, measured as $SEL_{diff-25}$, for 1.5-year-old spruce seedlings from week 37 until week 49. From week 43 seedlings were storable.

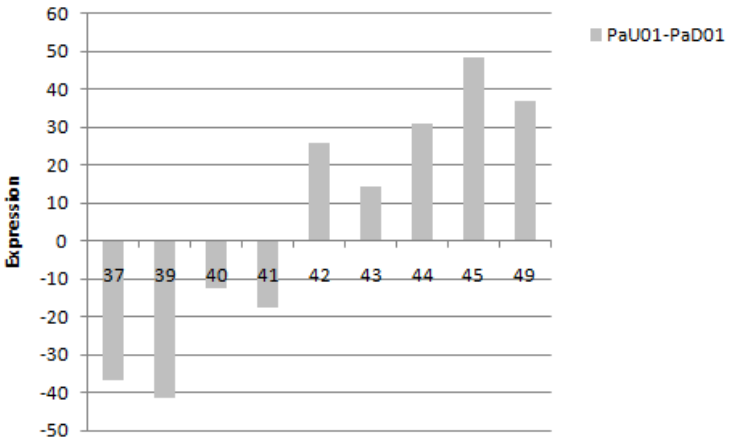

Figure 9. Freezing tolerance, measured as gene expression, for 1,5-year-old spruce seedlings from week 37 until week 49. From week 42 seedlings were storable.

Frost tolerance indicators

In order to identify more frost tolerance indicators that could reinforce the existing ColdNsure test NSure performed microarray analysis and Next Generation Sequencing (NGS) on non frost tolerant and frost tolerant seedlings from the first trial. About 40 genes were analysed that were upregulated during hardening and three genes that were downregulated (Figure 10). In week 14, the spruce seedlings, started to lose their freezing tolerance (Figure 10). NGS revealed more potential candidates than the ones we have analysed, showing that this is powerful technique to identify indicators when compared to the microarray analysis.

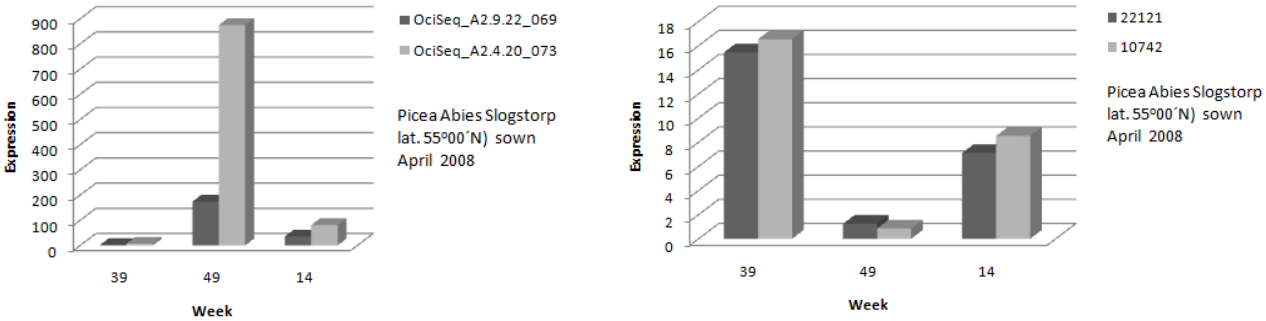

Figure 10. Frost tolerance indicators, measured as gene expression from buds from 1-year-old spruce seedlings outdoor. Left, two upregulated genes. Right, two down regulated genes.

Most of the candidate frost tolerance genes picked up in the first trial (31 in total) were validated in a second trial. Most of these genes showed a similar pattern, but six upregulated genes were discarded. In case for forteen upregulated genes a clear switch is observed between week 41 and 42 (Figure 11) correnspondingto the ColdNsure assay, which demonstrated that seedlings were frost tolerant from week 42 (Figure 9). The remaining eight upregulated genes are induced upon hardening, but the expression goes up slowly and therefore it is more difficult to distuinguish non frost tolerant seedlings (week 41) from frost tolerant seedlings (week 42, data not shown). Like in the first trial, the three downregulated genes are downregulated when seedlings become frost tolerant (Figure 11). The identification of strong up- and downregulating genes can be used in the future to reinforce the current ColdNsure test.

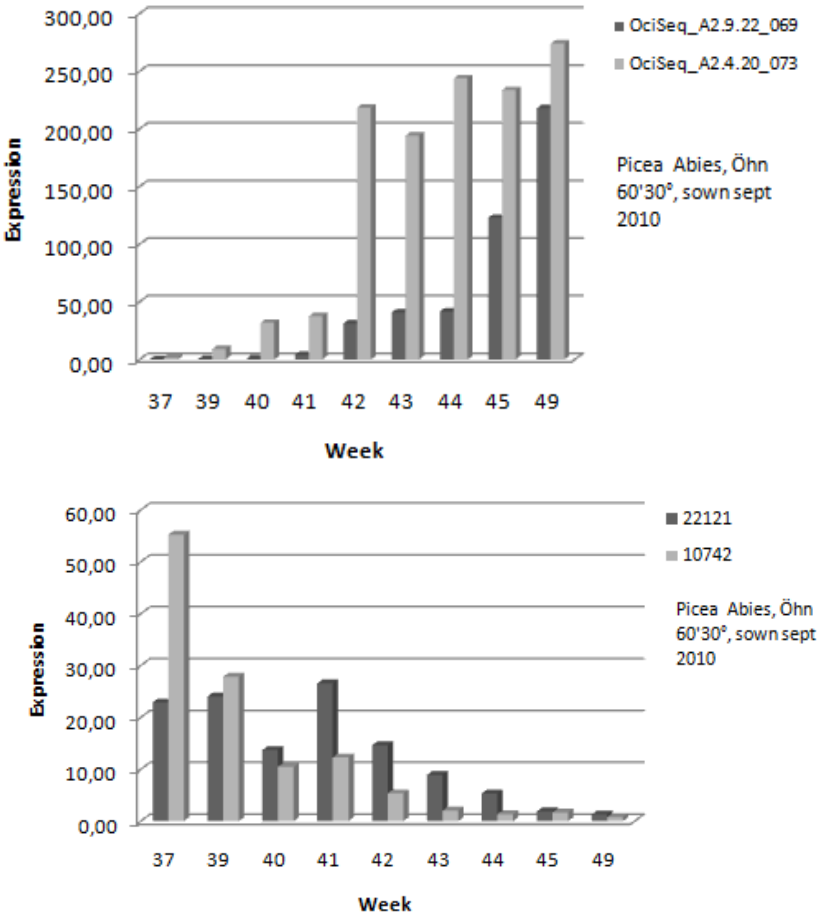

Figure 11. Validation of frost tolerance indicators, measured as gene expression from buds from 1,5-year-old spruce seedlings outdoor. Top, two upregulated genes Bottom, two downregulated genes. All four genes are also depicted in figure 10. From week 42 seedlings were frost tolerant according to the ColdNsure test.

Post storage results

The regrowth tests in the first trial showed that 40 % of the spruce seedlings put into frozen storage in week 39 did not survive the storage period (Figure 12). The root growth capacity after storage for seedlings put into storage in week 7 and 10 was considerably lower than for seedlings put into storage at an earlier date (Figure 12).

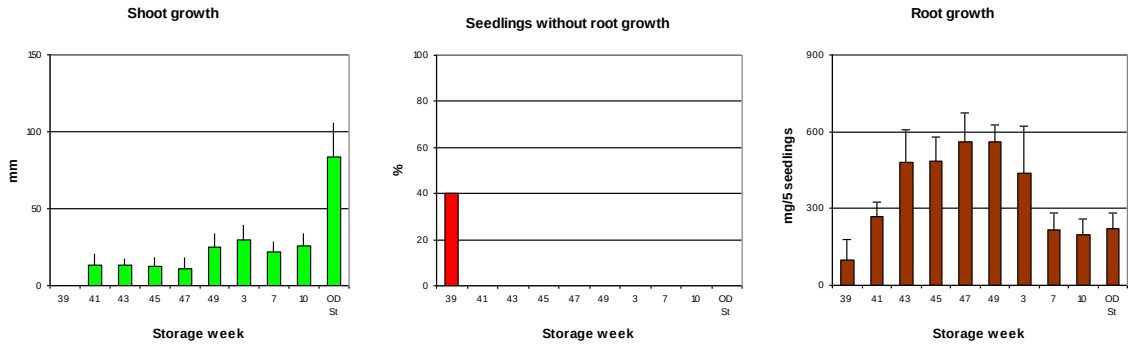

Figure 12. Shoot growth, mm, (left), root growth, mg/5 seedlings, (mid) and seedlings without root growth, %, (right) for 1-year-old spruce seedlings in a RGC-test May 18 – June 8 2009. N=25 (left and mid) N=5 (right)

The regrowth tests in the second trial showed that all seedlings put into storage in week 37 and one third of the spruce seedlings put into frozen storage in week 39 did not survive the storage period (Figure 13). Seedlings put into storage before week 42 did not produce a new shoot in the growth test. The main reason for lack of shoot growth was that de apical bud was damaged (Figure 14). These results correspond with the ColdNsure results. According to the SELdiff method, seedlings were storable from week 43, which is late when compared to the physiological results.

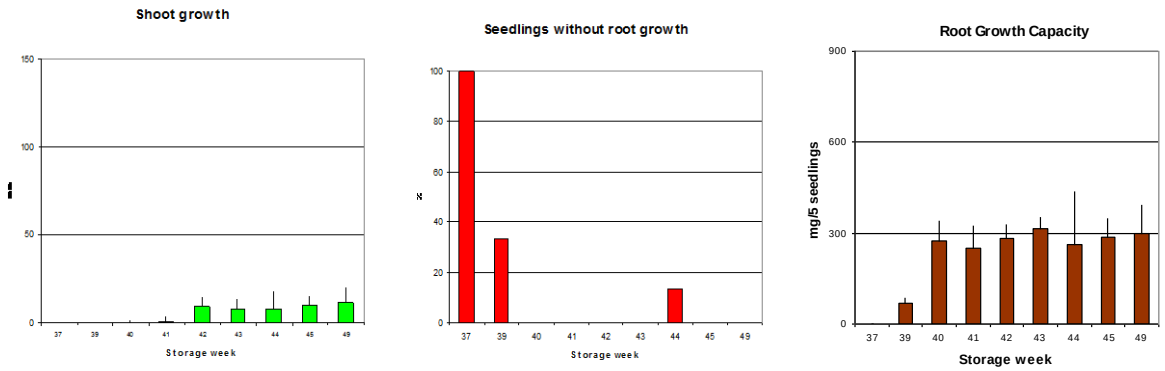

Figure 13. Shoot growth, mm, (left), root growth, mg/5 seedlings, (mid) and seedlings without root growth, %, (right) for 1-year-old spruce seedlings in a RGC-test Jan 13 – Feb 3 2011. N=25 (left and mid) N=5 (right)

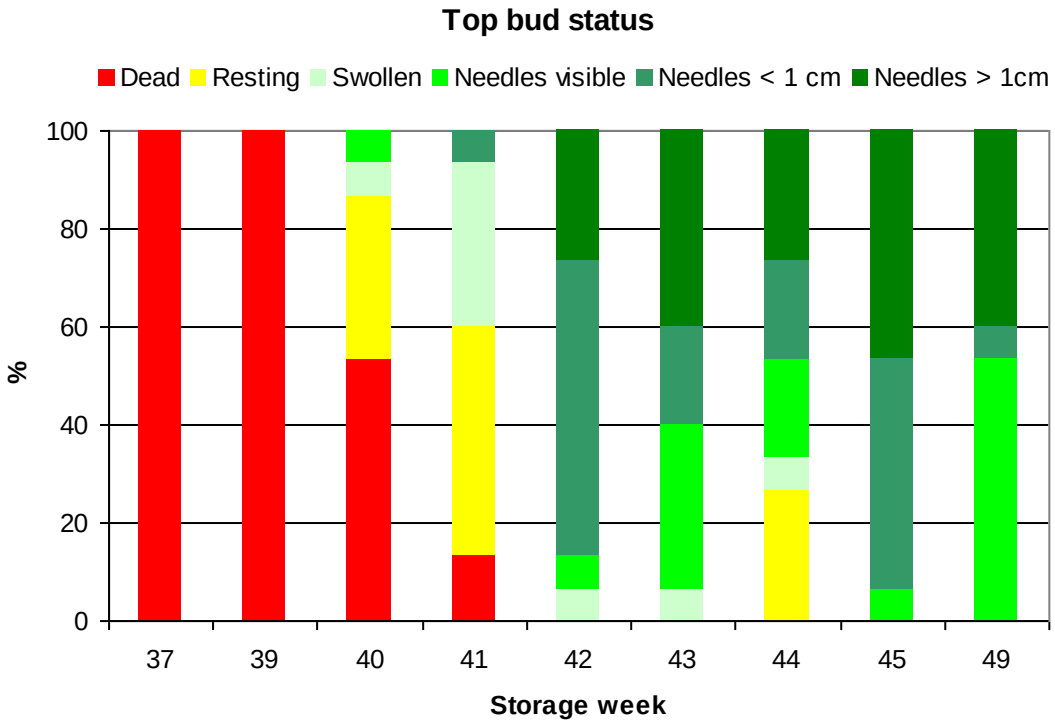

Figure 14. Status of the apical (top) bud of 1.5-year-old spruce seedlings at the end of a 3-week regrowth test performed in Jan 13 – Feb 3 2011. N=25

Vitality indicators

In the first trial seedlings were sampled at week 3 and week 18 during frozen storage. In order to identify vitality genes, microarray analysis and NGS sequencing was performed on non frost tolerant (week 39) and tolerant (week 45) seedlings. In total thirty-one genes turned out to be interesting (Figure 15). It should be noted that NGS revealed more potential candidates, but only a selection has been analysed. Due to dehardening, expression of some vitality indicators dropped during storage (Figure 15). However, even at the end of the storage period affected seedlings (week 39) could be discriminated from healthy frost tolerant seedlings (week 41 and up).

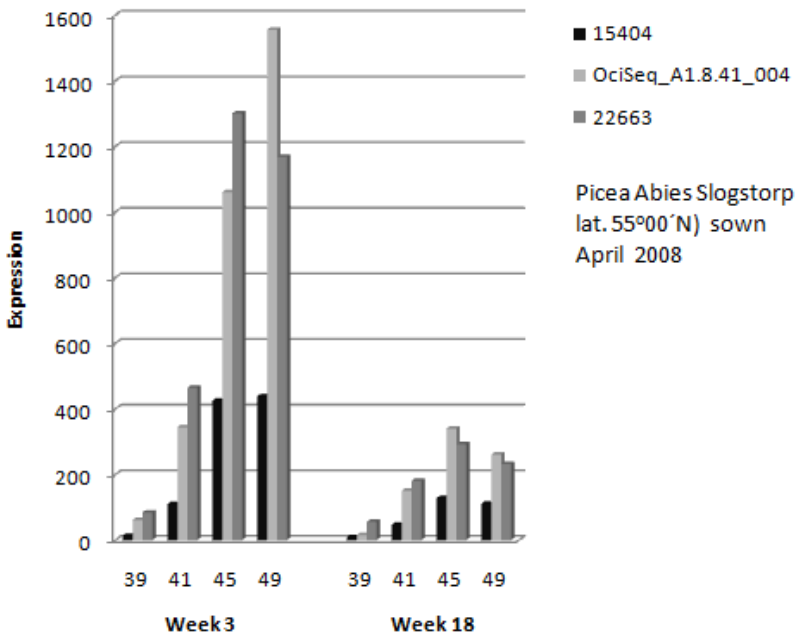

Figure 15. Vitality indicators, measured as gene expression from buds from 1-year-old spruce seedlings during frozen storage in week 3 and 18. From week 39 seedlings were frost tolerant according to the ColdNsure test.

Twenty eight vitality indicators originating from the first trial were validated in a second trial. Since the second trial was performed just before the end of the project, only samples for gene expression analysis from frozen stored seedlings were taken at the end of the trial (week 3). A very clear switch between non frost tolerant (week 37 – week 41) and frost tolerant seedlings (week 42 – week 49) was observed for seventeen genes (Figure 16). The remaining eleven genes showed little or no difference. Remarkably, we encountered differences in expression levels of some genes which is probably due to difference in provenance. In the future a third trial will be performed with different provenances to check whether the difference in expression levels is due to the provenance.

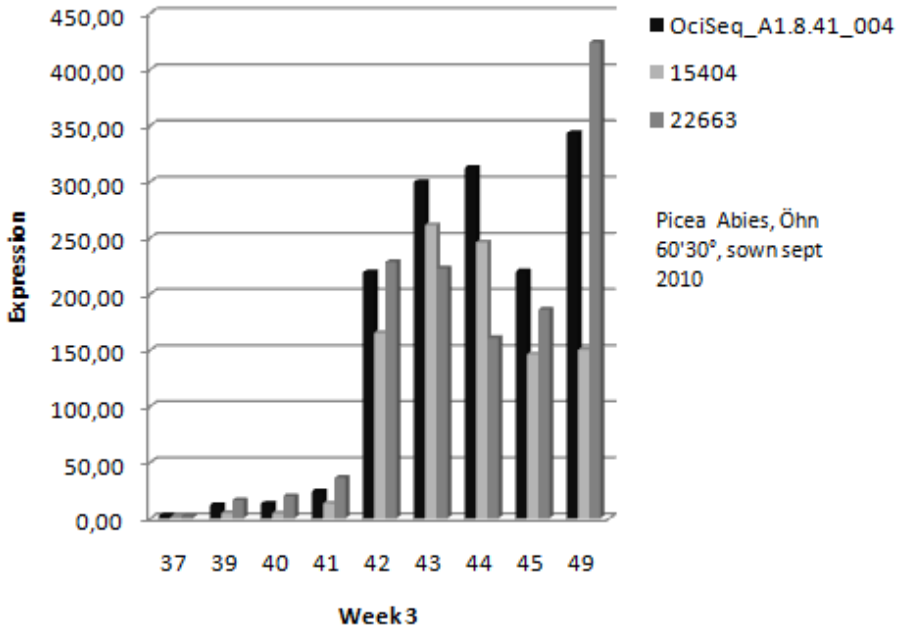

Figure 16. Vitality indicators, measured as gene expression from buds from 1,5-year-old spruce seedlings during frozen storage in week 3. All three genes are also depicted in figure 15. Seedlings were frost tolerant from week 42 according to the ColdNsure test.

Conclusions from freezing tolerance/vitality trials

In the first trial Dalarna Research Centre produced seedlings at different levels of freezing tolerance and thereby different levels of storability. This was also achieved in the second trial for spruce. Thereby Dalarna Research Centre delivered tissues samples from a range of different storability levels for both spruce and pine for further gene expression analysis. These stored seedlings showed a broad range of vitality (dead, damaged, totally vital).

NSure identified for spruce multiple frost tolerance and vitality indicators which can be used to reinforce the current ColdNSure test and to develop a prototype vitality test. Before lancing the prototype vitality test, NSure wants to perform a last validation with multiple spruce provenances. In case for pine several frost tolerance indicators were identified that can be used to support the ColdNSure test. For pine a number of vitality indicators has been identified, but NSure aims to find additional indicators. The NGS method would be recommendable. The current indicators should be validated in the future.

LN-treatment pine

For Scots pine the long night (LN) treatment on open land is commonly used to stop shoot elongation of early sown pine provenances in order to form a short and sturdy seedling with qualities reminding of a two-year-old seedling. LN-treatment is usually started in May and continues for three weeks. Today there is no practical way to measure the response of the seedlings and the nurseries are forced to use

extra long treatment to be sure to get an effect. Much would be gained if LN-treatments could be adjusted to the response of the seedling. This experiment with LN-treatment of pine was done in order to map the reactions of LN-treatment on bud set and growth. Parallel with morphological measurements Whatmancard samples were taken for analyses of gene expression to figure out if this could be a tool for prediction of seedling status during LN-treatment. The results from morphological measurements are presented below. These will be used to select the critical samples to analyse for gene expression.

Material and Methods

The trial concerning long night treatment on pine was conducted in 2009. The LN-treatment was applied on seedlings (Gotthardsberg, 57°30´) sown in March the same year started on May 8. Different lengths, 0, 7, 14, 21 and 28 days, of LN-treatment were tested. Each treatment consisted of approximately 1 050 seedlings in 10 container units. Height and bud status was recorded at start and then weekly for the whole period of 4 weeks. 30 seedlings were taken at day 14, 21 and 28 from both the control environment outdoors and the LN-treatment and put in growth forcing climate in a greenhouse. At day 54 the height and bud status of these seedlings were noted. Whatmancard samples (consisting of material from 15 seedlings) were taken from both treatments at start and then twice weekly during the 4 week test period. Buds were sampled for later analysis of genes at the start of the LN-treatment and after 2 and 4 weeks of LN-treatment.

Results

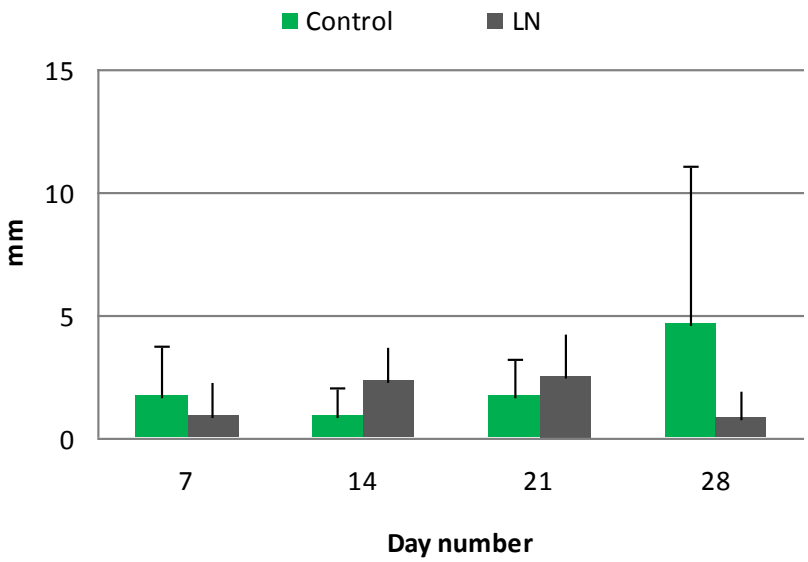

Figure 17. Height increment, mm, for pine seedlings (provenance Gotthardsberg, 57°30') during LN-treatment indoors in May and for control seedlings grown outdoors. N= 30

Height increment had ceased at day 28 of the LN-treatment (Figure 17). Seedlings grown outdoors (day 14 and 21) with normal day length did not increase their height as much as the seedlings grown indoors at long nights. This was probably due to a lower outdoor temperature.

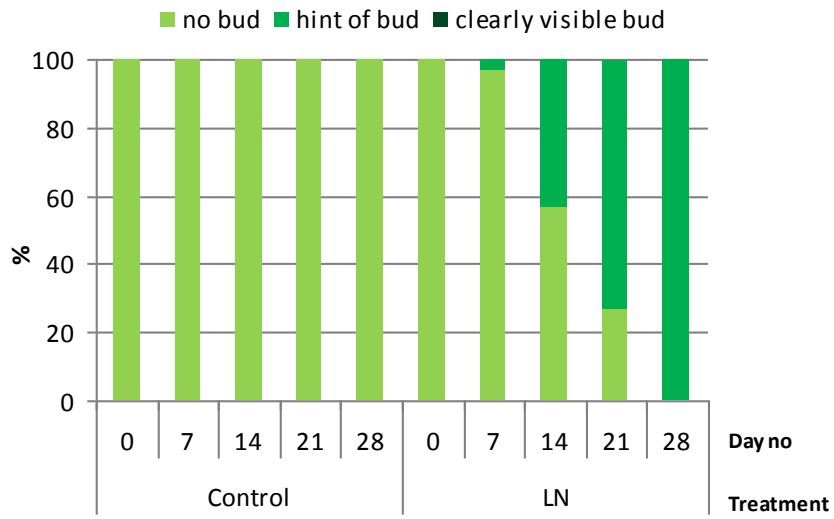

Figure 18. Bud status subjectively classified as a) no bud visible/tangible b) hint of visible/tangible bud and c) clearly visible/tangible bud for pine seedlings (provenance Gotthardsberg, 57°30') during LN-treatment indoors in May and for control seedlings grown outdoors. N= 30

Buds were initiated and subjectively visible/tangible already after 14 days on approximately 40 % of pine seedlings subjected to long nights (Figure 18). After 3 weeks almost 80 % of the seedlings had tangible buds and after 4 weeks all the seedlings had formed tangible buds.

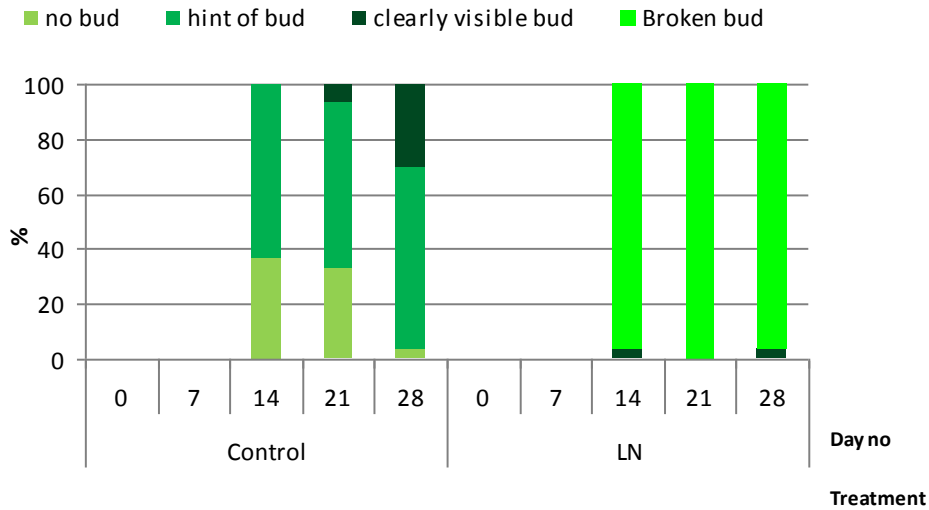

Figure 19. Bud status subjectively classified at day 54 as a) no bud visible/tangible b) hint of visible/tangible bud and c) clearly visible/tangible bud for pine seedlings (provenance Gotthardsberg, 57°30'). The seedlings had either been LN-treated in greenhouse for 14, 21, 28 days and then subjected to growth promoting climate in greenhouse or grown outdoors for 14, 21 and 28 days before being subjected to growth promoting climate in greenhouse (control). N= 30

Seedlings that were LN-treated for 2 to 4 weeks did initiate bud formation (Fig. 18) and after exposure to growth promoting conditions for 26 to 40 days these buds were broken and the seedlings were actively growing (Fig. 19). The control seedlings that had been cultivated outdoors under ambient day lengths and put into growth promoting conditions in greenhouse, at the same time as the LN-treated seedlings, had started to initiate buds at day 54 (Fig. 19).

Conclusions from LN treatment of pine

In this trial, by treating pine seedlings with different periods of long nights we were able to deliver tissue samples showing successive decrease of growth and increasing frequency of buds.
Although the physiological results looked promising, NSure decided not to continue due to the lack of several control bud samples making it difficult to find specific LN indicators. In the future NSure together with Dalarna Research Station aim to apply for a follow-up project.

LN-treatment spruce

LN-treatment of spruce seedlings is used to speed up dormancy induction and make them frost tolerant and ready for storage at an earlier date in the autumn. The practical procedure is to cover the seedlings with black curtains for approximately 16 hours per day during 3-4 weeks. This procedure is used for all batches irrespective of provenance. Much would be gained if LN treatments could be adjusted to the response of the seedling. The response is dependent on several factors such as quality of the LN equipment, duration of treatment, provenance of the seedlings, time of the year etc. Even though treatment duration is long seedlings often resume growth. This may cause severe problems later in the production chain. Logistically there is yet another problem correlated to the LN treatment; since numerous batches need the treatment at the same time nurseries experience logistical problems. A better utilization of the existing equipment would improve nursery logistics radically.
Much would be gained if LN treatments could be adjusted to the response of the seedling. This experiment with LN-treatment of spruce was done in order to map the reactions of LN-treatment on bud set and growth. Parallel with morphological measurements Whatmancard samples were taken for analyses of gene expression. This is done to figure out if gene expressions could predict seedling status during LN-treatment. Below, results from morphological measurements are presented, these are the starting point for analyses of gene expression.

Material and Methods

The two trials concerning long night treatment on spruce were conducted in 2009, starting July 24 and Aug 31. The first LN-treatment was applied on seedlings (Maglehem, 54°00') sown in July the previous year and the second LN-treatment was applied on seedlings (Runesten, 54°00') sown in April the same year. Different lengths, 0, 7, 14, 21 and 28 days, of LN-treatment were tested. Each treatment consisted of approximately 1 050 seedlings in 10 container units. Height and bud status was recorded at start and then weekly for the whole period of 4 weeks. 30 seedlings were taken at day 14, 21 and

28 from both the control environment outdoors and the LN-treatment and put in growth forcing climate in a greenhouse. At day 60 the height and bud status of these seedlings were noted. Whatmancard samples (consisting of material from 15 seedlings) were taken from both treatments at start and then twice weekly during the 4 week test period. Buds were sampled for later analysis of genes at the start of the LN-treatment and after 2 and 4 weeks of LN-treatment.

Results

Height increment for spruce seedlings subjected to long nights in late July/early August had ceased after 3 weeks LN-treatment. The spruce seedlings subjected to ambient day lengths were still actively growing at this time of the year (Fig. 20). Height increment for seedlings subjected to long nights in September did almost cease already after 2 weeks LN-treatment. The height growth for the seedlings subjected to ambient day lengths outdoors did also decrease at this time of the year and had almost stopped (Fig 21).

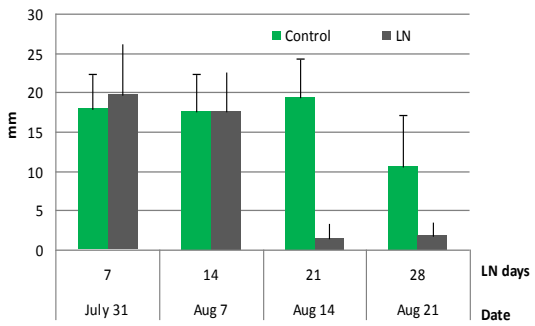

Figure 20. Height increment, mm, for 1-year-old and 15 cm high spruce seedlings (provenance Maglehem, 54°00') during LN-treatment performed July 24 – August 21 indoors and for control seedlings grown outdoors. N= 30

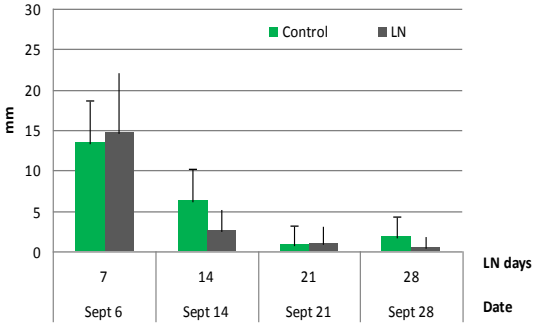

Figure 21. Height increment, mm, for 5-month-old and 25-cm-high spruce seedlings (provenance Runesten, 54°00') during LN-treatment performed August 31 – September 28 indoors and for control seedlings grown outdoors. N= 30

Buds were initiated and subjectively visible/tangible already after 7 days for approximately 60 % of the spruce seedlings that were subjected to long nights in late July/early August (Fig 22, top left). After 3 weeks LN-treatment almost all of these seedlings had tangible buds and after 4 weeks almost all the seedlings had formed clearly visible buds (Fig 22, top left). Seedlings subjected to ambient day lengths outdoors (Fig 22, bottom left) had also started bud formation at this time of the year but not a single one of the control seedlings had formed clearly visible buds and only approximately 60% of these seedlings had tangible buds in mid-August, i.e day 28 (fig 22, bottom left).

For the seedlings subjected to long nights in September the buds were initiated and subjectively visible/tangible after 14 days for approximately 70 % of spruce seedlings (Fig 22, top right). After 4

weeks all these seedlings had formed visible/tangible buds but not one of the seedlings had yet formed clearly visible buds (Fig 22, top right). Seedlings subjected to ambient day lengths outdoors at this time of the year, late September, had also started bud formation, but only 60% of these seedlings had tangible buds (Fig 22, bottom right).

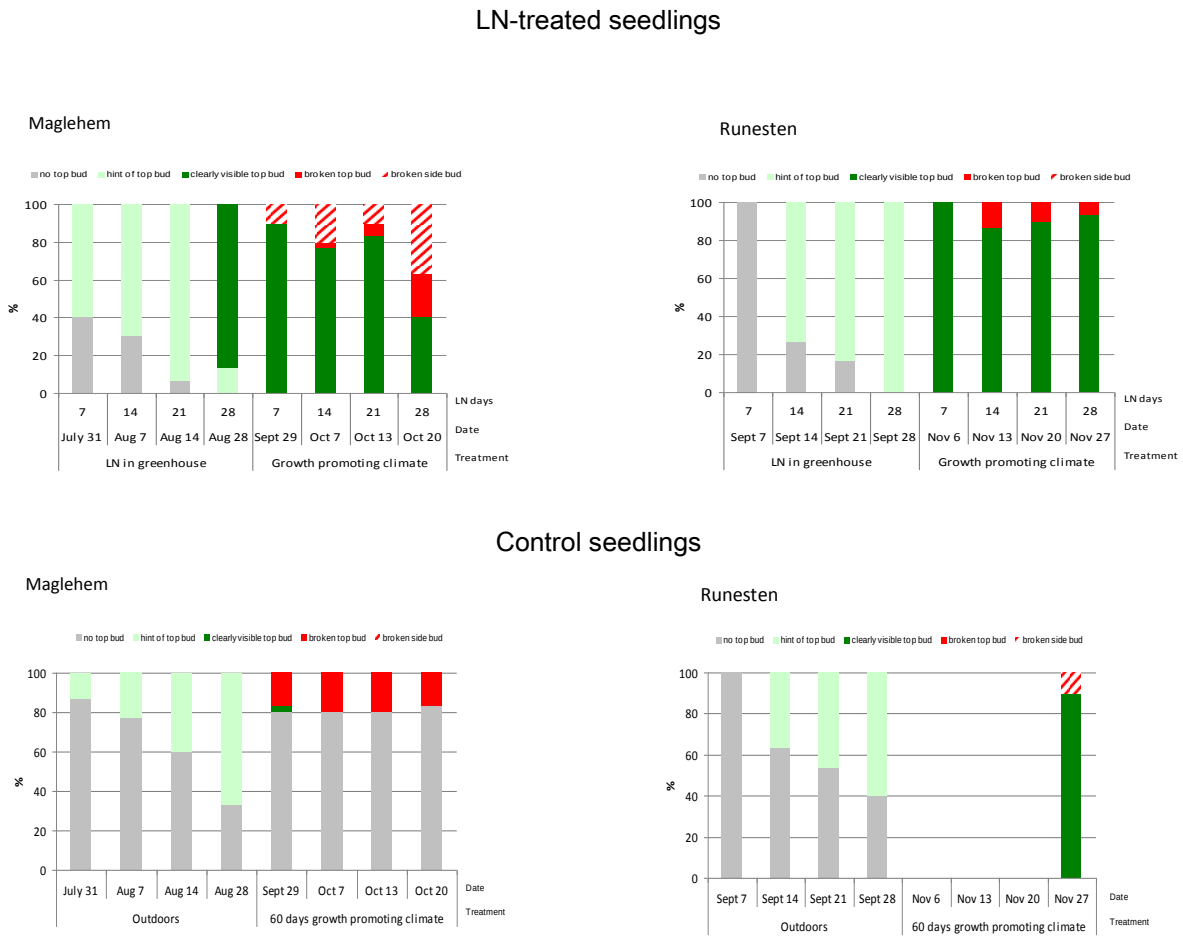

Figure 22. Bud status subjectively classified as a) no top bud tangible b) hint of tangible top bud c) clearly tangible top bud d) broken top bud e) broken side bud for:

Top left: 1-year-old and 15-cm-high spruce seedlings (prov Maglehem, 54°00′) during LN-treatment indoors in August and for the same kind of seedlings LN treated for stated number of days and then put into growth promoting climate in greenhouse for 60 days. N=30

Top right: for 5-month-old and 25-cm-high spruce seedlings (prov Runesten, 54°00′) during LN-treatment indoors in September and for the same kind of seedlings LN treated for stated number of days and then put into growth promoting climate in greenhouse for 60 days. N=30

Bottom left: for 1-year-old and 15-cm-high spruce seedlings (prov Maglehem, 54°00′) grown outdoors and in growth promoting climate in greenhouse for 60 days. N=30

Bottom right: for 5-month-old and 25-cm-high spruce seedlings (prov Runesten, 54°00′) grown outdoors and in growth promoting climate in greenhouse for 60 days. N=30

LN indicators

In order to identify LN indicators, NGS was performed on Runesten seedlings that were LN-treated for 14 or 28 days. Quantification of the expression of the different genes was determined by counting the sequence tags (per contig, per RNA sample). The transcriptome of both samples were compared and the genes that were differentially expressed were reported. Over >500 genes were identified that were upregulated and a similar amount of down regulated genes.

From this list about 14 potential LN indicators were selected and gene expression was determined in all control and LN-treated seedlings from both provenances (Figures 23 & 24).

In case of Maglehem seven genes were highly expressed after a 4 week LN treatment, while expression in the control seedlings was low (Figure 23). Interestingly, genes that are used in the ColdNSure to predict frost tolerance (PaU01, PaU02 and PaU03) were also upregulated during a LN treatment. This demonstrates that a LN-treatment speed up the dormancy induction and frost tolerance. The expression patterns match with the height increment and bud status studies shown in figure 20 & 22.

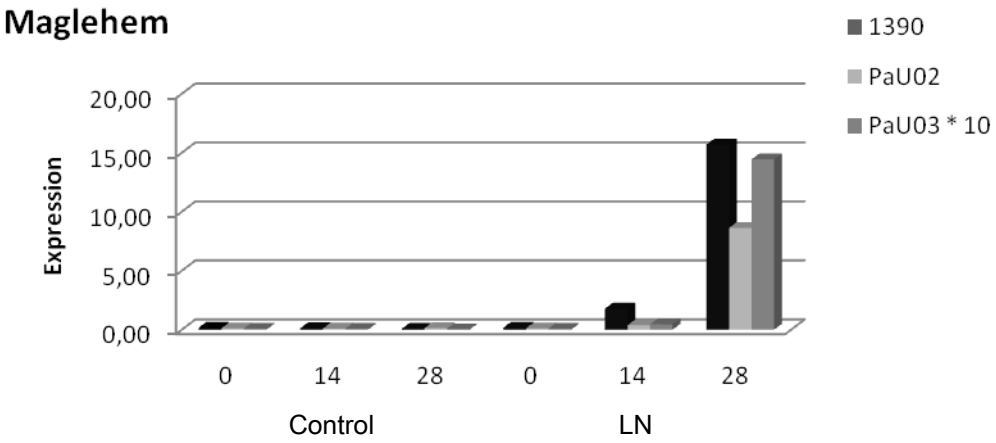

Figure 23. Effectiveness of a LN treatment, measured as gene expression from buds from Maglehem seedlings grown outdoor (control) or LN treated.

A LN treatment for Runesten in September turned out to be needless, ColdNSure genes and LN indicators were not only upregulated in LN-treated samples, but also in the control seedlings (Figure 24). However looking at the expression of *PaU03* level it seems that 2 week LN-treated samples are a bit further than the control seedlings (Figure 24). The expression patterns match with the height increment and bud status studies (Figure 21 & 22).

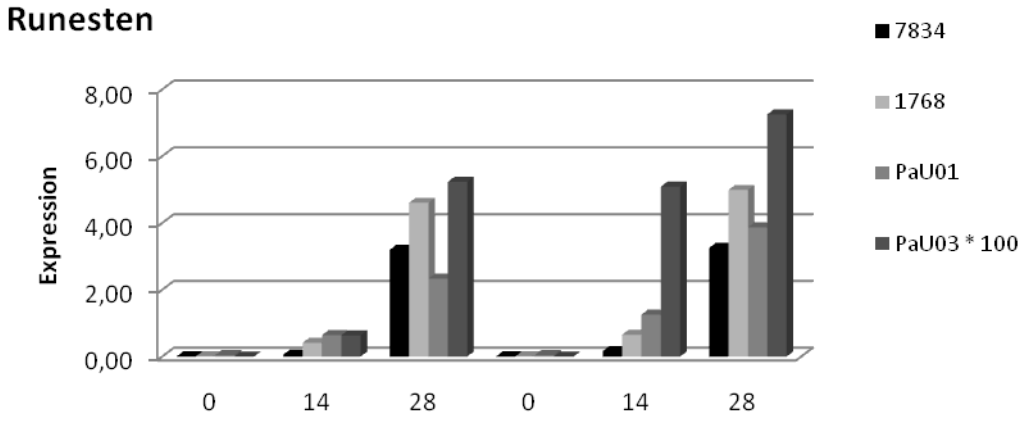

Figure 24. Effectiveness of a LN treatment, measured as gene expression from buds from Runesten seedlings grown outdoor (control) or LN treated.

Conclusion from LN treatment of spruce:

In this trial, by treating spruce seedlings with different periods of Long nights we were able to deliver tissue samples showing successive decrease of growth and increasing frequency of buds and buds of different stages of development. This gave the project a good opportunity to detect gene expression patterns. In addition to the project goals i.e producing tissue material of different physiological status we have discovered not earlier reported nor expected reactions from LN-treatment.

The seedlings used for the early LN treatment (Maglehem) had already started to form buds at the start of the LN-treatment and they also formed clearly visible buds within the 4-week-period that they were LN-treated. The younger seedlings of the other provenance Runesten that were LN-treated one month later (September) had not started bud initiation at the LN-start and did not form clearly visible buds. However all these seedlings had tangible buds after 4 weeks with long nights. The difference in morphology between the two provenances at the LN-start was not expected. Both provenances are from the same latitude and it would be expected that they react in the same way. The difference in age may have influenced the response to the LN-treatment.

Not expected, the buds formed during the LN-treatment turned out to be less stable the longer the LN-treatment lasted. This effect was most pronounced in the “early” LN treatment of the 1-year-old Maglehem seedlings. A strange result, since the height increment ceased only after a 21 day LN treatment and the longer the LN treatment the more buds are set. Furthermore, frost tolerance and dormancy related genes are upregulated from day 14. Most probably, the 28 day LN treated seedlings of Maglehem are not fully in rest yet and putting them in a strong growth promoting climate induces flushing. The seedlings that undergo a shorter LN treatment, still had to develop their bud before they even could flush. Therefore a lower percentage of the seedlings flushes in comparison to the 28 day LN treatment. The Runesten seedlings did flush a bit, but probably this is less, because it is later in the season. This is line with the fact that gene expression of frost tolerance and dormancy genes is not only induced in the LN treated seedlings but also in the control seedlings. Taken all this into account, we should question the regrowth experiment results.

The gene expression profiles match with the height increments studies performed in Maglehem and Runesten. The LN indicators show that a LN treatment of Maglehem during that time period was effective. In case for Runesten seedlings, which were treated in a later time period, a LN treatment turned out not be effective as the control seedlings were also becoming dormant/frost tolerant. This could also deduced from the expression data as the expression of genes related to dormancy/frost tolerance were also upregulated in the control seedlings.

Fungal infections

Lophodermium seditiosum infect pine seedlings during autumn, but the symptoms mostly do not appear until the year later. Species specific primers from the ribosomal ITS region that can detect presence of *Lophodermium* are available, but unfortunately it is not possible to distinguish between dead or alive material. In order to identify living occurrence inside needles it is necessary to grow out the fungi, a time consuming procedure.

Botrytis cinerea is a fungus that regularly infects seedlings in nurseries in especially humid circumstances such as in storage and during a LN-treatment. According to the inventory most nurseries encounter problems with Botrytis in comparison to other pathogens. A molecular test to detect this fungus would be of great of value.

Material and Methods Lophodermium seditiosum

Since it is difficult to inoculate seedlings artificially with *L. seditiosum* we used seedlings originating from a lot that contained infected seedlings from Lugnets nursery in 2008/09. In spring 2009 many of the thawed seedlings (Gotthardsberg 7707-31030) looked detrimental. From one storage box 20 seedlings were selected that looked detrimental and 20 seedlings that looked healthy. From each seedling approximately half of the needles were cut and homogenized in liquid nitrogen and stored in -80ºC. A part from the homogenised needles was used for PCR on genomic DNA with species specific primers. From each seedling 15 left over pieces were surface sterilised and checked on outgrow of the fungi. The mycelium growth was confirmed by PCT on genomic DNA using species specific primers.

Results Lophodermium seditiosum

Using PCR, DNA from *L. seditiosum* was found to be present in all 28 homogenised needles. PCR on genomic DNA isolated from the living mycelia growing out from the needles, revealed that *L. seditiosum* was present in all seedlings. However based on morphology of the mycelium *L. seditiosum* was identified in 9 of the 28 seedlings. As it is not possible to distinguish dead or alive *L. seditiosum* with the current used genomic PCR, NSure aimed to reveal genes that are expressed during the infection process. Nevertheless NSure decided not continue with this research due to the lack of scientific literature, lack of sequence information and the higher demand from the nurseries for a Botrytis test.

Material and methods Botrytis cinerea

Surface sterilized pine germlings were transferred and grown in micro-centrifuge tubes. To induce sensibility to infection half of the germlings were predisposed by stressing them 5h at 40º C, in dark while the others were left unstressed. Half of the stressed and half of the unstressed seedlings were inoculated with *Botrytis cinerea* while the rest served as un-inoculated controls. After inoculation each micro-centrifuge tube was transferred to 32 ml test tubes which were sealed to keep the humidity high. The tubes were kept in ambient daylight.

One week after inoculation, seedlings were controlled for infection under a stereomicroscope and the upper part of the germlings were homogenised in liquid nitrogen by pooling them by 5 based on their infection rate and treatment. This experiment was performed three times, but unfortunately seedlings from the second experiment thawed during the shipment to NSure. Experimental setups for validation tests and samples were taken in Svenska skogsplantors nursery Lugnet.

Results Botrytis cinerea

None of the un-inoculated germlings showed any signs of infection. The inoculated seedlings showed diverse levels of infection (from not visual infected seedlings to severe infected seedlings), providing good start material to identify indicators that can be used to detect active Botrytis in pine seedlings.

With help of a literature based study thirty-four indicators were selected of which thirteen pine specific and twenty-one Botrytis specific. The expression of these genes was studied in pine seedlings that were either inoculated with Botrytis or a control solution. At the end six Botrytis specific genes turned out to be successful to determine the infection rate. A criterion of 6 phases was developed to determine the stage of infection of the fungus. To validate this criterion a blind test was performed (Figure 25 and table 1). The non-inoculated seedlings were easily identified as well as the various stages of infection (Figure 25 and table 1). Subsequently Svenska Skogsplantor sampled on Whatman cards suspicious pine seedlings. Validation of these cards failed, because of low RNA yield. In the near future NSure will investigate the best manner of sampling. Next to measurements on RNA level, NSure is also able to detect Botrytis at DNA level which can be used as reinforcement.

Development phase and concluding remarks

The work concerning development of prototype molecular tests is mainly done by NSure. However both Dalarna University and SUAS were engaged in the work. The developmental phase has been somewhat delayed, since the research phase needed more work than expected and the experiments are season depended.

In case for spruce, a successful validation experiment has been performed to validate the identified frost tolerance and vitality genes. Multiple indicators were identified that can be used to either reinforce the existing ColdnSure test, but also for development of a vitality test. Coming season NSure wants to perform a second validation with more provenances in order to fine-tune the test.

The identified frost tolerance and vitality genes for pine still need to be validated. NSure together with Dalarna Research Institute aim to perform a validation next season.

Multiple LN indicators were identified in spruce that can be used to determine the effectiveness of a LN treatment, but they are not yet validated. Unfortunately due to lack of material, the search for LN indicators in pine was not pursued. In spruce and pine hardly any scientific research is performed to study the effect of a LN treatment, particularly not at molecular level. Therefore NSure together with Dalarna Research Station want to apply for a project. Within this project, we would able to develop the tests further.

The prototype test to detect Botrytis in pine seedlings needs to be optimized. NSure expects to do this with help of Svenska Skogsplantor.

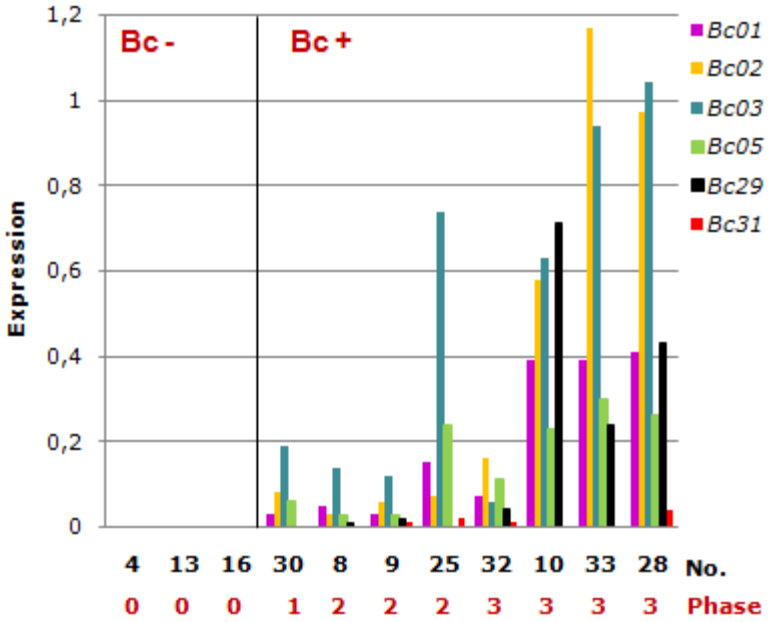

Figure 25. Blind test. Gene expression measured from pine seedlings inoculated with Botrytis (+) or a control solution (-). Background details of the samples (numbers depicted in black) can be found in table 1. The criterion is constituted of 6 phases. Phase 0 defines not infected, phase 1 potentially infected, phase 3-6 infected with an increasing rate.

Table 1. Visual inspection of samples used in the blind test

No.	Stress	*B. cinerea*	Visual inspection
4	+	-	No infection
13	-	-	No infection
16	-	-	No infection
30	+	+	No sign of infection
8	+	+	Little mycelium on 1 seedling
9	+	+	No sign of infection
25	-	+	No sign of infection
32	+	+	No sign of infection
10	-	+	No sign of infection
33	-	+	mycelium on all seedlings
28	-	+	mycelium on all seedlings