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Abstract

The use of so-called micro-plant propagation have become a well-adapted cultivation technique in Swedish forestry nurseries. Although this production method has many advantages, a commonly occurring problem is that the young plants, after transplantation in the nursery, have an underdeveloped root system when the shoot part have reached desired length. Currently used methods to control the plant growth in a desired way involves long-night treatment and exposure to different stresses such as drought or nutrient deficiency. Unfortunately, long-night treatment requires expensive equipment and stressing might cause injury to the plants. Alternative methods to control plant growth are therefore sought after by the forestry industry.

One of these alternative methods could potentially be the use of plant growth regulators (PGRs) which have been used for a long time in the agricultural sector, mainly to reduce the risk of lodging in cereals. There is however a key difference between the agricultural sector and the forestry industry – all plants grown as crops are members of the angiosperms, unlike the forestry plants which are dominated by gymnosperms. Research on PGRs have so far been focused on angiosperms exclusively. In this study, the effects of the substance trinexapac-ethyl on two economical important species of trees, Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*), were examined. Trinexapac-ethyl works by inhibition of multiple steps in the biosynthesis of gibberellin, an important plant hormone for length growth.

Trinexapac-ethyl was applied in the form of Moddus Start (250 g L^{-1} of a.i., Syngenta AG) in dosages of 0.3, 0.6 or 1.2 L ha^{-1}, either all at once or divided into two equally large amounts. There was three different occasions of applications: right after transplantation to cultivation cassettes, when desired shoot length had been reached and four weeks after the first treatment (if any). Both species were analysed by measurements of shoot length and stem diameter, weighing of the root and shoot parts separately and comparing their dry matter proportion, as well as ocular screening of any morphological changes. The frequency of crown buds in *P. sylvestris* was also determined and *P. abies* was examined via microscopy analysis.

The results indicate none or very modest growth regulating effect of the trinexapac-ethyl when applied to *P. sylvestris*. The substance did however have a more profound effect on the *P. abies* – growth regulating effect was observed for both shoot length and stem diameter in two out of three trial lanes, and all treated groups had an increase in their root/shoot proportion. Meanwhile, microscopy analysis revealed no harmful effects on the buds of *P. abies*. No other toxic effect of the trinexapac-ethyl could be observed on any of the species. Trinexapac-ethyl therefore seem to have potential as a PGR for the *P. abies*, but the substance needs further evaluation before any large-scale use.

Keywords: GA, gibberellin, Norway spruce, PGR, phytohormone, *Picea abies*, *Pinus sylvestris*, plant growth regulator, Scots pine, trinexapac-ethyl.

Sammanfattning

Användandet av s.k. mikroplantor har blivit en allt vanligare metod på svenska skogsplantskolor. Även om det finns många fördelar med denna produktionsteknik förekommer det dock problem med att de omplanterade färdigodlade plantorna har ett alltför svagt utvecklat rotsystem när skottdelen nått målhöjd på plantskolorna. Idag används metoder som långnattsbehandling och stressning av olika slag, t.ex. exponering för vatten- eller näringsbrist, för att kontrollera plantornas tillväxt. Tyvärr kräver långnattsbehandling dyr utrustning och det är riskabelt att utsätta plantorna för vatten- eller näringsbrist. Alternativa tillvägagångssätt för att styra tillväxten eftersöks därför av skogsindustrin.

En av dessa alternativa metoder skulle kunna vara användandet av tillväxtregulatorer som sedan länge har etablerat sig inom jordbrukssektorn, främst för att minska risken för liggsäd. Det finns dock en viktig skillnad mellan jord- och skogsbruk – samtliga grödor tillhör de gömfröiga växterna medan skogsbruket domineras av nakenfröiga växter. Forskning om tillväxtregulatorer har hittills enbart fokuserat på gömfröiga växter. I detta arbete undersöktes effekten av substansen trinexapak-etyl på två ekonomiska viktiga trädslag, tall (*Pinus sylvestris*) och gran (*Picea abies*). Trinexapak-etyl fungerar genom att hämma flera steg i gibberellinsyntesen, ett växthormon viktigt för plantors höjdtillväxt.

Trinexapak-etyl applicerades i form av Moddus Start (250 g L^{-1} aktiv substans, Syngenta AG) i doser om 0,3, 0,6 eller 1,2 L ha^{-1}, antingen allt på en gång eller uppdelat i två lika stora givor. Behandlingarna utfördes vid tre olika tidpunkter: strax efter omplantering till odlingskassetter, när önskad skotthöjd hade nåtts samt fyra veckor efter första behandlingen (om någon sådan fanns). Båda arterna analyserades genom mätning av såväl skottlängd som stamdiameter, separat vägning av rotsystemet och skottdelen vars torrsubstansvikt (TS-vikt) sedan jämfördes, samt okulär bedömning av eventuella morfologiska skillnader. Frekvensen kronknoppar i *P. sylvestris* bestämdes och *P. abies* analyserades via mikroskopering.

Resultaten visar på inga eller mycket små tillväxtreglerande egenskaper av trinexapak-etyl då *P. sylvestris* behandlades. Substansen hade emellertid starkare påverkan på *P. abies* – tillväxtreglerande effekt observerades på såväl skottlängd som stamdiameter i två av tre försöksled, och samtliga grupper hade en ökning av rot/skott-kvot. Mikroskoperingen påvisade inte någon skada på knopparna i *P. abies*. Inga andra toxiska effekter av trinexapak-etyl kunde identifieras på någon utav arterna. Det tycks alltså finnas potential att använda trinexapak-etyl som tillväxtregulator för *P. abies*, men mer utvärdering av substansen måste ske innan den kan börja användas i någon större skala.

Nyckelord: GA, gibberellin, gran, *Picea abies*, *Pinus sylvestris*, tall, tillväxtregulator, trinexapak-etyl, växthormon.

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Abbreviations

ABA	Abscisic acid
ACC	1-aminocyclo-propane-1-carboxylic acid
a.i.	Active ingredient
ARF	Auxin Response Factor
Aux/IAA	Auxin/Indoleacetic acid
CK	Cytokinin
DM	Dry matter
GA	Gibberellins
GAI	GA-Insensitive
GID1	Gibberellin Insensitive Dwarf1
GID2	Gibberellin Insensitive Dwarf2
IAA	Indoleacetic acid
PBS	Phosphate-buffered saline
PGR	Plant growth regulator
RGA	Repressor of GA_{1-3}
RGL1	RGA-Like1
RGL2	RGA-Like2
RGL3	RGA-Like3
SCF	SKP1, Cullin, F-box
s.d.	Standard deviation
SLY1	Sleepy1
TIR1/AFB	Transport Inhibitor Response 1/Auxin signaling F-box protein

1 Introduction

1.1 Background

The plant nurseries of the forestry industry have a diverse number of cultivation techniques, some of which includes the use of micro-plants at the incipient of the production line (Larsson, 2017). This method of cultivation use cassettes of small individual pot holes in which the seeds are planted and germinated in. After the seedlings have reached desired size, they are later transplanted to larger pots. Micro-plant propagation has the advantage that it's convenient to work with and it's also a very surface efficient method compared to larger containerized plants. However, this cultivation technique has one relatively frequent occurring problem facing the nurseries after the transplantation of the plants – fairly many plants have an underdeveloped root system when the shoot has the right length for plantation in the woods or for storage at the nursery during winter. A strong and healthy root system is of course crucial for the plant to function properly since it's responsible for the uptake of water and nutrients, gives the plant anchoring to the growing medium and act as a storage facility for the plant's photosynthate. If the plants are given extra time to develop a strong root system, the shoot part is at risk of becoming too high, and the plants will be difficult to handle during transportation, winter storage and planting in the woods.

There is in general two different ways used today in the forestry sector to control the shoot growth in a desired way (Larsson, 2017). One of them is to stress the plants which can be achieved by exposing the plants to drought, nutrient deficiency or by mechanical means. The other method – and the one most used today – is to manipulate the plants photoperiod. This is done by extending the plants length of night i.e. shortening the time that the plants are exposed to sunlight by covering them with blackout cloth. Shortening of the photoperiod makes the plant initiates

winter hibernation which inhibits shoot growth while stimulating further root growth. Although the long-night treatment is an effective method, it's also rather time consuming and relatively expensive. Because of this, alternative methods are still sought by the industry.

In the agricultural as well as the horticultural sector, plant growth regulators (PGRs) have been widely used since the first discovery of them (some nicotine based derivatives) in 1949 (Rademacher, 2000). For instance, 84% of the winter wheat grown in the UK are treated with PGRs (Berry *et al.,* 2004). The purpose of PGRs in cereal crops is to reduce the risk of lodging. Lodging is a problem in cereals since the yield can be lowered with as much as 80%, while at the same time reducing the quality of the grain, increasing the drying cost and delaying the harvest.

1.2 Purpose

Since the use of PGRs are so adapted in agriculture and horticulture, it would be interesting to investigate a possible use in the forestry production as well. This study aimed to evaluate the effects of the PGR trinexapac-ethyl on *Pinus sylvestris* as well as *Picea abies*, two species of great economical values for the Swedish forestry industry.

2 Theory

2.1 A Brief Overview of Phytohormones

Plants have a wide and diverse set of hormones, including well known groups such as auxins, cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA) and ethylene (Campbell *et al.,* 2014). Other more recently discovered plant hormones also exist such as the brassinosteroids, jasmonates and strigolactones. The nature of phyto-hormone systems is very complex since each hormone have several effects on the plant. The opposite phenomena, i.e. multiple hormones only affect a single process, can also occur throughout interaction between the substances, thereby making the plant hormone systems even less predictable. Furthermore, other factors than the hormone concentration also influence the final output of the plants growth and development. These other factors include the development stage as well as the location of the plant.

2.1.1 Gibberellins

One of the most important hormones with respect to length growth in plants are the gibberellins, as seen in figure 1 (a). This chemical class includes more than 100 different substances occurring naturally along the plant kingdom (Campbell *et al.,* 2014). The actual number found in any given plant species is however many times lower. Only a few GAs (e.g. GA_1, GA_3, GA_4 and GA_7) are bioactive (Davière & Achard, 2013). The majority of the GAs are either precursors or metabolites of biological active GAs. GAs stimulates plant growth by increasing both cell elongation and cell division (Wittwer & Bukovac, 1958). The hormones also have other effects in plants such as inducing flower development, promoting fruit setting as well as breaking dormancy in buds, tubers and seeds. It should be mentioned that

GAs have effect not only on angiosperms but also on gymnosperms. For instance, GAs have been shown to increase the female flower production in conifers species (Almqvist & Ekberg, 2002).

Early studies on certain pea mutants led to the conclusion that GAs act as an "inhibitor of an inhibitor", in which plants have an inherent growth restraining factor which the GAs can overcome (Harberd *et al.*, 2009). Further genetic research of mainly *Arabidopsis* and rice during the last decade have revealed increased insight into the GAs mechanism of action on plants (Davière & Achard, 2013). Several proteins important for the GAs effects have been identified. In *Arabidopsis*, one of these identified proteins includes the soluble nuclear GA receptor GID1 (Gibberellin Insensitive Dwarf1) which the GAs can bind to through hydrogen bonds. When this occurs, the GID1 alters its conformation and enclose the GA in its binding site.

Another key component in the GAs physiological function is the growth inhibitor protein group called DELLAs, which are named after one of the protein's domains (Davière & Achard, 2013). The DELLA proteins are important intracellular GA repressors, inhibiting the vast majority of the effects caused by the GAs. In *Arabidopsis*, five DELLA proteins are synthesized (GAI, GA-Insensitive; RGA, Repressor of $GA_{1\text{-}3}$; RGL1, RGL2 and RGL3, RGA-Like1, 2 and 3 respectively). Together, these proteins inhibit processes such as growth, floral formation and germination of seeds.

The GAs mechanism of action works by the entanglement of GAs, GID1 and DELLAs (Davière & Achard, 2013). As the GA binds to GID1 to form the GA-GID1 complex, further complex formation can occur through binding to the DELLAs, hence forming a GA-GID1-DELLA complex. Degradation of free DELLAs are in turn stimulated by this complex. This implies that if GAs are absent, the DELLAs degrading complex will not form and hence, the concentration of free DELLAs builds up. An accumulation of free DELLAs would repress the response associated with GAs, i.e. have a growth retarding effect. To understand how the DELLAs break down, there is however a third key component involved, namely the F-box proteins.

The F-box group includes proteins such as SLY1 (Sleepy1) in *Arabidopsis*, and GID2 (Gibberellin Insensitive Dwarf2) in rice (Davière & Achard, 2013). Proteins of the F-box are part of yet another complex group, the so-called SCF (SKP1, Cullin, F-box) E3 ubiquitin-ligase complexes. These complexes are responsible for attaching polyubiquitin chains to proteins which enables proteasomes (more

precisely the 26S proteasome) to degrade them. Destruction of free DELLAs would in turn reduce their growth-inhibitory effect on the plant. The overall growth stimulating effect of GAs is thus caused by the activation of the 26S proteasomes which degrades the growth-retarding DELLA proteins.

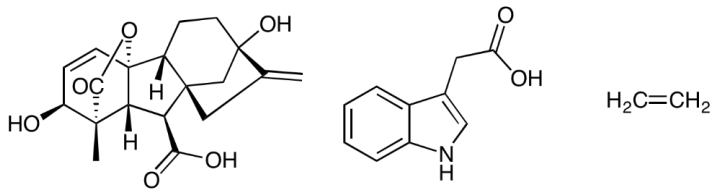
2.1.2 Auxins

Another essential hormone class regarding plant growth is the auxins, especially the indoleacetic acid (IAA) as seen in figure 1 (b), which is the primary natural occurring one (Campbell *et al.,* 2014). In young, still developing shoots, these substances will stimulate cell elongation. The growth stimulating effect of the auxin hormones is normally between a concentration range from approximately 10^{-8} to 10^{-4} M. At higher auxins levels, ethylene production might be induced which has a restraining effect on the cell elongation.

Biosynthesis, inactivation and transport pathways are all responsible for regulating the auxins level in the tissue of the plant (Lavy & Estelle, 2016). The mechanism of auxins is enabled due to three key protein groups; the auxin co-receptors TIR1/AFB which is yet another member of the F-box family, the transcriptional repressor Aux/IAA as well as the transcriptional factors ARF. When auxin is present in the nucleus of the cell, both broad as well as specific responses of the plant can occur due to the above stated protein groups.

2.1.3 Ethylene

Ethylene, figure 1 (c), is a simple, gaseous hydrocarbon that is produced in plants under stressful conditions such as drought, infection etc. (Campbell *et al.,* 2014). As stated above, ethylene has a negative effect on the elongation of the cells. Furthermore, the substance is also known to cause the so called “triple response” in plants during the seedling stage. The three effects of this response are retardation of stem elongation, thickening of the stem as well as shoot bending inducing horizontally growth.



(a) GA_3 (b) IAA (c) ethylene

Figure 1. Chemical structure of some phytohormones important for plant growth.

2.1.4 Abscisic Acid and Related Compounds

There exist more substances in plants with growth retarding effect than ethylene. One of the more well-known examples is abscisic acid (ABA), but other substances such as podolactone, asparagusic acids etc. have also been shown to act as natural growth inhibitors (Kefeli, 1978). Not only are the stem elongation supressed by these compounds, but also seed germination, opening of buds etc. It's therefore not surprising that the highest concentration of these naturally occurring growth inhibitors are found in dormant organs such as tubers and autumn buds of woody plants.

2.1.5 Cytokinins

Cytokinins (CKs) represent the last member of the major phytohormone class. The major task of the CKs, in combination with other plant hormones, is to regulate cell division (Kamínek, 2015). As the other hormone systems, the CKs also have other functions such as stimulation of metabolic sink formation, senescence of leaves, halting of apical dominance etc.

2.1.6 Practical Application of Phytohormones

The study of phytohormones is of great academic interest but has also been extremely important in the agricultural sector as well as other industries working with plant materials. Both naturally occurring as well as synthetic plant hormones have been found to be useful in many different types of applications. For instance, the discovery of the synthetic auxin 2,4-D was one of the greatest game changer for weed control in the history of agriculture since it effectively kills broad-leaved plants while leaving members of the grass family relatively intact (Overbeek, 1948). The compound therefore enabled selective weed control in crops such as cereals and sugarcane to name a few. Furthermore, plant hormones have been used to force some crops (e.g. pineapple) into flowering and initiate fruit formation, induce root growth in cuttings as well as keeping fruits such as apple and citrus from falling of the tree prematurely. The compounds have also been used to create larger, sugar enriched fruits than would normally form. Yield increase can also be achieved not only by larger fruits, but also due to enlargement of the leaf area and other photo-synthetic active tissues (Wittwer & Bukovac, 1958). As an example, GA treatment of forage crop and vegetables such as celery has shown to increase the yield with up to 40%. Another profound effect of hormone treatment is when seedless varieties of grapes are being sprayed with GA. Not only does the fruit size (and therefore yield) increase, but also the length of the pedicles, see illustration in figure 2. This results

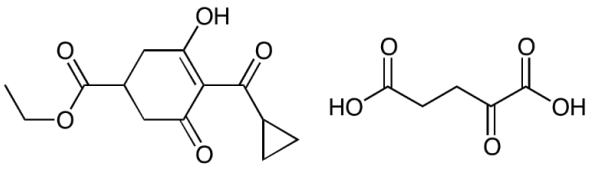
in a more promiscuous cluster which in turn leads to less need of cluster-thinning or girdling for achieving high quality grapes. Also, the more openly growth structure of the cluster enables more efficient pest control.

Figure 2. Illustration of the morphological effects of GA on seedless varieties of grapes. Part of untreated plant to the left, treated to the right. (Illustration: Emil Bengtsson, SLU, drawn after photography in Wittwer & Bukovac, 1958)

2.2 About the PGR of Choice – Trinexapac-ethyl

Trinexapac-ethyl is a relatively recent discovered PGR, launched on the market in 1992 (Rademacher, 2015). The substance belongs to the chemical class of acylcyclohexanediones, see chemical structure in figure 3 (a). Trinexapac-ethyl interferes with multiple biosynthesis routes in the plant, however the most important for growth related effect is through the inhibition of later stages of GA formation. The GA biosynthesis route can be divided into three major phases with respect to at which site of the cell the reactions occurs and what kind of enzymes that participates (Rademacher, 2000). The first stage takes place in the proplastids, where terpene cyclases are used to form the GA precursor *ent*-kaurent. In the next stage of the GA biosynthesis route, *ent*-kaurent is oxidized into GA_{12}-aldehyde by the aid of monooxygenases. The site of action of this step is in the endoplasmatic reticulum. The final stage of GA formation primarily involves dioxygenases for further oxidation of the newly synthesized GA_{12}-aldehyde, although some oxidation reaction requires monooxygenases instead. These oxidation reactions mainly occurs in the cytosol, and together with further hydroxylation reactions leads to the

formation of different GAs. The trinexapac-ethyl, as well as the other PGRs of the acylcyclohexanediones group, inhibits these last steps of the GA biosynthesis route. The trinexapac-ethyl's mechanism behind this is by being an analogue of the substance 2-oxoglutaric acid, see figure 3 (b), which is a co-substrate of the dioxygenases needed for the oxidation reactions after the GA_{12}-aldehyd formation. Being an analogue to this co-substrate means that the trinexapac-ethyl has enough similar molecular structure as the 2-oxoglutaric acid to bind to the dioxygenases and replace the needed co-substrate, thereby halting the oxidation reactions. According to enzyme kinetic data, it seems likely that the trinexapac-ethyl works in a competitive way, in other word it competes with the intended co-substrate 2-oxoglutaric acid for binding to the dioxygenases.



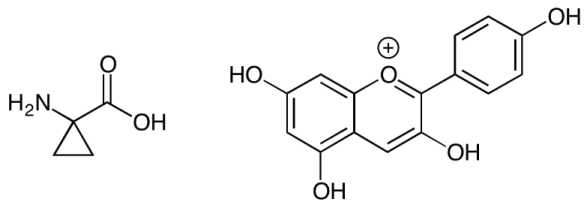
(a) trinexapac-ethyl (b) 2-oxoglutaric acid

Figure 3. The trinexapac-ethyl together with the co-substrate it mimics.

More specifically, the main reaction halted by the trinexapac-ethyl and its related compounds of the acylcyclohexanediones family after the GA_{12}-aldehyd has formed seems to be the conversion to GA_1 from GA_{20} (Rademacher, 2000). This reaction occurs by hydroxylation of the 3β-carbon atom. Also, the later hydroxylation step at the 2β-carbon which converts the GA_1 to GA_8 is another main target step that is being halted.

Another biosynthesis system that trinexapac-ethyl interfere with is the ethylene production in the plant (Rademacher, 2015). This is achieved in a similar way as the inhibition of GA biosynthesis, the trinexapac-ethyl mimics the co-substrate ascorbate of the enzyme ACC oxidase. Without the ascorbate, the ACC oxidase can't convert the substance ACC, seen in figure 4 (a), into ethylene. Furthermore, trinexapac-ethyl also retards the flavonoids synthesis by inhibition of the enzyme flavanone 3-hydroxylase. Similar to the GA related dioxygenases, this enzyme requires 2-oxoglutarate (the anion form of 2-oxoglutaric acid) as a co-substrate. Flavonoids are a diverse group of substances including sub-groups such as flavans, flavones and anthocyanins, as seen in figure 4 (b) (Strack & Wray, 1994). The flavonoid compounds have many different functions in plants. For instance, the anthocyanins have an important role when it comes to the coloration of flowers and fruits, hence enhancing pollination and seed dispersal. The anthocyanins – together

with other related flavonoids – also seem to play an important role in the plants defense against insect pests. Furthermore, flavonoid compounds have been shown to have microbiological activity (Bohm, 1994). Substances from many sub-groups of the flavonoids both induces as well as inhibits *Rhizobium* bacteria to start nitrogen fixation in legumes. It also seems that plants exposed to stressful condition such as fungal attack change its composition of flavonoids compounds.



(a) ACC (b) pelargonidin

Figure 4. (a) The ethylene production related compound ACC. (b) The anthocyanin pelargonidin, a flavonoid compound.

The overall physiological effect of trinexapac-ethyl is in other words to lower the plants levels of GAs and ethylene, as well as some other substances not related to plant growth such as the flavonoids compounds. Though a plant respond to ethylene by reducing its stem growth, a lover level of this hormone would normally imply that the plant would grow a taller shoot i.e. the opposite of what is the intention of PGR application. However, it seems likely that this effect is countered and over-shadowed by the reduction of active GAs in the plant.

3 Materials and Methods

3.1 General Overview

The trinexapac-ethyl was applied in the form of Moddus Start (formula A17600C, Syngenta AG, Basel, Switzerland) to both *P. sylvestris* and *P. abies*. There was in total eight trial lanes (A – H) with different dosages and/or number of application times, see table 1 below for a summary.

Table 1. Summary of the trinexapac-ethyl treatments. ”X” implies that an application of the PGR was carried out whereas “-“ means no treatment. In trial lane E, F and G the dosage was divide equally between the two treatments. Trial lane H was used as control.

Trial lane	Total applied dosage of Moddus Start (L ha^{-1})	Number of applications	Time of applications		
			Right after transplantation to cultivation cassettes	When desired shoot length had been reached	4 weeks after first treatment
A	0.3	1	X	-	-
B	0.6	1	X	-	-
C	0.3	1	-	X	-
D	0.6	1	-	X	-
E	0.6	2	X	X	-
F	1.2	2	X	X	-
G	0.6	2	-	X	X
H (control)	0.0	0	-	-	-

The concentration of trinexapac-ethyl in Moddus Start was 250 g L^{-1} (24.2% w/w) and was diluted with water according to table 2. PHFIX5 (Sudau Agro GmbH, Erding, Germany) was added to the mixture to lower the waters pH from 8 to 5. This

was done in order to protect the active ingredient from complex bonding to the eventual ions present in the water.

Table 2. Ingredients of the spraying mixture.

Moddus Start dosage (L ha^{-1})	Moddus Start (mL)	PHFIX5 (mL)	Water (L)
0.3	1.125	3.00	3
0.6	2.250	3.00	3

The spraying mixture corresponds to 800 L ha^{-1} of solution for each trial lane. This rather high amount of water was used since the cassettes had a high density of plants, meaning that there was a lot of surface area for the spray mist to target. Since each cassette of the *P. sylvestris* has an area of 0.0828 m^2, and the corresponding area for the *P. abies* was 0.148 m^2, the amount of spraying mixture each species should receive per cassette was calculated as follows:

800 L ha^{-1} = 0.08 L m^{-2}, the equivalent for each cassette was then:
0.08 L m^{-2} x 0.0828 m^2 = 6.624 mL (*P. sylvestris*)
0.08 L m^{-2} x 0.148 m^2 = 11.840 mL (*P. abies*)

The spraying equipment used was a hand-held sprayer (Orion, Kwazar Corporation Sp. z o.o., Jaktorów, Poland) with a nozzle (Hardi 4110-10-dysa, Hardi International A/S, Alslev, Denmark) which gives approximately 7 mL s^{-1}. If all the spraying mist hits the cassettes, it means that the duration of the spraying of one cassette would be:

6.624 mL / 7 mL s^{-1} = 0.95 s (*P. sylvestris*)
11.840 mL / 7 mL s^{-1} = 1.69 s (*P. abies*)

It was however estimated that during the applications, approximately one third of the spray missed the cassettes. Assuming that 67% of the spray mist actually hit the target, then each cassette would have the following application time to get the right dosage of the PGR:

0.95 s / 0.67 = 1.41 s (*P. sylvestris*)
1.69 s / 0.67 = 2.52 s (*P. abies*)

An attempt to improve the precision of the treatment dosages was made by treating 3 sets of *P. sylvestris* cassettes for approximately 5 seconds at the time rather than 1.41 seconds each. A similar procedure was applied for the *P. abies*, where 3 sets of cassettes were sprayed for about 8 seconds in total.

3.2 Treatments

The first treatment was carried out in a greenhouse between 7 and 8 a.m. at the 21th of June (photo in figure 5). Both *P. sylvestris* and *P. abies* had dry foliage and moist root substrate during the spraying. *P. sylvestris* remained in the greenhouse whereas *P. abies* was moved to outdoor cultivation after the application. After approximately 4 weeks (at the 28th of July) both plant populations were screened for visual effects of the trinexapac-ethyl. Due to the unusually cold summer weather, all the *P. abies* that had been included in the project had had an extremely low growth rate (including the control). Therefore, the original *P. abies* were discarded and replaced with non-transplanted plants. Consequently, only the following trial lanes were possible for the added non-transplanted *P. abies* seedlings: C, D, G and H.

Figure 5. Newly treated *P. sylvestris* on the 21th of June 2017, together with the spraying equipment used. (Photo: Martin Larsson, Svenska Skogsplantor)

At the 29th of July, the second spraying was performed, between 2 – 3 p.m. in the greenhouse. The original *P. sylvestris* was treated as planned, and the recently added *P. abies* was also sprayed with the same amount of PGR as *P. sylvestris*. Both species had trial lane C, D and G treated with the PGR, *P. sylvestris* also had trial lane E and F sprayed in addition. All the plants were directly transported to outdoor cultivation after the spraying. To protect the plants from rain, which might interfere

with the uptake of the trinexapac-ethyl, the plants were placed under a protecting roof for the first 12 hours after the treatment.

One month later, at the 28th of August the last treatment was carried out. As for the other treatments, this one was also conducted in the greenhouse, around 3 p.m. Only trial lane G was sprayed at this occasion (for both species), and the plants were then placed for outdoor cultivation.

3.3 Analysis

The plants were examined with respect to shoot length, stem diameter, proportion between root and shoot part, frequency of crown buds (*P. sylvestris* only), microscopy analysis (*P. abies* only) as well as screening for morphological changes. None of the plants located at the outer part of the cassettes were measured since they might be influenced by confounding factors such as higher light levels, different microclimate etc. Also, individuals of *P. sylvestris* shorter than 7 cm and individuals of *P. sylvestris* no taller than 10 cm were discarded since they exhibited abnormally low growth likely caused of other factors than the PGR, such as individual seed diversity, pests or diseases. The frequency of plants possessing abnormally low growth appeared to be similar for each trial lane.

The statistical analysis conducted for both the shoot length and the stem diameter was a one-tailed z-test with the significance level (α) of 0.05 in which every trial lane was compared with the control trial lane H. The hypothesized mean difference was set to 0, which in other words means it was assumed that there was no significant difference between treated and untreated plants. The statistical analysis was conducted by using the Analysis ToolPak add-in for Microsoft Excel. The z-test was judged to be a reasonable statistical test method since the sample size (n) i.e. the number of plants measured, was considered large ($\geq$30) and thus assuming a normal distribution of the samples.

3.3.1 Shoot Length

All the plants were measured with respect to shoot length. The obtained values were rounded to closest 5 mm interval. The measurements were conducted by the use of a ruler.

3.3.2 Stem Diameter

As a measure of plant health, i.e. how robust and vigorous the plants were, the stem diameter was measured. Every second of the cassettes rows were used for this task. The measurements of the stem diameter were performed just above the site were the first root appeared. This was done by using an electronic caliper (Ironside 14137761, IP 67, Ironside International, Paris, France).

3.3.3 Root/Shoot Proportion

Determination of the root/shoot proportion (dry matter weight, DM-weight) were achieved by randomly selecting 30 plants from each trial lane and then cleaning the peat of from the root system. The plants were then dried at room temperature for two days and then cut at the transition zone between the root and the shoot. After the plants had been separated, they were put in an oven at 105 °C for about 24 hours to remove volatile water. When this step was completed, the plants root and shoot parts were weighed separately in groups of 30 based on their trial lane belonging. Since the plants were weighted in groups rather than individual, no standard deviation could be determined and hence no statistical analysis were carried out for this measurement.

3.3.4 Crown Budding Rate

All *P. sylvestris* were ocularly screened for crown buds. See figure 6 below for comparisons between juvenile and crown buds. The *P. abies* lacks this juvenile-crown budding trait, and therefore this examination step could not be applied for this species.

Figure 6. Comparison between juvenile bud (left) and crown bud (right) on *P. sylvestris*. Note that the crown bud is divided into smaller subunits, enabling the plant to branch. (Photo: Emil Bengtsson, SLU)

3.3.5 Morphological Effects

Both *P. sylvestris* and *P. abies* were examined if any morphological effects (except length and stem diameter) of the PGR could be noticed. Properties of the plants such as colouration, general structure etc. were evaluated. All trial lanes were inspected during this step.

3.3.6 Microscopy Analysis

Some plant specimens were taken for further investigation using light microscopy. The aim was to screen the plants for effects on the cellular level by measuring the length as well as the number of cells. Another purpose with the microscopy studies was to see if the PGR could have affected the health and/or development of the plants buds. The condition of the buds was interesting to evaluate since they are crucial for the survival of the plant – if the buds dies or get damaged, the growth of the whole plant will be halted since the meristem is located in the bud.

Because of practical reasons, such as time limitation, but also due to lack of any notable growth-related effect on the *P. sylvestris*, the *P. abies* was examined exclusively in this step. Only the trial lane with the most apparent shoot length retardation – that is trial lane D – was screened and compared with the control trial lane H. Two plants of each examined trial lane were randomly selected.

The preparation of the plant samples for microscopy analysis began with fixation and wax-embedding based on an *in situ* hybridization protocol for *P. abies* published by Karlgren (Karlgren *et al.,* 2009). The initially step was to divide the shoot part in segments of 5 cm, starting from the top of the plant, and then removing the needles and eventual side shoots. Obviously, the bottom segment was sometimes shorter than 5 cm since the shoot part ends at that zone. In order to make the plant material practical to work with during the examination, the segments were divided into 1 cm long sub-segments. Figure 7 below illustrates the sectioning of the *P. abies*. The sub-segments were then put in a fixation liquid (see Appendix 1 for full list of ingredients). As a back-up if the fixation liquid wasn't able to penetrate the whole plant sample, one of each sub-segments of each zone were cut in half transversely to expose the inner parts directly to the fixation liquid. When all of these steps were completed, the plant segments were put in a vacuum chamber for 1 hour to further increase the uptake of the fixation liquid. The plant material was then put in new fixation liquid and once again put in a vacuum chamber, this time for 24 hours. After that, the plant material was put in a 70% EtOH solution until further preparation was conducted.

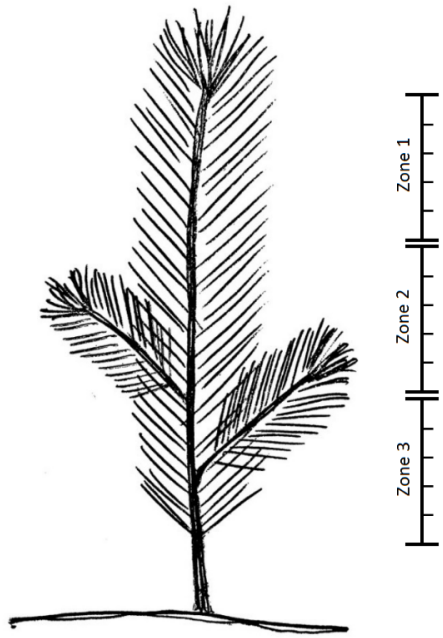


Figure 7. Principle drawing of the sectioning of the *P. abies*, here divided into three 5 cm segments (denoted as "zones") starting from the top. Note the 5 sub-segments marked in each zone. (Illustration: Emil Bengtsson, SLU)

Approximately 3 weeks later, the samples were removed from the EtOH solution and then put in a container for further processing in a tissue processor (Leica TP1020). The tissue processor enables liquid wax penetration of the plant samples and the samples were treated for approximately 12 hours. When completed, the embedding of the solid wax procedure started. This step was made by using a wax-embedding device (HistoCore Arcadia H, Leica Biosystems Nussloch GmbH, Wetzlar, Germany) at 60 °C. Directly after the samples had been moulded, they were put on cooling at 4 °C overnight for solidification of the wax. A few days later, the first slicing of the plant material began. The samples were planed in 70 µm strips (seen in figure 8) using a microtome (Type HM 355S, Microm International GmbH, Dreieich, Germany) and then moved to water/heat treatment for stretching on microscope slides.

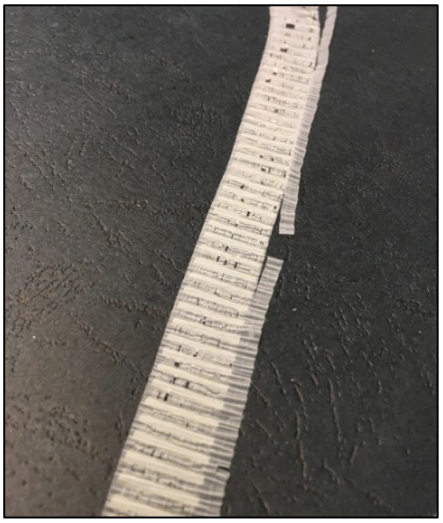


Figure 8. Recently sliced wax-embedded plant material. (Photo: Emil Bengtsson, SLU)

One week later, the final preparations before the microscopy examination was conducted. These last steps were dewaxing of the samples followed by staining of the specimen. This included the plant material to be put in multiple solutions for different extent of time. The dewaxification agent used was Histoclear (National Diagnostics, Atlanta, GE, USA) and the staining colour used was Toluidine Blue (Sigma, St Louis, MO, USA). See summary of dewaxification/staining preparation schedule in table 3. The plant samples were ready for microscopy analysis after the dewaxification/staining treatment. The microscope use was a Carl Zeiss, model Axio Scope.A1, together with the software ZEN 2012 (Blue edition, Carl Zeiss Microscopy GmbH, Jena, Germany).

Table 3. Schedule of the different steps used in the preparation of the plant material during dewaxification/staining treatment.

Step number	Solution	Duration (minutes)
1	Histoclear	10
2	Histoclear	10
3	EtOH, 100% (v/v)	1
4	EtOH, 100% (v/v)	1
5	EtOH, 90% (v/v)	1
6	EtOH, 80% (v/v)	1
7	EtOH, 60% (v/v)	1
8	EtOH, 40% (v/v)	1
9	PBS, 1x	1
10	Toluidine Blue, 0.05 % (m/v)	5
11	*Milli-Q* water	5

4 Results

Figure 9 and 10 below shows how the plants looked during the end stage of the experiment. A summary of the effects of trinexapac-ethyl on the quantitative traits measured is reviewed below in table 4 and 5 for each species separately. More detailed results for each single property examined (including non-quantitative ones) are found further down.

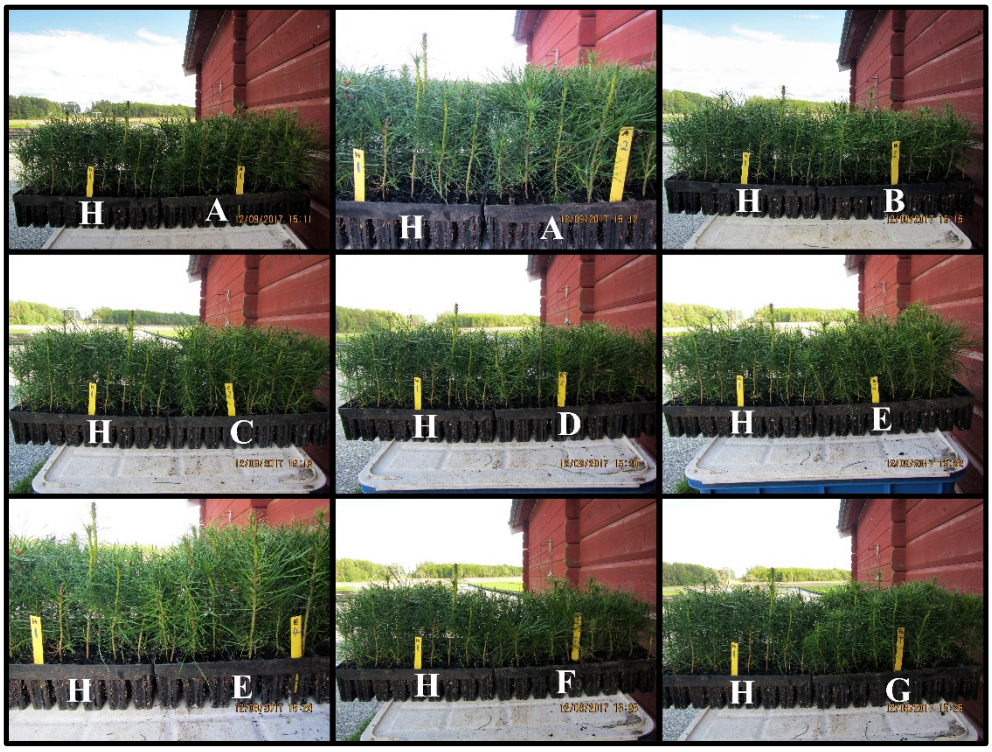


Figure 9. Appearance of the *P. sylvestris* during the end stage of the experiment. Characters indicates which trial lane each cassette belong to. Note that there is a close-up photo of trial lanes A – H and A – E i.e. the ones with significant increases of shoot length. (Photos: Martin Larsson, Svenska Skogsplantor)

Table 4. Summary of results of the quantitative properties measured in *P. sylvestris*. The cross-symbol (†) in trial lane A and E indicate significant difference but in the "wrong" extreme, i.e. treated plants had a significantly increase of shoot length. See the discussion part for more details. See table 1 for timing of the PGR application.

	Trial lane							
	A	B	C	D	E	F	G	H (control)
Moddus Start dosage (L ha^{-1})	1 x 0.3	1 x 0.6	1 x 0.3	1 x 0.6	2 x 0.3	2 x 0.6	2 x 0.3	0 x 0.0
Shoot length, significant difference	Yes†	No	No	No	Yes†	No	No	–
Stem diameter, significant difference	No	No	No	No	No	No	No	–
Root/shoot proportion (mg mg^{-1})	0.38	0.38	0.37	0.43	0.37	0.37	0.37	0.39
Crown buds frequency (%)	5.33	1.43	3.90	1.32	2.50	3.51	3.57	10.39

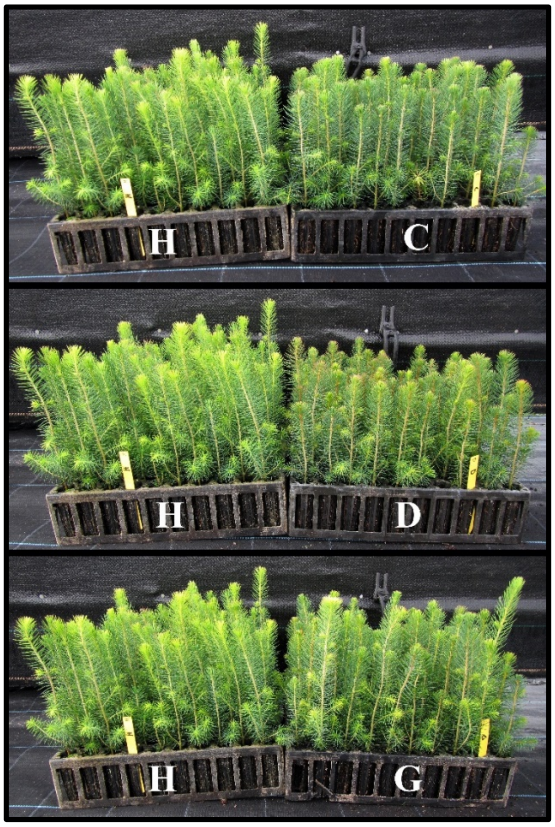


Figure 10. Appearance of the *P. abies* during the end stage of the experiment. Characters indicates which trial lane each cassette belong to. (Photos: Martin Larsson, Svenska Skogsplantor)

Table 5. Summary of results of the quantitative properties measured in *P. abies*. See table 1 for timing of the PGR application.

	Trial lane			
	C	D	G	H (control)
Moddus Start dosage (L ha^{-1})	1 x 0.3	1 x 0.6	2 x 0.3	0 x 0.0
Shoot length, significant difference	Yes	Yes	No	–
Stem diameter, significant difference	Yes	No	Yes	–
Root/shoot proportion (mg mg^{-1})	0.33	0.35	0.32	0.30

4.1 Shoot Length

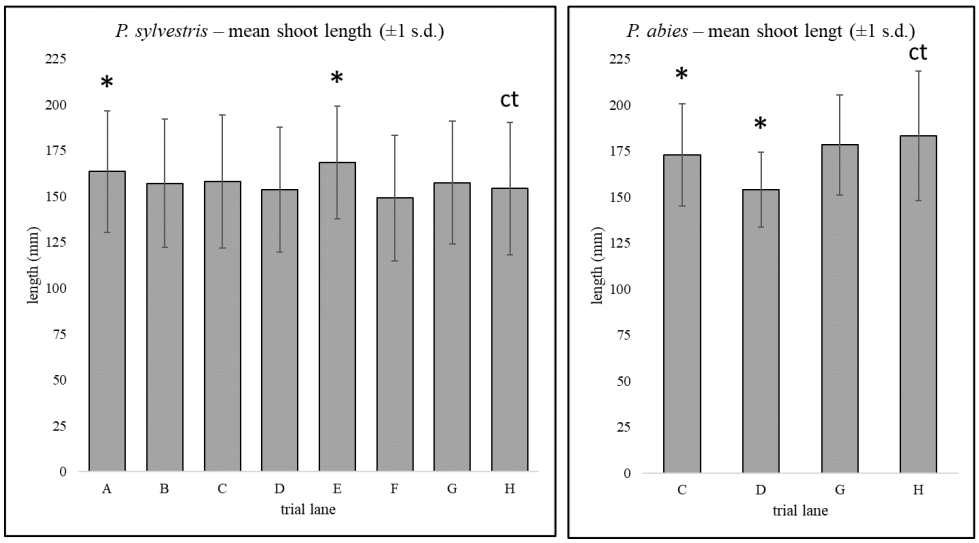


Figure 11 and 12. Graphical presentation of the mean shoot length of each trial lane for both species. Asterisk (*) indicates statistical significance difference if compared to the control trial lane H, marked as "ct".

Table 6. Shoot length of *P. sylvestris*.

	Trial lane							
	A	B	C	D	E	F	G	H (control)
Mean shoot length (mm)	163.7	157.2	158.1	153.8	168.6	149.1	157.6	154.3
s.d. (mm)	33.2	34.9	36.1	34.1	30.6	34.1	33.5	36.0
Shoot length difference (%)	+ 6.09	+1.88	+2.46	-0.32	+9.27	-3.37	+2.34	-
n (number)	75	70	77	76	80	57	56	77
P-value	0.047	0.308	0.255	0.467	0.004	0.199	0.293	-
Significant difference in shoot length	Yes	No	No	No	Yes	No	No	-

Tabell 7. Shoot length of *P. abies.*

	Trial lane			
	C	D	G	H (control)
Mean shoot length (mm)	173.1	153.9	178.4	183.3
s.d. (mm)	27.7	20.4	27.2	35.1
Shoot length difference (%)	-5.56	-16.04	-2,67	-
n (number)	95	97	93	89
P-value	0.014	0.000	0.149	-
Significant difference in shoot length	Yes	Yes	No	-

4.2 Stem Diameter

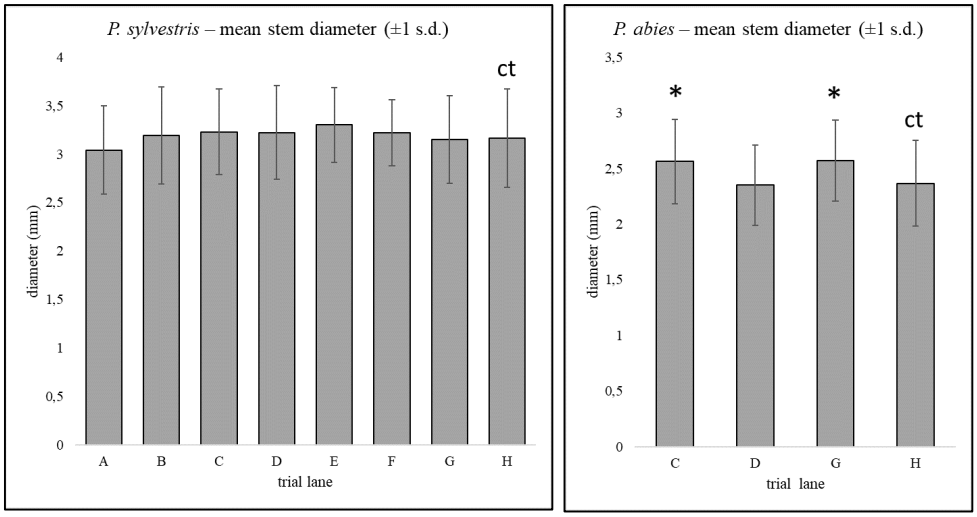


Figure 13 and 14. Graphical presentation of the mean stem diameter of each trial lane for both species. Asterisk (*) indicates statistical significance difference if compared to the control trial lane H, marked as “ct”.

Table 8. Stem diameter of *P. sylvestris*.

	Trial lane							
	A	B	C	D	E	F	G	H (control)
Mean stem diameter (mm)	3.042	3.191	3.230	3.224	3.303	3.223	3.150	3.164
s.d. (mm)	0.454	0.501	0.441	0.485	0.387	0.342	0.563	0.506
Stem diameter difference (%)	-3.86	+0.85	+2.09	+1.90	+4.39	+1.86	-0.44	-
n (number)	50	44	55	54	54	38	38	52
P-value	0.100	0.396	0.236	0.268	0.057	0.256	0.443	-
Significant difference in stem diameter	No	No	No	No	No	No	No	-

Table 9. Stem diameter of *P. abies.*

	Trial lane			
	C	D	G	H (control)
Mean stem diameter (mm)	2.563	2.350	2.569	2.368
s.d. (mm)	0.378	0.360	0.363	0.387
Stem diameter difference (%)	+8.23	-0.76	+8.49	-
n (number)	58	59	58	49
P-value	0.004	0.406	0.003	-
Significant difference in stem length	Yes	No	Yes	-

4.3 Root/Shoot Proportion

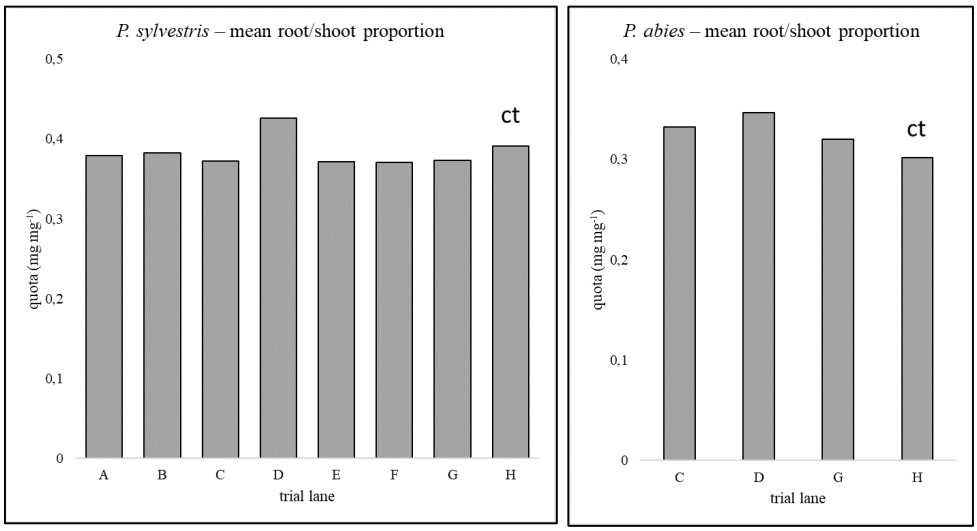


Figure 15 and 16. Graphical presentation of the mean root/shoot proportion of each trial lane for both species. Note that no statistical analysis were conducted for this measurement. The control trial lane H is marked as "ct".

Table 10. Root/shoot proportion in *P. sylvestris*, mean of 30 plants per trial lane.

	Trial lane							
	A	B	C	D	E	F	G	H (control)
Root DM-weight (mg)	20.3	18.5	17.7	22.8	20.7	19.7	19.0	19.4
Shoot DM-weight (mg)	53.6	48.4	47.6	53.5	55.7	53.2	50.9	49.6
Root/shoot proportion (mg mg^{-1})	0.38	0.38	0.37	0.43	0.37	0.37	0.37	0.39

Table 11. Root/shoot proportion in *P. abies*, mean of 30 plants per trial lane.

	Trial lane			
	C	D	G	H (control)
Root DM-weight (mg)	11.3	9.7	10.5	9.5
Shoot DM-weight (mg)	34.0	28.0	32.8	31.5
Root/shoot proportion (mg mg^{-1})	0.33	0.35	0.32	0.30

4.4 Crown Budding Rate

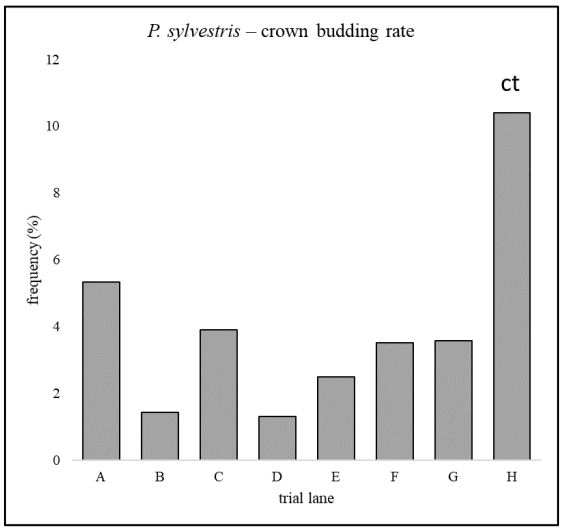


Figure 17. The rate of crown buds in *P. sylvestris* visualised. Notice that the vertical axis of the graph is limited to 12%. The control trial lane H is marked as "ct".

Table 12. Crown budding rate of *P. sylvestris.*

	Trial lane							
	A	B	C	D	E	F	G	H (control)
Frequency of crown buds (%)	5.33	1.43	3.90	1.32	2.50	3.51	3.57	10.39
n (number)	75	70	77	76	80	57	56	77

4.5 Morphological Effects

No visible differences between the trial lanes of *P. sylvestris* could be noticed by ocular inspection. This was however not the case regarding the *P. abies*. Some of the plants in the treated trial lanes revealed some different morphological structures when compared to the untreated ones. One of these differences was the angle of the needles relative to the stem, where the treated plants appeared to have a wider angle (i.e. more horizontal direction), as seen in figure 18. It was also noticed that the length of the fir needle seemed to be shorter for the treated plants.

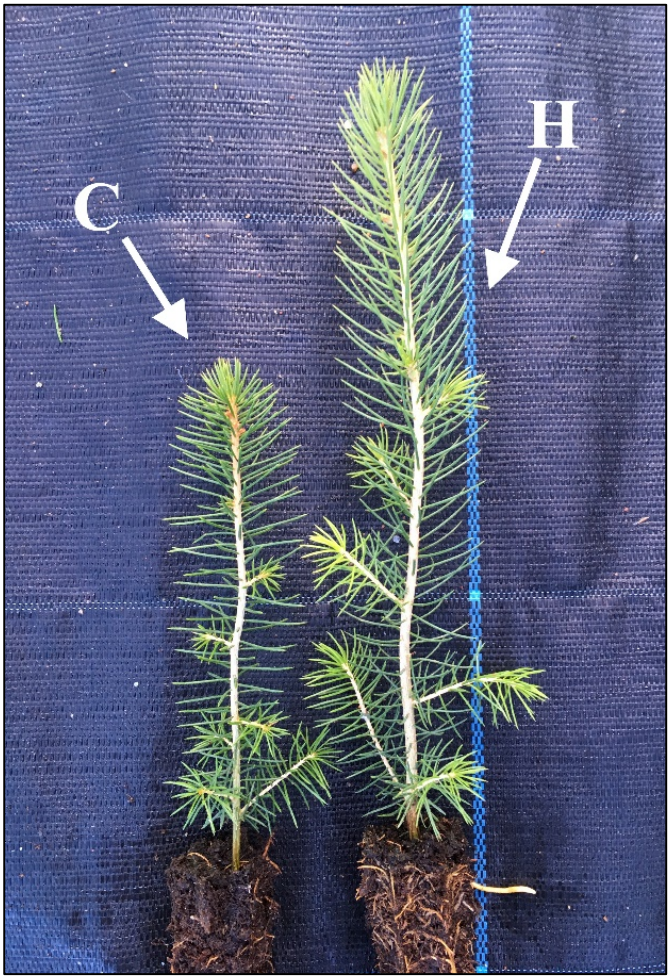


Figure 18. Comparison between two samples of *P. abies*. Characters indicates which trial lane each plant belong to. (Photo: Emil Bengtsson, SLU)

4.6 Microscopy Analysis

Unfortunately, it was practically impossible to do any cell length measurement or cell count of the plant samples due to indistinct transitions between the cells as seen in figure 19 and 20. One possible explanation to the lack of cell border sharpness might be because the plants had already developed highly lignificated tissues. A tissue with a high degree of lignification would by itself have cells that are difficult to distinguish from each other, but it would also impede a smooth slicing of the microtome.

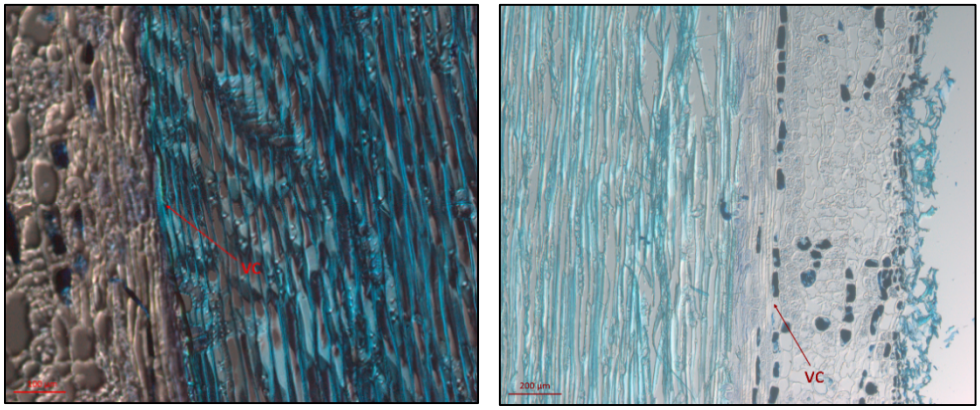


Figure 19 and 20. Photos showing the obscure borders between the cells located in the stem. The plant samples used in both photos are from the control trial lane H. *Abbreviation(s): vc – vascular cambium.*

The plant samples of the bud part had however enough satisfied quality to enable examination of the bud health, probably due to less lignified tissue. No visible differences could be recognized at the cellular level when comparing the buds of treated with non-treated plants, see figure 21 and 22 for comparison. As the photos shows, both plants seems to have healthy, well-developed meristem, pith, needle primordia and scales.

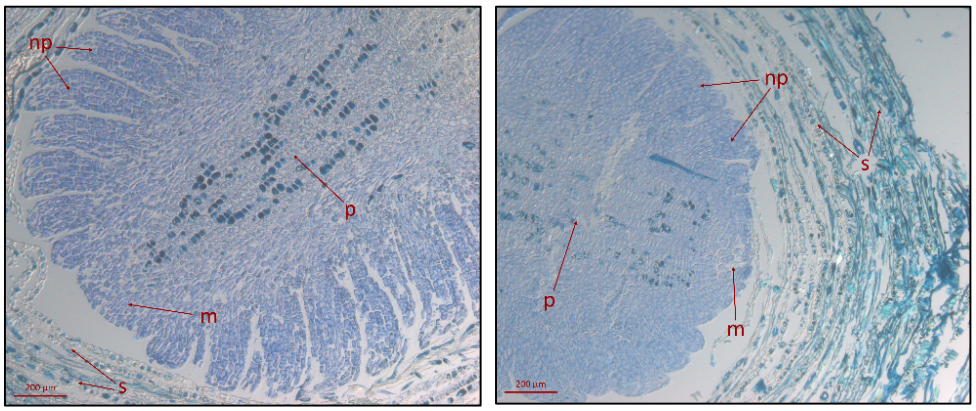


Figure 21 and 22. Plant buds from trial lane H (left), and trial lane D (right). *Abbreviation(s): m – meristem, np – needle primordia, p – pith, s – scales.*

Further evaluation of the piths adjacent to the buds did not show any differences between treated and untreated plants. As shown in figure 23 and 24, it seems that the different cells and structures have a very similar appearance.

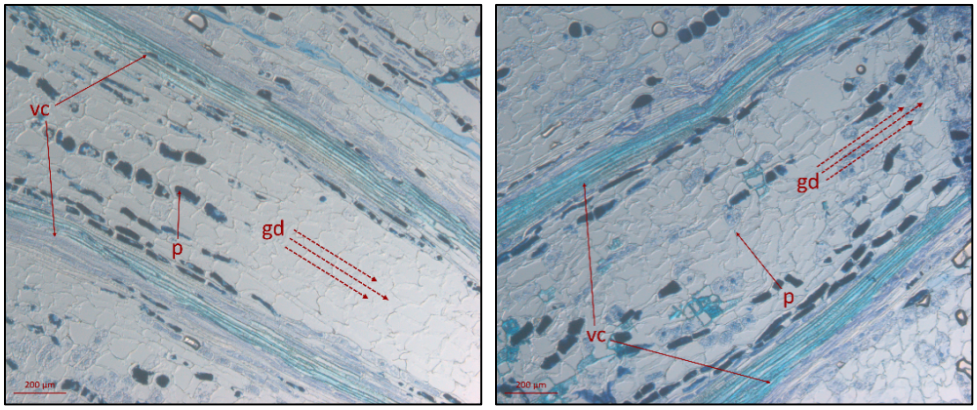


Figure 23 and 24. Piths located near the buds. No notable differences between the control trial lane H (left) compared to trial lane D (right). *Abbreviation(s): gd – growth direction, p – pith, vc – vascular cambium.*

5 Discussion

5.1 Summary of Results

Trinexapac-ethyl have been shown to retard stem growth in perennial ryegrass (*Lolium perenne*) with 13% when applied at dosages of 200 g ha^{-1} (Chastain *et al.*, 2014). The growth regulating effects are stronger at higher dosages. At rates of 600 g ha^{-1}, growth reduction of 28% have been reported.

No growth restraining effect of the PGR could however be demonstrated on *P. sylvestris*. Measurements of the shoot length revealed statistical significant increase in trail lane A (1 x 0.3 L ha^{-1}) and E (2 x 0.3 L ha^{-1}) when compared to the control trial lane H. None of the other trial lanes had significant differences. Regarding the stem diameter, no significant differences could be demonstrated for any of the trial lanes. This suggest that the trinexapac-ethyl doesn't have any growth inhibiting effect on the *P. sylvestris*.

The quota between root and shoot is of interest since it gives an indication how developed the root system is relative to the shoot part. As stated in the introduction part, plants with a well-developed root system without too long shoot part are desired in nursey production of forestry plants. For *P. sylvestris*, no obvious effect on the root/shoot proportion could be determined, the ratio seems to be similar in every trial lane. All trial lanes hade a slightly decrease of the quota, except trial lane D (1 x 0.6 L ha^{-1}) which had somewhat higher value when compared to the control trial lane H. It's however unlikely that the treatment for trial lane D could affect the plants root/shoot ratio to any notable extent since that trial lane did not have statistical significant difference in neither shoot length nor stem diameter.

The percentage of crown buds of *P. sylvestris* is interesting since plants that have developed these tend to get a better start when transplanted from nursery to field. This is due to the enabling of branching of the tree, which gives better competitive advantages over weeds, and generally contributes to a more robust plant. Although no statistical analysis was carried out regarding the crown budding rate, it seems like the treated plants had a lower rate of these. This was a bit unexpected since the crown buds can form if the plant is exposed to stressful conditions. Although a PGR treatment might be a very mild stressful element, it nevertheless adds some extra stress compared with no treatment at all. No obvious relationship between the actual dosage received by the plant and the crown budding rate could be determined. For instance, trial lane F (2 x 0.6 L ha^{-1}) had a higher frequency of crown buds than trial lane B (1 x 0.6 L ha^{-1}).

Moving on to *P. abies*, there was significant reduction of shoot growth in two of the trail lanes: C (1 x 0.3 L ha^{-1}) and D (1 x 0.6 L ha^{-1}). The effect was stronger in trial lane D, with a shoot growth reduction of about 16%, than in trial lane C which had a reduction of approximately 6%. This result is reasonable since trial lane D received twice as much trinexapac-ethyl than trial lane C and both were treated at the same time. A shoot growth reduction of 16% is between the reported reduction range of perennial ryegrass, suggesting that the trinexapac-ethyl works about as effective in gymnosperms as in angiosperms. Trial lane G (2 x 0.3 L ha^{-1}) had no significant shoot length retardation even though it received the same amount of PGR at the same time as trial lane C, and then another equally large dose approximately four weeks later. The reason why this was the case is not clear, but maybe the trinexapac-ethyl interfere in a greater extent with the ethylene production in the later application, thereby reducing the amount of it. A lower level of ethylene would – as previously stated in the theory chapter – stimulate shoot growth.

The effect on the stem diameter growth on *P. abies* yielded significant increase in trial lane C and G, but not in D. This was a somewhat unexpected result since trial lane D showed the strongest shoot growth retardation. It would be reasonable that the plants with lowest shoot length growth also would had the thickest, most robust stem as well. One possible explanation to this result could be that the trinexapac-ethyl also retards the growth rate of the vascular cambium, thereby also making the shortest plant the thinnest one.

The results from the root/shoot proportion in *P. abies* showed that all treated trial lanes got a higher quota than the untreated one, which of course is a desired property of the PGR. The same pattern as for the shoot length appeared between the trial lanes, i.e. the effect was strongest in D, followed by C and finally G. This indicate

that the shoot growth was reduced in a greater extent than the root growth, which seems to be a reasonable result.

Trinexapac-ethyl appears to have altered the morphology of some individuals of *P. abies*. The shortening of the needles further advocates that the PGR indeed had growth regulating effect on *P. abies*. It is not clear whether or not the change in the plant architecture have any effect on its field performance.

Judging from the results of the microscopy analysis, it doesn't seem that the trinexapac-ethyl affect the bud health in any negative way. This is of course a good property of the PGR since the buds is crucial for the plants survival. Neither did the pith of the plant showed any signs of damage on the cellular level. It should be noticed that the sample size for this microscopy evaluation was very small (only four plants tested). Caution should therefore be taken before any generalized comment of bud or pith health are stated.

5.2 Conclusion

Based on the results of this study, it seems that the trinexapac-ethyl had no retardation properties on the growth of *P. sylvestris*, and hence no practical application. Factors such as uptake of the PGR, metabolic inactivation etc. may poses an explanation to why the substance failed to inhibit shoot growth. Environmental aspect such as temperature etc. can of course also affect the results. Another possible explanation to the absence of any evident effect of the PGR in *P. sylvestris* might be because of too low dosages. If this scenario is likely or not is difficult to say since the sensitivity against trinexapac-ethyl differ greatly between plant species. As an example, the dosage of Moddus Start for use in wheat and barley is 0.3 L ha^{-1} and 0.6 L ha^{-1} respectively, either all at once or divided into equally large amounts at two separated occasions. For use in dicotyledonous crops such as oilseed rape, the dosage is considerably larger. With the equivalent PGR Circle (250 g L^{-1} of trinexapac-ethyl, Syngenta AG), the dosage goes up to 1.5 L ha^{-1}. In this experiment, the total amount of applied PGR ranged from 0.3 to 1.2 L ha^{-1}. Comparing this dosage range with a trinexapac-ethyl sensitive crop i.e. wheat, the *P. sylvestris* received normal to very high dosages of trinexapac-ethyl. If, however the species is compared to crops such as oilseed rape, the plants received fairly low dosages of the PGR. This implies that it's possible that a growth regulating effect of the trinexapac-ethyl on *P. sylvestris* might reveal at higher dosages than the ones used in this study. It's also more likely that adverse, toxicological effects will occur as well. A higher amount of PGR applied would of

course also be more expensive and consequently not as appealing for the forestry nurseries to use. The reduced frequency of crown bud formation on treated plants should also be taken into consideration before any use of the substance in the forestry production.

Trinexapac-ethyl showed growth regulating properties on the *P. abies*, although the results on this species had some non-coherent elements. There is potential for practical use of the PGR at the forestry nurseries during cultivation of the *P. abies*, but of course more research need to be carried out to examine dosage levels, time of application(s) etc. It should also be affirmed that the stem growth is not halted in such an extent that the trees have a reduced stamina when they are being planted in the woods. The fact that no toxicological effects could be demonstrated on any of the species is of course a good trait of the trinexapac-ethyl.

5.3 Further Work

It would be of great interest to evaluate the uptake of the PGR, especially in the *P. sylvestris* due to the lack of effects. If the substance was not taken up properly, there might be reasons to further test the trinexapac-ethyl with different combination of adjuvants. Conversely, if the substance indeed was absorbed adequately, it would suggest that the trinexapac-ethyl indeed has no use in cultivation of *P. sylvestris*.

Whether or not the growth restraining effect of trinexapac-ethyl failed to occur in *P. sylvestris* due to inhibition of ethylene production could probably be tested with an ethylene releaser e.g. ethephon (trade name Cerone, Bayer Crop Science) added to the PGR mixture. If a test is conducted in which plants are treated with either trinexapac-ethyl, ethephon or both of these combined, and the trial lanes treated with the mixed PGRs shows synergistic rather than additive growth retardation, it indicates that the trinexapac-ethyl in some cases fails to retard the plant growth due to its inhibition of ethylene production. Observation of synergistic effects would also suggest that trinexapac-ethyl might still have some potential use for cultivation of *P. sylvestris* if combined with an ethylene releaser.

Since the trinexapac-ethyl interferes with the flavonoids synthesis, it would probably be a good idea to investigate if other metabolic pathways are affected before any large-scale use. Since conifers produce a variety of secondary metabolites, especially terpenes for the resin, an interference with these systems could potentially make the plants more susceptible to insects and pathogens.

Studies on the cellular effects in the vascular cambium would be extremely interesting since it may explain why the stem diameter is not coherent with the shoot length retardation in *P. abies*. Another crucial evaluation would of course be on how the plants performs in the field. It's important to know if properties such as winter survival rate have been affected by the trinexapac-ethyl treatment.

Although the microscopy analysis was successful with respect to evaluation of bud health, measurement of the cell length and cell count proved to be impossible for the plant samples used in this experiment. The length and number of cells might be possible to analyse if samples from younger plant tissue were used since they would have a lower degree of lignification. That type of tissue would probably enable a smoother slicing by the microtome as well as having clearer transition between the cells. In other words, it would have been better to analyse the plants relatively shortly after the trinexapac-ethyl had been applied. Embedding of plastic rather than wax would further be an improvement of this measurement.

In any of the measurements, it would also be a good idea to carry out the studies in a controlled environment such as a phytotron. This is due to the fact that even though the PGR can have an impact on the plants growth, the effect might be hidden in disguise because of cold weather condition etc. This would halt the growth independent on a PGR present or not. Another improvement for further studies on the effect trinexapac-ethyl on *P. abies* and/or *P. sylvestris* would be to have a more accurate application method of the PGR than the one used in the experiment. An evenly sprayed PGR treatment might have more coherent effects on the plants.

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Appendix 1

The ingredients of the fixation liquid were the following:

- *Milli-Q* water, 450 mL
- 5 M NaOH, approximately 4 droplets
- Paraformaldehyde (K28401505, Merck KGaA, Darmstadt, Germany), 25 g
- Tween 20 (9005-64-5, Merck KGaA, Darmstadt, Germany), 1 droplet
- 10x PBS with pH 7.0, 50 mL

Whereas the 10x PBS (phosphate-buffered saline) in turned consisted of the following substances (the volumes below is for 1 L prepared PBS):

- *Milli-Q* water, 640 mL
- 5 M NaCl, 260 mL
- 1 M Na_2HPO_4, 70 mL
- 1 M NaH_2PO_4, 30 mL

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