Nitrogen uptake and assimilation during Norway spruce somatic embryogenesis

Investigating the role of glutamine

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Abstract

Sweden is currently experiencing shortage of Norway spruce (*Picea abies*) seeds due to e.g. irregular flowering and the recent year's outbreaks of pests and pathogens destroying cones and seeds. Therefore, the forest sector is investigating alternative ways of propagating Norway spruce plants to secure Norway spruce regeneration and sustain the genetic diversity.

Somatic embryogenesis (SE) is a vegetative method that can be used for *in vitro* propagation of Norway spruce plants. Plants produced through the SE technique could potentially lead to; i) shorter time from recent tree breeding improvements to reforestation. ii) preserving genetic diversity in managed forests, while maintaining genetic gains from the tree breeding. SE can also be used as a mean to cryo-preserve valuable genetic material and to study embryo development. Unlike the seed embryo development, which is dependent on the surrounding tissue for nutrients, the entire SE development is undeniably dependent on the culture conditions; light, temperature, gaseous environment, media amendments and nutrient composition. I have used SE as a research tool to generate material for studying uptake, and utilization of carbon and nitrogen during Norway spruce embryo development.

My research has contributed new evidence that media supplied glutamine is essential for growth during the process of Norway spruce somatic embryogenesis. I found that media supplied with glutamine is important for maintaining the pool of free amino acids, the building blocks of e.g. DNA, proteins and chlorophyll. In addition, glutamine present in the growth medium assisted in alleviating stress-related metabolic pathways - the alanine aminotransferase pathway and the GABA shunt. Furthermore, the findings from my work can aid the on-going development for an industrial production system for Norway spruce plants through SE.

My research project has been a collaboration between Swedish university of agricultural science (SLU) and the forest company Sveaskog, specifically the seed and plant division Svenska Skogsplantor.

Keywords: Amino acid, Assimilation, Carbon, Germination, Glutamine, Nitrogen, Norway spruce, Somatic embryogenesis, Utilization

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Preface

There is a re-written version of the popular sea shanty “The drunken sailor” covering the main findings from a thesis about conifer breeding (Prescher, 2007). The thesis version of the shanty is named “The seed orchard song”, with the lyrics: *Hooray, the seed is needed (x 3). For more growth in forests.*

Unfortunately, today Norway spruce is on the verge of shipwreck in terms of providing enough elite seed material for Swedish forestry. Thus, in this thesis I will further explore an alternative way of fortifying Norway spruce plant propagation through the process of somatic embryogenesis. So, my version of the shanty lyrics goes like: *Hooray, somatic embryogenesis is needed (x3). For more growth in forests.*

Dedication

My family, and especially my late grandfather Carl-Erik.
Och min Iris, för alla de kvällar mamma inte kunde säga god natt.

You are human and fallible.
Jane Eyre, by Charlotte Brontë

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Carlsson, J., Svennerstam, H., Moritz, T., Egertsdotter, U., Ganeteg, U.* (2017). Nitrogen uptake and assimilation in proliferating embryogenic cultures of Norway spruce - investigating the specific role of glutamine. *PLoS ONE*, vol 12 (8), e0181785. https://doi.org/10.1371/journal.pone.0181785

II Carlsson, J., Egertsdotter, U., Ganeteg, U., Svennerstam, H.* (2018). Nitrogen utilization during germination of somatic embryos of Norway spruce - revealing the importance of supplied glutamine for nitrogen metabolism. (under revision for *Trees*)

III Carlsson, J., Svennerstam, H.* The essential nature of exogenously supplied glutamine for Norway spruce somatic embryogenic cell culture. (Manuscript)

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The contribution of Johanna Carlsson to the papers included in this thesis was as follows:

I Carlsson contributed to the experimental design and had primary responsibility for planning and conducting the sampling, experimental work, and data analysis and had main responsibility for writing.

II Carlsson had overall responsibility for the experimental design and planning of the study, and had primary responsibility for conducting the sampling, performing the experimental work, analysing the data, and writing the paper.

III Carlsson contributed to the planning of the study, sample acquisition, data analysis, and writing.

Abbreviations

All abbreviations are explained when they first appear in the text.

1 Introduction

1.1 Forest ecosystems and forestry

Globally, forest ecosystems are renewable hubs used for numerous products and materials and consist of a diverse mixture of tree species from the two plant groups angiosperms and gymnosperms.

Angiosperms have traits such as an endosperm tissue within the seeds and the development of fruit bodies that contain the seeds, which distinguishes them from gymnosperms (Davis & Schaefer, 2011). The evolutionary separation between gymnosperms and angiosperms has been dated to around 300 ± 50 million years ago. Gymnosperms are a group of plant species with an ancient origin from the late Carboniferous geological period (358.9–298.9 million years ago) and were the group that dominated among land-living plants during the Mesozoic geological period, around 252 to 66 million years ago. Fascinatingly, gymnosperms have survived waves of extreme climate changes, which for other species living on the earth have led to mass extinction. Presently, four sub-lineages make up the group of gymnosperms – cycads, *Ginkgo*, gnetophytes, and conifers (reviewed in Wang & Ran, 2014).

In many countries today, forestry is an important sector contributing to biological, economic, and social values. There is a growing awareness of the need to think and act to increase the sustainability of our resources so that they can be used by future generations. In addition, rising awareness leads to the development of new sustainable management methods and forest-related products and at the same time leads to efforts to preserve biological and genetic diversity in the forests (Carnus et al., 2006; Lelu-Walter et al., 2013).

Sweden is one of the top five major export countries of forest products (e.g. paper, pulp and lumber). With forest covering of more than 50 % of the total land area, forests are of great value and forestry is one of the most important sectors for the Swedish economy. Around 80 % of the total standing volume on

productive forest land is represented by two conifers – the Scots pine (*Pinus sylvestris*) and the Norway spruce (*Picea abies* (L.) Karst) – and the remaining volume consists of a mix of deciduous tree species and a few other non-native conifers (SLU, 2017). Therefore, if Swedish forestry shall continue to play a central role in the global forestry sector and the production of forest-related products, new areas including biotechnology, bioenergy, and building material need to be explored. In addition, advances to meet the strongly increasing demands for forest conservation and biodiversity are needed, work which has already been initiated within the tree breeding programmes.

1.2 Forest improvement by tree breeding

Forest tree breeding has a long history in Sweden, starting in the 1930s with the selection of plus trees (Eriksson et al., 2006). Selection of plus trees is the basis of forest tree breeding, which aims to improve the tree species traits and qualities, i.e. growth, disease resistance, or wood properties. Today, the tree breeding programmes are governed by the Forestry Research Institute of Sweden (Skogforsk). One of the greatest challenges for the breeding has been, and still is, to identify, prioritise, and select these traits. The breeding efforts must balance the plus tree selection considering features with huge prediction uncertainties such as future climate change and anthropological values of the forest. Furthermore, conifer tree breeding is also difficult because the breeding cycles spans over almost 20 years, which is a long period compared to agricultural crops (Eriksson et al., 2006; Prescher, 2007; Rosvall & Mullin, 2013).

1.2.1 Norway spruce seed orchards

From the plus tree selections used in the breeding programmes, grafts have been used for establishing seed orchards with the purpose of producing saleable seeds for seedling production in nurseries (Eriksson et al., 2006). Since the 1950s, when the first seed orchards were established, there have been two more rounds of seed orchard installations in Sweden, and the latest one (the third round), will be completed in 2018 (Remröd et al., 2003). This third round of seed orchards has been estimated to deliver seeds with around 25 % better gain compared to seeds collected from the local wild stand (Rosvall et al., 2003). The production of Norway spruce seedlings for forest regeneration in Sweden in 2016 and 2017 was around 170 and 200 million seedlings, respectively (Skogsstyrelsen, 2017; 2018). The drive for meeting the thriving and increasing demands for forest products is one of the reasons why seed orchards and

propagation materials are still so important (Remröd et al., 2003; McKeand et al., 2006; Westin & Haapanen, 2013).

It is difficult to establish and manage Norway spruce seed orchards. First, it can take almost two decades for a new seed orchard to grow and mature to an age where the mother trees produce flowers (strobili) and subsequently cones containing seeds. Thus, seed orchards are always 20 years behind the latest improvements from the breeding programme. Second, the mature mother trees flower irregularly, about every 4 to 7 years (Lindgren et al., 1977; Crain & Cregg, 2018). Treatments such as the application of hormones (e.g. gibberellin; Almqvist, 2007), root pruning, and tree strangulation (for a review see e.g., Crain & Cregg, 2018) have shown promising results, with increased numbers of flowers during years when flowering occurs. However, direct treatments to initiate conifer flowering and the factors controlling flowering in Norway spruce have not been identified. Third, the seed orchards have been suffering from occasional outbreaks of pests (e.g. *Dioryctria abietella* and *Eupithecia abietaria*). In addition, during the last decade a fungus named *Thekopsora areolata* has destroyed huge amounts of the seed harvest (Almqvist & Rosenberg, 2016; Capador et al., 2018). These complications have, in combination, seriously affected the seed production and the seed supply for plant propagation (Almqvist et al., 2010;). Therefore, in addition to the necessity to propagate trees with enhanced traits for the forest sector, alternative techniques to maintain plant production for reforestation are required (Park, 2002; Nehra et al., 2005; Rosvall, 2011).

1.2.2 Vegetative plant propagation

The concept of vegetative propagation of forest plants has been debated longer than the acute problem with Norway spruce seed production has existed. This is mostly because vegetative propagation gives an opportunity for faster transfer of genetically valuable material from tree breeding to industrial forestry. Presently, vegetative propagation of plants via rooted cuttings is the most common method used to produce valuable genetic material (Park, 2002; Lelu-Walter et al., 2013). However, in Sweden this method has been used only on a very small scale, with seedlings originating from cuttings contributing to less than one per cent of the total plant production (Sonesson et al., 2001). Another form of vegetative propagation for large-scale plant production is the use of somatic embryogenesis (SE) (Park, 2002; Lelu-Walter et al., 2013). The method of SE (the development of a plant or embryo from somatic cells and not the germ cells) has shown great potential for the mass production of Norway spruce plants, although the method requires further technical development before it can

be used on a large industrial scale (Park, 2002). The implementation of SE in conifer plant production and its advantages and disadvantages have previously been reviewed (Grossnickle et al., 1996; Park, 2002; Nehra et al., 2005; Andersson & Lindgren, 2011; Lelu-Walter et al., 2013). The reasons for using the specific process of SE are to multiply genetically important germplasm at a fast rate, to store valuable germplasm as embryogenic lines using cryopreservation until field tests are completed, and to provide an external monitoring tool for observing embryo development, which normally occurs in isolation within the seed. In combination, these uses benefit the forest industries by providing the flexibility to shift or including new tree breeding objectives and to quickly get the material out into the forest, as well as to supervise and maintain the genetic diversity.

The technique for large-scale propagation is costly, mainly due to the intensive manual labour required, and therefore there has been no real success in producing plantlets via SE. Even so, efforts are currently underway to develop a system combining liquid cultures for the multiplication of cell culture, an automated embryo-harvesting system, and a germination platform, which four Swedish forestry companies together with a biotech company have been aiming to industrialise to reduce production costs (personal communication, D. Pacurar[1]). Hence, basic knowledge about all aspects of embryo development, e.g. nutritional needs, metabolic pathways, signalling pathways, etc., supports the goal of an entirely automated SE process.

1.3 Plant reproduction and embryogenesis

During their lifecycles, both gymnosperms and angiosperms alternate between the gametophyte stage (haploid; one inherited copy of each chromosome either from the mother or father) and the sporophyte stage (diploid; two copies of each chromosome, one from both parents), which is referred to as "an alternation of generations". Thus, the number of chromosomes is the principal difference between the two plant generations. Gymnosperms exist as sporophytes for much of their lifecycle, whereas the haploid generation only occurs during meiosis where the gametes (i.e. the cells involved in sexual reproduction) are produced. The beginning of a new plant life starts with the fusion of two gametes (a sperm and an egg), which generates a diploid zygote. The zygote develops into an embryo, and this developmental process in the embryo is called zygotic embryogenesis (ZE). There are dissimilarities between

[1] Daniel Pacurar, project leader for the SE automatization system at SweTree Technologies, Umeå.

angiosperm and gymnosperm embryogenesis regarding, for example, the formation and development of the embryos (see e.g. reviews by Raghavan & Sharma, 1995; Smertenko & Bozhkov, 2014; Winkelmann, 2016; and references therein). This thesis deals with Norway spruce, and therefore in the following sections I will focus on describing embryogenesis in gymnosperms with a special emphasis on conifers.

1.3.1 Conifer seed development - zygotic embryogenesis

Pollination

The pollen cone and the ovulate cone (megastrobilus) are the production sites of two types of spores, microspores and megaspores, respectively. From the microspore, pollen grains are matured and eventually sperm cells are formed. In the ovule, the megaspore develops in to a megagametophyte and eventually differentiates into the egg cell. Gymnosperms have the ability to produce multiple egg cells, referred to as archegonia. Pollen grains land the surface of the megastrobilus mostly via wind, and the pollen grain passes into the ovule through small microscopic holes on the seed surface called micropyles. Once inside the ovule, the pollen grain releases the sperm. Conifer sperm do not have flagella and are thus immobile, but the sperm can be transferred to and fused with the egg via a pollen tube that emerges from the pollen grain a few days up or weeks after the pollen grain entered the ovule. Subsequently, the zygotic embryo develops inside the ovule and a seed is formed (Owens et al., 1998; Raven et al., 2005).

After pollination

The embryogenesis of conifers after pollination is illustrated in Fig 1, which includes a detailed terminology of the process. Singh (1978) described and divided the process of embryogenesis in gymnosperms according to three phases:

i. Proembryogeny – all steps before the elongation of the suspensor.

ii. Early embryogeny – the steps throughout and after the elongation of the suspensor and until the formation of the root meristem.

iii. Late embryogeny – the steps including the formation of both the root and shoot meristem and the continued tissue development of the embryo.

Proembryogeny (i)

The phase of proembryogeny begins when the egg's nucleus divides and initially forms four free nuclei (i.e. plural for nucleus; the organelle in the cell containing most of the organism's DNA). After a number of cell divisions, in which the primary proembryo gradually separates into two primary layers (tiers) of cells, a basal embryo plan is formed. One tier is designated to form the embryo mass and later the body, and the upper tier will become the embryo-supporting arrangement, the suspensor (von Arnold & Clapham, 2008; Winkelman, 2016).

Early embryogeny (ii)

In the phase of early embryogeny, the embryonal mass is formed and the suspensor elongates. Common for species belonging to the *Pinaceae* family is the occurrence of cleavage polyembryogeny, i.e. the proembryo splits and multiple embryo masses arise. However, only one of the embryo masses will develop and become the seed embryo, and the other embryo masses will degenerate (Pullman & Bucalo, 2014). As an exception, tree species belonging to the *Picea* genus do not seem to have this polyembryogenic feature (Buchholz, 1942; Dogra, 1967).

Late embryogeny (iii)

In the final phase, late embryogeny, the basal and apical meristems in the embryonal mass start to form. The basal meristem will form the root and the apical meristem will form the shoot. Also, during embryo maturation the embryo suspensor undergoes programmed cell death (Smertenko & Bozhkov, 2014).

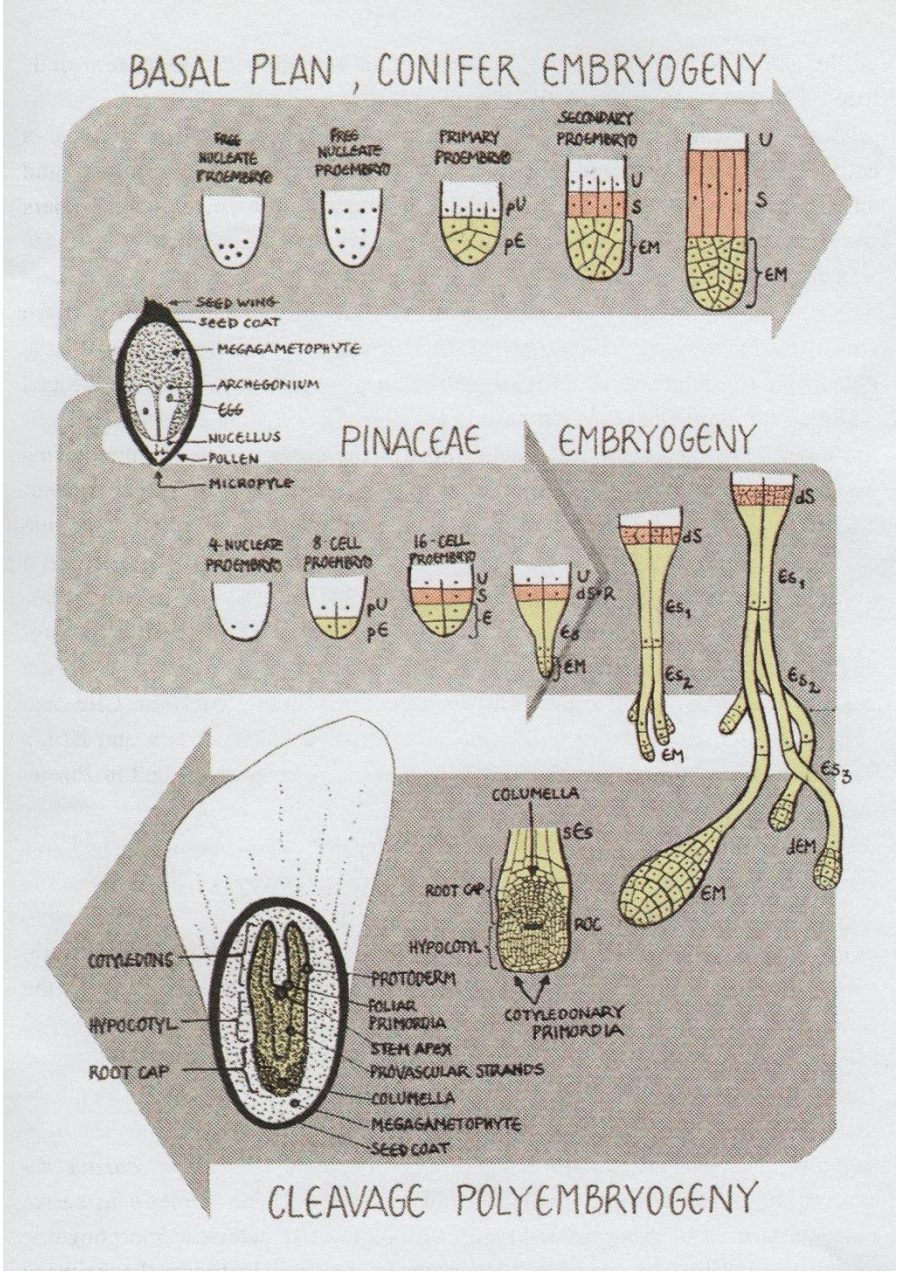

Fig 1. Illustration of the embryogenesis of conifers after pollination, i.e. the pollen enters the seed thru the micropyle. Primary upper tier, pU; primary embryonal tier, pE; upper tier, U; suspensor tier, S; embryo mass, EM; dysfunctional suspensor tier (Rosette tier), dS=R; embryonal suspensor tier, Es_x; degenerating embryo mass, dEM; root organisation centre, ROC; secondary embryonal suspensor cells, sEs. Reproduced with permission from Ulrika Egertsdotter, Research Notes 52, doctoral thesis, 1996.

1.3.2 Embryo development in the laboratory

History

The embryo culture technique for propagating plants is over 100 years old and refers to the sterile cultivation of excised zygotic embryos in a nutrient medium with known supplements of chemical elements. The technique was initially performed using several species from the *Brassicaceae* family, where mature embryos were germinated into plantlets on a nutrient medium supplemented with sucrose (reviewed by Raghavan, 2003). Additionally, efforts in pursuing a way of propagating orchids via embryo culture techniques were made due to (i) restricted import of orchid plants, (ii) the desire to develop new hybrids, and (iii) the difficulties of successfully germinating orchid seeds. The small size of the seed makes it sensitive to abiotic and biotic factors such as fungi or algae competition and risk of seed dehydration (Knudson, 1922).

Sixty years ago, regeneration of a plant embryo from a single somatic cell was achieved in carrot (*Daucus carota*) (Steward et al., 1958), and more than thirty years later the first conifer somatic embryo (Chalupa, 1985; Hakman et al., 1985) was developed using the SE process (described in more detail in the following section).

Somatic embryogenesis

A somatic cell contains a complete number of chromosomes and is produced through the process of mitosis, i.e. the duplication of a cell.

The process of SE is the development of somatic embryos from somatic cells. However, the exact mechanism through which the transformation from a somatic cell to an embryonic cell occurs is not known. Plant somatic cells can be totipotent, i.e. the plant cell has the capacity to differentiate into any cell type (Verdeil et al., 2007). SE is commonly thought to be induced when totipotent somatic cells are subjected to high concentrations of the plant hormone auxin (Braybrook & Harada, 2008; Angoshtari et al., 2009) or to stress such as dehydration, temperature, or heavy metal ions (reviewed by Fehér et al., 2003).

SE in Norway spruce as a model system for research

The SE method can be divided into different developmental stages, from the pro-embryo to the plantlet (illustrated in Fig 2). For Norway spruce, the zygotic embryo is used for the induction of an embryogenic culture that is composed of pro-embryogenic masses (PEMs). The embryogenic culture is proliferated to produce PEMs, which are subsequently used for the propagation of SE plantlets

via early embryo differentiation and maturation followed by germination and plant formation (von Arnold et al., 2002; Pullman et al., 2003; von Arnold & Clapham, 2008).

For conifers, different standard SE protocols have been developed for different species and SE phases. The growth media used in SE contain nutrient additives, for example micro- and macro elements, vitamins, plant growth regulators, carbohydrates, osmotic compounds, and amino and/or organic acids (George & de Klerk, 2008). Early efforts in optimising culture media composition and growth conditions were of the "trial-and-error-approach", but these have contributed to advanced protocols for proliferation maintenance and for the production of mature somatic embryos (Bozhkov et al., 1993; Kaul & Hoffman, 1993; Pullman & Bucalo, 2014). Nonetheless how these additives influence the SE process is not completely understood and requires further study. Another approach for improving SE has been "learning from seeds", i.e. studying the biochemistry, physiology, physical environment, and gene expression patterns of zygotic embryos during development (Pullman & Bucalo, 2014; Winkelmann, 2016).

The similarities between conifer ZE and SE have been considered to be one of the most promising features for monitoring and studying embryo development. However, there is a major difference between the two types of embryogenesis; SE involves the proliferation of PEMs by supplying external plant growth regulators, whereas ZE takes place inside a seed coat surrounded by a nourishing tissue called the megagametophyte. Therefore, storage compound accumulation during embryo maturation in addition to the nutrients provided in the growth media is crucial and essential during the SE process (Hakman, 1993; Goldberg et al., 1994).

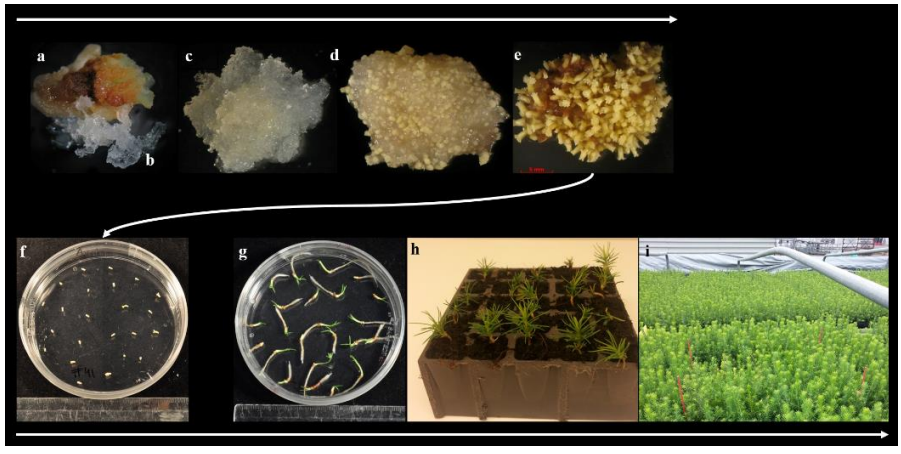

Fig 2. (a) The proliferation of somatic cells is induced from a zygotic embryo placed on a medium supplemented with the plant growth regulators (PGRs) auxin and cytokinin at room temperature and in darkness. (b) The initiated cell culture of PEMs. (c) Proliferating cell culture of PEMs maintained on medium including the same PGRs as used for initiation. The medium is exchanged every second to third week, which gives the possibility to multiply the cell culture at this phase. (d) For induction of embryo maturation, PEMs are moved to a pre-maturation medium for depletion of the remaining auxin and cytokinin and kept at room temperature and in darkness. The cell culture is transferred to a maturation medium supplemented with the PGR abscisic acid for the final development of mature somatic embryos. (e) It takes about 8–9 weeks for mature somatic embryos to develop. The embryos are harvested and normally undergo a period of desiccation before the last SE phase of germination. (f-g) The germination sometimes starts under low intensity light, which is gradually increased to higher light intensities. Somatic embryo germination is achieved on a medium without PGRs, and it takes generally around 6 weeks before the plantlet has a well-developed root and shoot capable of surviving the transition from the *in vitro* to *ex vitro* environment. (h) The plantlet is transplanted to a peat-based substrate and placed in a growth chamber for adaptation to non-sterile conditions and for further growth and development. (i) At this stage, the plantlet can be moved to a forest plant nursery where final acclimatisation for a life in the forest takes place.

1.4 Basis for plant growth and development

Essentially everything a plant requires completing its lifecycle is provided by light energy, carbon dioxide (CO_2) and the uptake of water and mineral nutrients. From these essential compounds, the plant can create all biomolecules needed for growth, development, and reproduction. A basic overview is illustrated in Fig 3.

1.4.1 Carbon

Carbon (C) is central to all known living organisms, and it is the fundamental element in all plant biochemistry and forms the backbone of numerous biomolecules, including carbohydrates, proteins, nucleic acids, and lipids (Ribas-Carbo et al., 2010).

Heterotrophs, e.g. animals, are organisms that need to consume other organisms to obtain C and energy, as opposed to autotrophs, e.g. plants, that use light energy to assimilate C from the air and to produce their own energy. This energy is generated via photosynthesis, assimilating C from CO_2 into the cell biochemistry, e.g. into sugars. Sugars can be metabolised to other biomolecules and can be used to produce adenosine triphosphate (ATP), the energy currency of all organisms. The production of biomolecules and storage of energy in ATP is a two-step process, starting with glycolysis and ending with respiration. However, glycolysis can in principle run independently of respiration whereas respiration is dependent on substrates originating from glycolysis.

In the cell, glycolysis is a process that initially breaks down sugars to generate several C-metabolites and pyruvate, the primary substrate for the citric acid cycle – which is the hub for substrates used for the biosynthesis of, for example, amino acids (AAs), nucleic acids, and lipids – and for oxidative phosphorylation via the electron transport chain, i.e. production of energy, (Huppe & Turpin, 1994; Nunes-Nesi et al., 2010; Krasavina et al., 2014). After glycolysis, the substrate for respiration can enter the citric acid cycle either through direct transport into the mitochondria as the three-C molecule pyruvate or as malate, which after entering the mitochondria is converted back to pyruvate via malic enzyme (Koning, 1994; Wang et al., 2017). During respiration, the captured energy fixed in the chemical bonds of pyruvate is released through several oxidation steps in the citric acid cycle. The energy released in the citric acid cycle is transferred to three other molecules – nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide, and ATP – which are more cell-suitable forms for the storage and transfer of energy.

In contrast to autotrophic plant tissues that can produce and store energy through photosynthesis and C assimilation via the Calvin cycle, heterotrophic SE culture systems are dependent on the addition of exogenous sugar for C and energy. In the early years of developing plant embryo culture, among the first factors investigated was the importance of sugars for efficient embryo growth (reviewed by Raghavan, 2003). Today, sugars (e.g. sucrose, fructose, maltose) are standard additives for *in vitro* applications, with sucrose being most commonly used (Yaseen et al., 2012).

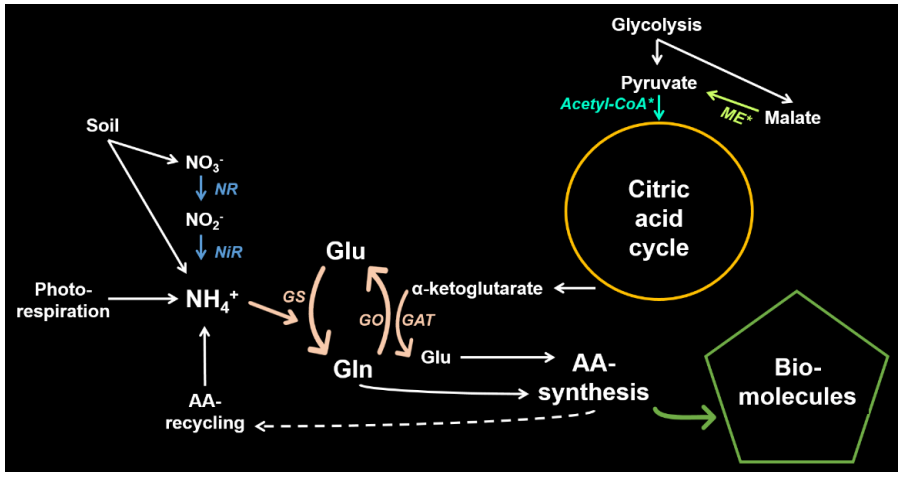

Fig 3. Simplified overview of assimilation of N originating either from photorespiration, uptake from the soil solution, or from recycling of amino acids (AAs), via the GS/GOGAT pathway, the linking point to the assimilation of C from the glycolysis and citric acid cycle, and the further synthesis of AA used for production of biomolecules. nitrate reductase, NR; nitrite reductase, NiR; glutamine synthetase, GS; glutamate-2-oxoglutarate aminotransferase, GOGAT; malic enzyme, ME.

1.4.2 Nitrogen

Nitrogen (N) is a necessary component of many biomolecules, including proteins, DNA, and chlorophyll. Even though the atmosphere is rich in N (78 % dinitrogen gas, N_2), most living organisms cannot access N in this physical state, and therefore N is considered a limiting factor for growth in many ecosystems (Näsholm et al., 2009; Bernhard, 2010; Nunes-Nesi et al., 2010; Britto & Kronzucker, 2013; Nacry et al., 2013). Thus, N nutrition studies in plant research often aim at understanding key aspects of plant N use efficiency (NUE) in a restrictive environment, for instance the boreal zone and/or cold ecosystems. Several aspects of NUE, such as N uptake, metabolism, allocation, and remobilisation, have been extensively studied from molecular, biochemical, physiological, and ecological perspectives both in natural settings and in anthropic systems, i.e. in agricultural and forest management. Generally, such research shares a common goal to optimise plant NUE to increase yield and quality and to minimise the negative environmental impacts of N fertilisation (Masclaux-Daubresse et al., 2010; Britto & Kronzucker, 2013; Nacry et al., 2013).

There are two inorganic forms of N that are accessible to plants, nitrate (NO_3^-) and ammonium (NH_4^+). Plants take up NO_3^- from the soil, and nitrite and NH_4^+ are produced via a two-step enzymatic reduction by the enzymes nitrate

reductase (NR) and nitrite reductase (NiR), respectively. This reduction is costly for the plants with respect to the energy required for the reactions (Lam et al., 1996; Krapp, 2015). Besides being an assimilation product of NO_3^-, plants can take up NH_4^+ directly from the soil. In photosynthetic tissues, NH_4^+ is also released as a by-product of photorespiration.

N uptake is often studied with respect to N source preference, which is defined as the N source taken up in the highest relative amounts or at the highest rates in different settings, or with respect to growth/yield parameters (Nacry et al., 2013). In addition, uptake of a N source can be influenced by so-called N repression, such as that described in the roots of *Arabidopsis thaliana*, where NO_3^- uptake has been shown to be repressed by the addition of external AAs, mainly by glutamine (Gln) (reviewed by Nacry et al., 2013; Gent & Forde, 2017, and references therein). Also, NH_4^+ uptake, transportation, and assimilation have been speculated to be regulated by other N sources (reviewed by Glass et al., 2002). Throughout this thesis, preference is defined as the N source contributing the most to the total N uptake and subsequent N assimilation into the free AA pool. It is therefore not necessarily the case that a preferred N source with respect to uptake is providing highest growth rates.

Assimilation of NH_4^+ into primary AA metabolism occurs via the enzymatic pathway of glutamine synthetase/glutamate-2-oxoglutarate aminotransferase (GS/GOGAT). In the GS/GOGAT pathway, NH_4^+ is incorporated into the two AAs glutamate (Glu) and Gln (Lea & Ireland, 1999; Forde & Lea, 2007), and together these two AAs represent the core of AA metabolism.

In angiosperms, AA metabolism is governed by the production of Glu and Gln in the chloroplasts of photosynthetic cells, which is controlled by the GS2/Fd-GOGAT cycle. Beside the plastid GS2 enzyme, the GS1 enzyme has been found in the cytosol (McNally et al., 1983; Ireland & Lea, 1999). For conifers, the only identified enzyme is GS1, but it has been found to exist in two isoforms, GS1a and GS1b. Both GS1a and GS1b have been found in the cytosol, but linked to photosynthetic and non-photosynthetic cells in different tissues, suggesting that the two isoforms have the same roles as the angiosperm GS1 and GS2. The gene expression of the two isoforms has been observed at different phases during *Pinus* embryogenesis (Cantón et al., 1999; Avila Sáez et al., 2000; Avila et al., 2001; Pérez Rodríguez et al., 2006; Cánovas et al., 2007).

An alternative way by which NH_4^+ may be assimilated is via glutamate dehydrogenase (GDH), which converts α-ketoglutarate and NH_4^+ into Glu, and this reaction is reversible. However, it is not clear whether GDH contributes to N assimilation or under which conditions the enzyme carries out assimilation of NH_4^+. GDH in plants has a K_m for NH_4^+ ranging from 10 to 80 mM (Stewart et al., 1980), inferring that the cellular levels of NH_4^+ must be very high for the

reaction to run in the assimilatory direction. A study following the assimilation of isotopically labelled NO_3^- and NH_4^+ in rice coleoptiles under both aerobic and anaerobic conditions suggested, based on the labelling pattern, that NH_4^+ assimilation mostly occurred via the GS/GOGAT pathway, whereas NO_3^- assimilation (following nitrate reduction to NH_4^+) could operate via GDH (Fan et al., 1997). Additionally, GDH has also been proposed to have a role of supporting the supply of C for the citric acid cycle through the deamination of Glu during C limiting conditions e.g. in darkness (Robinson et al., 1992; Aubert et al., 2001; Miflin & Habash, 2002). However, the general consensus is that the main role of GDH is to deaminate Glu, i.e. NH_4^+ release (for reviews see e.g. Dubois et al., 2003; Forde & Lea, 2007; Masclaux-Daubresse et al., 2010).

Beside the inorganic N sources available in the soil, there is also a considerable pool of organic N in the soil, including compounds such as free AAs, peptide-bound and protein-bound AAs, and organic acids. Over the last two-decade attention has been given to the aspect of plant organic N uptake from the soil (see the review by, e.g. Näsholm et al., 2009; Tegeder & Masclaux-Daubresse, 2018) and several transporters involved in AA uptake from the soil have been identified in *Arabidopsis thaliana*, e.g. lysine histidine transporter 1 (Hirner et al., 2006; Svennerstam et al., 2007), lysine histidine transporter 6 (Perchlik et al., 2014), amino acid permease 1 (Lee et al., 2007), and amino acid permease 5 (Svennerstam et al., 2008). Subsequent incorporation of AAs taken up into the N metabolism is suggested to occur through a series of transamination reactions rather than de-amination reactions (Persson et al., 2006; reviewed by Näsholm et al., 2009).

Both inorganic and organic N are commonly added to *in vitro* growth media (Bozhkov et al., 1993; George & de Klerk, 2008; Pullman & Bucalo, 2014). Historically, adding single or mixed AAs to growth media has its origin in a growth performance study of barley (*Hordeum vulgare*) embryos, showing increased dry mass and N content after the addition of various organic N compounds (reviewed by Raghavan, 2003). Growth media for many *in vitro* applications, including SE, are designed to provide ample amounts of each nutrient without the interaction of microbes and fungi found in soil systems. Media for SE have historically been optimised mainly using a trial-and-error-approach (as previously mentioned in section 1.3.2, sub-section SE in Norway spruce as a model system for research). This led to the observed positive effects of organic N on SE for several species and different tissues (Kirby, 1982; Bozhkov et al., 1993; Pinto et al., 1993; Khlifi & Tremblay, 1995; Ogita et al., 2001; Vasudevan et al., 2004; Hamasaki et al., 2005; Pescador et al., 2012). However, these studies did not always aim to determine the possible underlying mechanisms and the physiology behind these observations.

Casein hydrolysate (CH) and/or Gln are commonly used as organic N sources for different stages of the SE process (Bozhkov et al., 1993; Barret et al., 1997; Pescador et al., 2012; Llébres et al., 2018), but it is not known why organic N is such a good supplement in these tissue culture systems. This suggests that further research and optimisation of, for example, N composition might be required for a more thorough understanding of N metabolism in plants grown *in vitro*. The emphasis of this thesis has therefor been on the role of Gln, a molecule with both assimilated N and C, during Norway spruce SE.

2 Aim and objectives

The aim of this thesis was to support the forest plant industry by contributing basic knowledge about a technique with the potential for large-scale Norway spruce plant propagation at a time when the seed orchards are facing lethal problems with pests and pathogens. Increased knowledge of C and N utilization during different stages of SE in Norway spruce is necessary in order to develop improved and cost-efficient methods for SE plantlet propagation.

With the specific aim of investigating the role of Gln in the process of SE, my work has been focused on the following objectives:

- To provide additional insights into N nutrition during the proliferation of PEMs and the germination of somatic embryos, with an emphasis on the uptake of different N sources and the subsequent incorporation of these sources into AA metabolism.
- To further our understanding of the importance of the C in Gln for the synthesis of biomolecules, energy production, and the link to N metabolism in PEMs.
- To investigate the C and N budget of germinating somatic embryos of Norway spruce and to determine the importance of stored versus C and N supplied in the medium during the germination phase.

3 Materials and methods

3.1 Reasons to study *in vitro*-grown plants

Experimental plant research is often, by necessity, performed under highly controlled environmental conditions, often with fixed parameters such as light intensity, day/night rhythm, and temperature. Results obtained from such experiments are not necessarily relevant for plants growing in natural ecosystems where the plants experience, among other things, weather changes, seasonal variations, and competition for resources. To determine how the results from *in vitro* experiments should be interpreted, and to what extent the results are relevant for natural conditions, is thus a major challenge. This is especially important to take into consideration when studying *in vitro*-grown plants because they have been kept under restricted and controlled growing conditions and have not experienced a natural environment. However, there are situations where studies of plants grown *in vitro* are required, and research fields and applications that use *in vitro*-grown plants include, for example, visualisation of plant morphology, tissue sampling, large-scale screening experiments and conservation of plant biodiversity (Engelmann, 2011; Reis et al., 2017; Tikkinen et al., 2018).

This thesis deals with C and N nutrition during Norway spruce SE, which is performed under sterile *in vitro* conditions. Previous studies have reported that the *ex vitro* growth performance of SE plantlets is affected by maturation treatments with, for example, polyethylene glycol and abscisic acid (Bozhkov and von Arnold, 1998; Högberg et al., 2001) and different light conditions during germination (Kvaalen & Appelgren, 1999; von Aderkas et al., 2015). Therefore, it is highly desirable to gain as much knowledge about the *in vitro* process as possible to increase the survival rate of the embryos and their performance upon transfer to natural conditions in greenhouses, in nurseries, and in forest settings.

3.2 Composition of growth media

The medium composition was a crucial aspect of the experimental set-ups used in this study. Choosing a medium might seem a trivial task because there are many reports in the literature aiming at developing culture protocols for optimum growth performance in areas such as initiation success, proliferation rate, and number of mature somatic embryos (George & de Klerk, 2008; Pullman & Bucalo, 2014). Consequently, the selection of experimental media must be made with consideration because the medium composition determines which questions can be asked and what conclusions can be drawn. For example, both the total amount and the proportions of supplied nutrients highly influence SE performance. Nutrients are often supplied as salts, which consist of more than one ion, thus it is important to avoid artefacts from, for example, too high of an ion concentration or an ion imbalance, which might be caused when trying to achieve ion ratio regulation (George & de Klerk, 2008; Niedz & Evens, 2008).

Therefore, the media used for the studies in this thesis were designed to enable studies of different SE phases and of tissues grown on different C and N sources and to minimize artefacts resulting from changes in the concentrations of other ions.

3.2.1 Proliferation media

The proliferation media used in the studies were designed to enable the comparison of PEM growth on different N sources and different N concentrations, as well as to enable the assessment of the relative importance of the different N sources, i.e. measuring the relative uptake of each N source and to what extent each N source is assimilated into the primary pool of total free AAs (**paper I**). The media also contain two C sources – sucrose and Gln – that can be taken up by PEMs and be assimilated into the free AA pool and enter the citric acid cycle (**paper III**) to produce metabolites that can be used as substrates for respiration.

The standard medium chosen for proliferation, also referred to as ½-LP (von Arnold & Eriksson, 1981), has been successfully used in the past for proliferation of Norway spruce PEMs. Choosing a previously studied medium (for PEMs) as a starting point facilitates comparison of previously obtained data. The media compositions of supplied N and C sources, including total N concentration and relative proportions of the N sources used in **papers I** and **III** are illustrated in Fig 4. Thus, in **paper I**, growing PEMs on PM#1 or PM#2 and on PM#3, PM#4, or PM#5 allowed for a comparison of PEM performance growing on the same N species but with different total N content. It also allowed for the comparison of PEM performance on PM#2 and PM#3, i.e. media with

the same total N content but with different N sources (i.e. only inorganic N sources in PM#2, whereas PM#3 contained both inorganic N and Gln N). Thus, this comparison gave an assessment of the importance of a specific N source rather than the total amount N given.

In **paper III**, comparing PM#1 (only sucrose as the C supplement) to PM#3 (both sucrose and Gln as the C supplements) enabled the investigation of Gln as a source of C and energy.

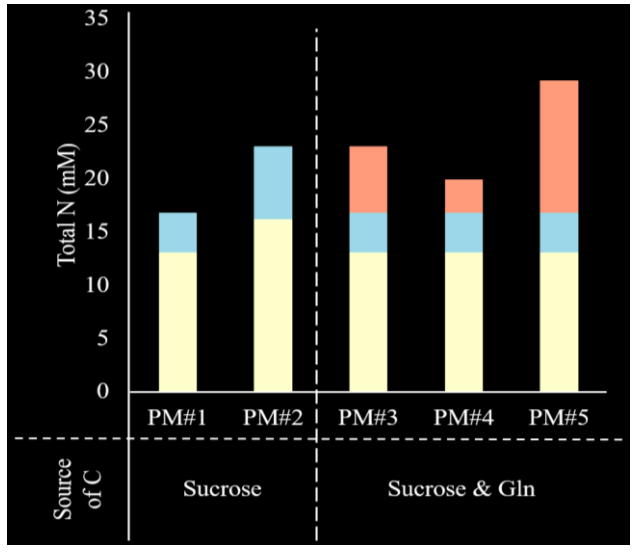

Fig 4. Experimental proliferation media compositions with inorganic and organic N – including fractions of the N sources in the form of NO_3^- (yellow), NH_4^+ (blue), and Gln (orange) – and supplied C sources used in **paper I** and **III**.

3.2.2 Germination media

As mentioned earlier in the introduction, a mix of organic N, such as CH, is commonly supplied to *in vitro* growth media. CH is a mixture of AAs, and depending on whether the CH is added to the medium before autoclaving or after autoclaving (sterile filtered), part of the N in CH might be present as NH_4^+ (e.g. as the result of AA degradation during autoclaving). One goal of this study was to use ^{15}N-labelled N sources to trace the uptake and assimilation of N from the different N sources provided. Obtaining ^{15}N-labelled CH would have been challenging, if not impossible, and very costly. In addition, even if obtaining ^{15}N-CH would have been possible, it would be impossible to determine the origin of each ^{15}N molecule taken up and further assimilated because CH is a mix of different N sources. Therefore, to enable this experiment, Gln was used instead of CH. To ensure that this change did not cause any unwanted effects on

germination, a small pilot study was performed comparing germination on media containing CH or Gln. No detrimental effects were observed from using Gln instead of CH.

In addition, Gln was chosen as the organic N source to test the hypothesis that Gln would be a preferred N source (as in **paper I**) and to assess whether Gln would have a similar importance for relieving metabolic constraints as suggested by the remarkably high level of alanine (Ala) present in the PEMs (**paper I**).

3.3 Analytical methods using ^{13}C- and ^{15}N-isotopes

This work was largely based on analytical methods using ^{13}C- and ^{15}N-isotopes, which are described here in the following sections.

3.3.1 Isotopes and isotopologues

Without going deeply into the chemistry and physics, a molecule consists of atoms attached to each other by chemical bonds. An atom is the smallest unit of matter and is made up of three particles – protons, neutrons, and electrons. The average mass of all the particles in the whole molecule is considered to be the molecular mass or molecular weight. An isotope of a compound has equal numbers of protons but not the same number of neutrons, and therefore different isotopes of a compound have different mass numbers (total numbers of protons and neutrons). Natural abundance is the relative amount of each possible isotope in a natural sample; for instance, the natural abundances of ^{13}C and ^{15}N are 1.06 % and 0.37 %, respectively (Meija et al., 2016). Hence, the average molecular mass of Gln, which has five C, ten hydrogen (H), two N, and three oxygen (O) atoms (Fig 5a) includes the natural abundance of ^{13}C, ^{2}H, ^{15}N, and ^{18}O isotopes. Therefore, it is possible to have different forms of, for example, isotopically labelled ^{15}N-Gln (Fig 5b-d).

Consequently, molecules can have the same molecular formula and structure but a diverse isotopic composition through the exchange of one or several atoms with an isotope or isotopes, and these are known as isotopologues. The monoisotopic mass and the isotopologues (m^{+1}, m^{+2}, etc.) illustrated in Fig 6 can be separated and detected with mass spectrometry techniques. For example, the actual monoisotopic mass of Gln is 146.0691 g^{-1} mol, and if Gln contains one or two ^{15}N atoms, the isotopologues would then be detected as 147.0662 g^{-1} mol (m^{+1}) or 148.0632 g^{-1} mol (m^{+2}), respectively. These isotopologues will thus have different properties that can be used for separation between the monoisotopic mass (the mass of the most abundant isotope, which in Fig 6 would be ^{14}N) and other isotopic peaks.

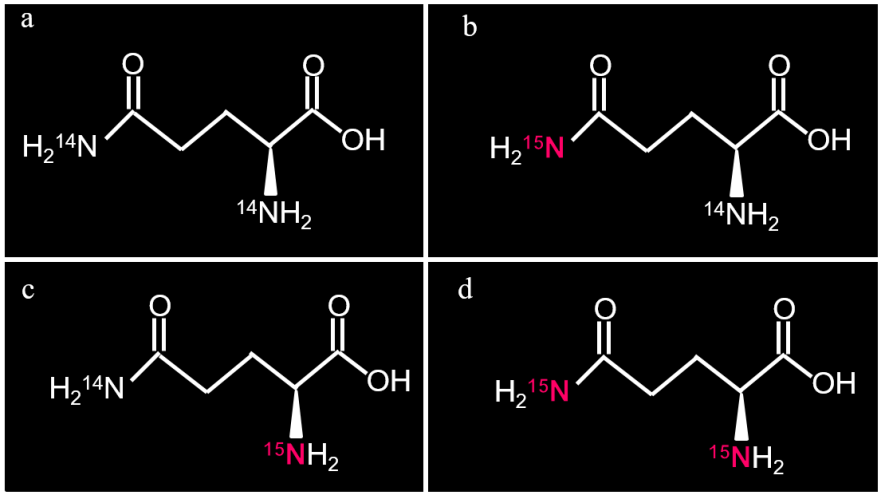

Fig 5. The molecular structure of Gln (a). The structures of three different forms of isotopically labelled ^{15}N-Gln, indicated by the dark pink ^{15}N atom (b–d).

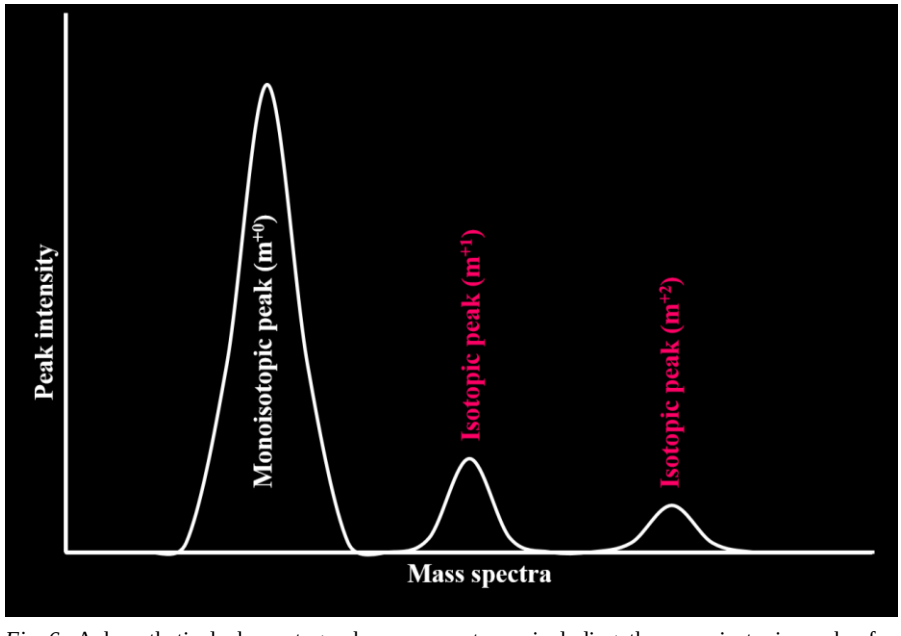

Fig 6. A hypothetical chromatograph mass spectrum, including the monoisotopic peak of a molecule and two isotopologue peaks (m^{+1} and m^{+2}) indicated by the dark pink text.

3.3.2 Reciprocal isotopic labelling

A nutrient such as Gln provided in a growth medium will mainly contain ^{14}N atoms. Using media supplemented with isotopically labelled ^{15}N-Gln enables the calculation of the mass fraction discrepancy of the exchanged source (or sources) with its isotopic source without changing the medium composition.

In this work, the replacement of molecules containing a certain isotope (in **papers I** and **II** ^{14}N, and in **paper III** ^{12}C) with a molecule containing another isotope (in **papers I** and **II** ^{15}N, and in **paper III** ^{13}C) is called "reciprocal isotopic labelling" (Fig 7), and this makes it possible to study the origin of a specific atom by separating it from a molecular source in an environment where the atom exists in several sources.

For instance, in a hypothetical growth medium, N atoms originate from four different molecules containing N (Fig 7a), and these N sources are then reciprocally labelled in four different medium aliquots (Fig 7b-e). The medium composition remains unchanged with respect to N concentration and the relative amounts of each N source and total N content, but the shift in atom mass in the different media enables calculations of different isotopologues.

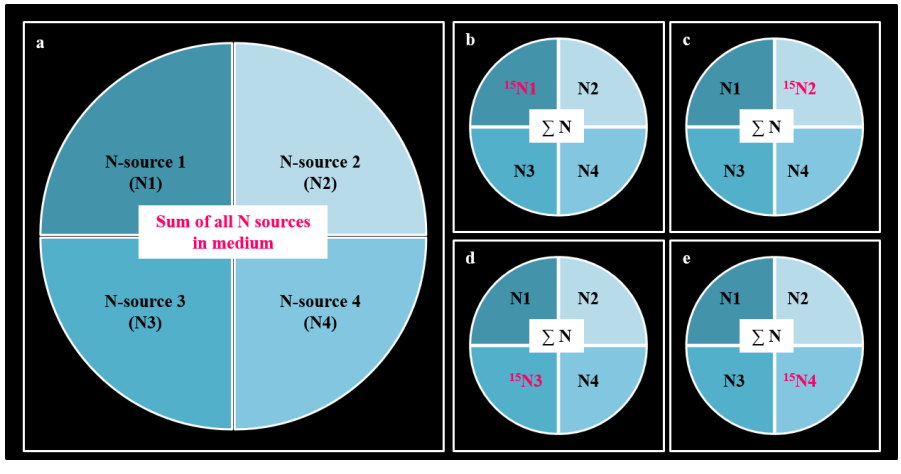

Fig 7. Illustration of a theoretical medium containing four different sources of N (a) and how the four N sources are reciprocally labelled, indicated by pink ^{15}N atom (b–e). The medium composition remains unchanged with respect to the relative amounts of each N source and the total N concentration.

3.4 Using ^{13}C and ^{15}N-isotopes to measure uptake, assimilation, and respiration

In the absence of the megagametophyte during cultivation of somatic embryos, the SE culture conditions must resemble the natural seed in terms of

nutrient compositions and plant growth regulators. Optimised culture practices are therefore very important for the development of somatic embryos of good quality and in large quantities. Hence, the focus in this work was directed towards C and N utilisation during different stages of SE in Norway spruce to study the contribution of C and N taken up from the medium into the biomass and then tracing C and N into the primary metabolism via assimilation.

3.4.1 Uptake

Plants take up nutrients mainly by the roots via i) simple diffusion, ii) passive transport, or iii) active transport (reviewed by, for example, White, 2012). Studies of nutrient uptake in *in vitro* culture systems provide an overview of how the growing tissue consumes the provided resources by measuring the uptake of specific C and N sources in the presence of all available sources in the growth medium. Uptake studies can be designed in different ways, and short-term incubation with isotopically labelled N sources will reflect gross uptake, i.e. total amount taken up, whereas long-term incubation provides an efflux component because the labelled N sources can be recycled in different pathways and N atoms might be released back into the medium. In addition, measuring uptake by analysing remaining N containing compounds in the medium, so called depletion experiments, will reflect net uptake.

Analysis of C and N using elemental analyser isotope ratio mass spectrometry (EA-IRMS) generates mass fraction data of atoms and both $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios. These data can be used to calculate the amount of total C and N and how much isotopic ^{13}C and ^{15}N have been taken up by the tissue (after the subtraction of the natural abundance).

3.4.2 Assimilation

To contribute to growth, the N taken up must be assimilated into AA metabolism (Ortiz-Lopez et al., 2000). To support growth, either as a C-backbone or as an energy source, the C that is taken up must be metabolised in the glycolysis pathway, in which C can be released as CO_2 through respiration (generating energy) or incorporated into core N and C metabolism by providing substrates for the citric acid cycle and for biosynthesis of, for example, AAs (Huppe & Turpin, 1994; Nunes-Nesi et al., 2010; Krasavina et al., 2014).

Both combined liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) analyses generate chromatographic and mass spectral data, including isotopologue information (Fig 6). This allows for the calculation of ^{13}C-labelled or ^{15}N-labelled

metabolites in a sample. However, to be able to perform the calculation, the sample material needs to grow on both non-labelled and isotopically labelled medium because the natural abundance of the isotope must be subtracted for every single molecule examined.

In all three papers presented in this work, LC-MS was used for targeted AA profiling. In addition, in **paper III**, GC-MS was used for analysing citric acid cycle metabolites.

3.4.3 Respiration

The energy requirements of a plant cell are mainly fulfilled by the energy produced during respiration and the electron transport chain, as described in the introduction section 1.4.1. Therefore, CO_2 respiration measurements can be used as a way of investigating tissue metabolic activity (Gonzalez-Meler et al., 2004). In the proliferation medium, the most abundant C source is sucrose, but given the possibility that Gln can also contribute as a source of C, the study performed in **paper III** was designed to investigate the relative contribution of these C sources as respiration substrates. To measure the respiration of the respective C sources, ^{13}C-labeled sucrose and Gln were added to PEM cultures, and the respiration output was analysed for the delta ^{13}C of CO_2 output, i.e. the fraction of $^{13}CO_2$ versus $^{12}CO_2$.

4 Results and discussion

4.1 N uptake and assimilation in Norway spruce PEMs (Paper I)

The studies in **paper I** aimed to increase our understanding of N nutrition in Norway spruce PEMs, with a special focus on the PEM uptake capacity of different N sources and the subsequent assimilation of these N sources into the free AA pool. Two hypotheses were formulated. The first was that the previously observed positive growth effect of Gln (Kirby, 1982; Bozhkov et al., 1993; Pinto et al., 1993; Khlifi & Tremblay, 1995; Ogita et al., 2001; Vasudevan et al., 2004; Hamasaki et al., 2005; Pescador et al., 2012) is due to the N species that is added rather than to an increase in the total N concentration. The second, based on previous studies showing that conifers preferentially take up AAs and NH_4^+ (Marschner et al., 1991; Kamminga-van Wijk & Prins, 1993; Kronzucker et al., 1996, 1997; Gessler et al., 1998; Malagoli et al., 2000; Öhlund & Näsholm, 2001, 2004; Persson et al., 2006; Miller & Hawkins, 2007; Metcalfe et al., 2011; Gruffman et al., 2013, 2014), was that the increased growth effect results from different uptake and utilisation capacities of the single N forms.

The goal was to trace ^{15}N in both the tissue and in free AAs, reflecting the uptake preference and assimilation capacity of the different N sources, respectively. This was achieved by designing the proliferation media as described in section 3.1.1, thus allowing for a comparison of PEMs grown on different total N concentrations and on different exogenously supplied N sources. The studies were performed in combination with the reciprocal set-up explained in section 3.2.2 supplying the growth medium with N sources labelled with the stable ^{15}N isotope.

The findings in **paper I** showed that PEMs grown with only inorganic N had significantly lower concentrations of free AAs, less biomass (Fig. 1, **paper I**), and visual signs of an unhealthy culture as compared to PEMs grown on Gln

supplied in the medium, which had a 15-fold higher concentration of total free AAs (Fig. 2, **paper I**), greater biomass, and visual signs of a healthy culture.

The study also confirmed the hypothesis that the growth effect is due to N source rather than an increase in total N concentration because addition of Gln had a more positive effect on growth than addition of corresponding amounts of inorganic N. The second hypothesis was also confirmed, that PEMs have a controlled N uptake with a preference for Gln over NH_4^+ and NO_3^-. In addition to confirming the two hypotheses, an additional observation about assimilation was made, showing that out of the total amount of assimilated N in the free AA pool, 64 % originated from Gln, 25 % originated from NH_4^+, whereas only 11 % originated from NO_3^-(Fig. 5, **paper I**).

For PEMs grown with exogenously supplied Gln, all added N sources were found to be assimilated into the free AA pool, but the contribution of the different N sources to the total N assimilation was not equal nor reflecting the fraction of provided N sources. This is indicative of active N metabolism, with functional NR, NiR, GS, and GOGAT enzymes, even though there seems to be a restriction on the NO_3^- assimilation enzymes. The assimilation of NH_4^+-N represents a greater fraction of the total N assimilated as compared to the fraction of NH_4^+ taken up, and this suggests the presence of an active GS enzyme and a functional GS/GOGAT pathway. An alternate way of assimilating NH_4^+ into the AA pool is via GDH, but as described in the introduction section 1.4.2, GDH is considered to be mainly involved in Glu catabolism. Although it cannot be ruled out, it is unlikely that GDH makes a major contribution to NH_4^+ assimilation.

As a final observation, exogenously supplied Gln also alleviated the seemingly inadequate AA metabolism of PEMs grown in the presence of only inorganic N sources, as evidenced by the reduced biomass growth of PEMs and the low concentration of free AAs in PEMs grown without Gln supplied in the media as compared to PEMs grown with Gln supplied in the medium. Because remarkably high concentrations of Ala were observed in PEMs grown in the presence of Gln (Fig. 3, **paper I**), this suggest that Ala biosynthesis was involved in alleviating these metabolic constraints (see also below, section 4.3, **Paper III**, and section 4.6).

4.2 Nitrogen utilization during germination of somatic embryos of Norway spruce (Paper II)

The germination stage is one of the bottlenecks for Norway spruce SE. To what extent stored versus supplied nutrients contribute to growth and development has not been extensively studied, which makes it difficult to optimise germination medium with respect to nutrient content. Therefore, a

deeper knowledge of the importance of C and N utilisation during somatic embryo germination is required. The studies in **paper II** were therefore performed to investigate the C and N budget for germinating somatic embryos of Norway spruce and to increase our understanding of N nutrition during germination. Two main aims were formulated – (i) to examine the importance of stored C and N versus C and N in the medium, and (ii) to characterise N metabolism in terms of N uptake and assimilation. Based on the findings regarding N uptake and assimilation in PEMs (**paper I**), a hypothesis was formulated that Gln is a significant N contributor to AA metabolism.

Similar to **paper I**, the goal with the experimental set-up in **paper II** was to study uptake preference and assimilation of the different N sources by tracing the ^{15}N into tissues and the assimilation of ^{15}N into free AAs. The differences from **paper I**, were i) the germination medium as explained in section 3.1.2, ii) the sampling was carried out over 24 days at five different time points, and iii) the light condition was set to low intensity red light to limit photosynthesis and promote root development. This set-up also had the advantage that it was possible to distinguish between C originating from the embryo and the medium because C from CO_2 fixation was minimised.

The germinant fresh biomass was found to increase throughout the study period, and the fresh weight increased 8-fold between day 1 and day 24 of germination. The germinant dry biomass was 3.5 times higher after 24 days of germination as compared to the initial mature somatic embryo starting weight. The fresh weight/dry weight ratio was lowest at day 1, and thereafter it increased and reached the highest observed ratio day 12, and then the ratio declined somewhat until day 24 (Table 2, **paper II**). The increased fresh biomass and low fresh weight/dry weight ratio found early in the study would suggest re-hydration because the somatic embryos were coming from a desiccation treatment before they were placed on germination medium.

Measuring uptake of nutrients can provide an overview of nutrient utilisation, and C and N concentrations in a plant (% w/w) are considered to be a representation of the overall C and N status of the plant. The C concentration in the germinant was reduced from 57 % at day 1 to 46 % at day 12 and remained stable between days 12 and 24 (Fig. 1a, **paper II**). The initially measured relatively high C concentration suggests that a considerable part of the C stored in the mature somatic embryo is in the form of lipids, which is the only group of biomolecules to have such concentrated amounts of C (Schmid & Ohlrogge, 2002). The stabilized C concentration observed between days 12 and 24 thus suggests that the storage lipids have been consumed and re-synthesised into other biomolecules with a relatively higher H, O, and N content. The C content on the other hand, increased 2.6 times overall during the 24-day study period,

but day 6 to day 12 no changes in biomass development were seen. Morphological changes were observed by imaging between day 6 and 12, and during this time the somatic embryos developed into a shape more resembling a seedling structure with distinct root and shoot parts. Why there was a standstill in C increase cannot be determined from this study, but one possible reason for this observation might be developmental processes linked with greater respiration, i.e. CO_2 release.

The study found increasing N concentration after 6 days of germination but no further change between days 12 and 24. The germinant had a pattern of increasing N content similar to the C content, but without the standstill between days 6 and 12. The uptake data also showed that already after 3 days more than 25 % of the total N content in the germinating somatic embryos originated from the N sources supplied in the medium. However, after 24 days 90 % of the total N taken up originated from N supplied in the medium.

In addition, the UPLC-UV and LC-MS analysis confirmed the hypothesis that Gln-N would be a significant contributor to the TFAA pool of a Norway spruce somatic embryo during the first stages of germination because 50 % of the assimilated N found in the germinants originated from exogenously supplied Gln (Fig. 4, **paper II**), thus being a significant N contributor.

Unlike the observation of high levels of Ala in **paper I**, this study did not find any unusually high levels of AAs related to metabolic stress pathways. Despite this, data from the LC-MS analysis showed an alternation of the assimilation pattern in the germinants over time, with the Gln:arginine (Arg) assimilation ratio decreasing from 2.7 at day 3 of germination to 1.5 at day 24, implying that the germinants were mobilising their N metabolism towards synthesising AAs with a high N to C ratio (Fig. 3 and Table S3, **paper II**). In line with the observation in **paper II**, increased concentrations of free Arg, Glu, and Gln have previously been reported for conifers as a result of hydrolyzation of storage compounds during seed germination (Gifford & Tolley 1989; King & Gifford 1997; reviewed by Cánovas et al. 2007). A hypothesis based on those results is that Norway spruce somatic embryos growing under red light accumulate N-rich AAs as a form of N storage until light conditions change, at which time hydrolyzation of storage reserves is required to facilitate photosynthesis. However, additional studies under white light are essential to confirm this.

Because mature somatic embryos lack the nutritious megagametophyte tissue, it is not surprising that the nutrition supplied by the medium is important during *in vitro* germination. Thus, the findings in **paper II** show that germinating somatic embryos under low-intensity red light conditions are highly

dependent on the nutrients provided in the medium for their growth and development.

4.3 Impact of exogenously supplied Gln on C metabolism during proliferation of Norway spruce PEMs (Paper III)

The findings in **paper I** – showing that Gln supplied in the medium was the main source of N and relieved metabolic constraints – motivated the studies carried out in **paper III** investigating aspects of energy, respiration, and metabolic constraints during PEM proliferation. The first two hypotheses were that exogenously supplied Gln would contribute with i) an already assimilated source of C for biomass production and ii) energy production through the respiration of α-ketoglutarate. The third hypothesis was that Gln is essential for PEM metabolism by relieving unidentified metabolic constraints.

The experimental set up in **paper III,** in contrast to the other two papers, had the goal to follow ^{13}C-labelled sucrose and Gln into the biomass, AAs, and energy-related metabolism. The medium used for proliferation was the same as **paper I**, as described in section 3.1.1, as well as the use of reciprocal labelling (explained in section 3.2.2). However, the interpretation of the pulse-chase labelling data in **paper III** was complex. The ratio of Gln:sucrose C in the standard medium was approximately 1:23. For the pulse-chase experiment, a ratio of 1:1 was used to ensure that Gln derived ^{13}C would be detectable in the compounds analysed. Consequently, the contribution of Gln C supplied in the medium to AAs and citric acid metabolites could be over-estimated if only looking at the processed data and figures.

In **paper III**, the exogenously supplied C sources' contributions to PEM C content were found to be 4.1 ± 0.2 % for Gln and 95.9 ± 0.8 % for sucrose, indicating that Gln is not important as a source of C for biomass production.

Moreover, although respiration experiments showed that Gln was functional as a respiration substrate for PEMs (Fig. 1, **paper III**), the amount of exogenously supplied Gln available in standard medium would not be great enough to represent a significant substrate for respiration. More importantly, Gln supplied in the medium was found to increase the PEMs' ability to respire C from sucrose supplied in the medium (Fig. 2, **paper III**). This finding suggests that the PEMs are experiencing some form of stress or metabolic constraint that is relieved by Gln.

The AA profile obtained in this study showed that PEMs grown in the presence of exogenously supplied Gln generally had a significantly higher level of AAs, and a particularly higher concentration of Ala, findings that are in line

with the results presented in **paper I**. However, there was no significant difference between the concentrations of tissue Gln in PEMs grown with or without Gln supplied in the medium (Fig. 3, **paper III**). This suggest that the internal Gln pool is consumed by metabolic processes, with one being Ala biosynthesis given its great abundance and that Ala-N was mainly derived from Gln-N taken up from the medium, as mentioned earlier in section 4.1 (**paper I**) and further discussed below in section 4.6.

The dominating source of Ala-C was sucrose and the predominant accumulation of ^{13}C in the Ala^{+3} isotopologue (Table S2, **paper III**) strongly suggests that Ala was produced from pyruvate (having three C atoms) directly after glycolysis via the enzyme alanine aminotransferase (AlaAT). Given the findings of **paper I**, the primary Ala N-donor is primarily Gln-derived Glu.

The high accumulation of Gln derived C in the $GABA^{+4}$ isotopologue (Table S2, **paper III**) is indicative of an active GABA-shunt, also suggesting a direct connection between exogenously supplied Gln C and the synthesis of GABA. However, on standard medium, where the concentration of Gln supplied in the medium is much lower than in the pulse-chase experiment, the GABA C backbone could be predominantly sucrose derived.

The data generated via GC-MS analysis, tracing sucrose C and Gln C into five citric acid cycle metabolites; citrate, α-ketoglutarate, succinate, fumarate, and malate, is presented as peak areas of ^{13}C excess (Fig. 5, **paper III**). Absolute concentrations were not measured, and consequently the only comparison of metabolite abundance possible is between treatments, and not with other metabolites. Unfortunately, pyruvate could not be analysed with the GC-MS method that was used, and this made the interpretation of the data more difficult. The GC-MS data showed that the capacity of PEMs to metabolise Gln-derived C was not affected by the absence or presence of exogenously supplied Gln. However, the presence of Gln in the growth medium did significantly increase the levels of sucrose C in three of the five metabolites – citrate, succinate, and malate. The absolute amount of malate also increased in response to medium amended with Gln. This suggest that malate is accumulating due to increased biosynthesis and/or decreased turn-over when Gln is supplied in the medium.

The combination of respiration experiments and GC-MS analysis in **paper III** showed that Gln supplied in the medium has a profound positive effect on sucrose metabolism, something that also translates into significantly improved growth (**paper I**). The targeted AA profiling clearly demonstrates the presence of two stress-related metabolic pathways – the sucrose-driven AlaAT pathway and the GABA shunt – both of which are dependent on Gln-derived Glu as substrate.

4.4 Uptake of provided N sources

4.4.1 The inorganic N sources

In the medium composition used in both **paper I** and **II**, NO_3^- was the most abundant N source during the proliferation and germination phases. However, both studies found that NO_3^- was the N source least utilized, i.e. the fraction of total NO_3^--N taken up (Fig. 4, **paper I**, and Fig. 2, **paper II**) was smaller than that provided in the medium, and the fraction of NO_3^--N assimilated into the free AA pool was smaller than the fraction taken up as NO_3^- (Fig. 5, **paper I**, and Fig. 4, **paper II**). This agrees with studies of conifers in natural conditions, which have been shown to have a preference for NH_4^+ (Marschner et al., 1991; Kamminga-van Wijk & Prins, 1993; Kronzucker et al., 1996, 1997; Gessler et al., 1998; Malagoli et al., 2000; Öhlund & Näsholm, 2004; Miller & Hawkins, 2007) and AAs (Öhlund & Näsholm, 2001, 2004; Persson et al., 2003, 2006; Metcalfe et al., 2011; Gruffman et al., 2013, 2014) compared to NO_3^-.

Another reason for the observed low NO_3^- uptake in PEMs and germinating somatic embryos of Norway spruce could be that there are constraints for uptake and/or subsequent assimilation, e.g. due to regulation of NO_3^- transporters and/or enzymes in the assimilation pathway. There are some reports in the literature regarding the characteristics of NO_3^- transporters in developing embryos, including those involved in NO_3^--N transport and those involved in N accumulation and storage (Chopin et al., 2007; Almagro et al., 2008; Léran et al., 2015). For conifers, high-affinity NO_3^- transporters (NRT2/NRT3) have been identified in pine roots and have been proposed to play a key role in the uptake and transportation of exogenously accessible NO_3^--N (Castro-Rodríguez et al., 2016, 2017). Wang et al. (2012) summarised the identified NO_3^- transporters for *Arabidopsis thaliana* and their physiological functions and regulations, and they showed that NO_3^- transporters are regulated by, for example, levels of NO_3^- and NH_4^+, limitations on the total N concentration, light intensity, sucrose, and diurnal rhythm.

In **papers I** and **II**, i.e. for both PEMs and germinants, NH_4^+ was taken up in greater amounts relative to what was provided in the growth media (Fig. 4, **paper I**, and Fig. 2, **paper II**). Currently, the consensus is that the mechanisms controlling NH_4^+ utilization i.e. uptake, transportation, and assimilation, are regulated by the downstream metabolites of N assimilation, i.e. Gln and Glu (reviewed by Glass et al., 2002). Because no uptake data from either PEMs (**paper I**) or germinants (**paper II**) grown on media supplied only with inorganic N were obtained in the studies presented within this thesis, it cannot be excluded that Gln addition had a down-regulating effect on NH_4^+ uptake. Nevertheless,

the findings in **paper I** show that PEMs grown on medium without the addition of Gln were unable to maintain functional AA metabolism, suggesting that the supplied Gln is vital for adequate AA metabolism in PEMs rather than having a negative impact on uptake of NH_4^+ (and NO_3^-). It could also be that for PEMs, the positive effect on metabolism overshadows any negative effects of Gln on NO_3^- or NH_4^+ uptake. Furthermore, in **paper II**, it was shown that despite increasing internal Gln concentration in the germinants over the study period (Fig. 3, **paper II**), the percentage of NH_4^+-N contributing to the total N assimilation was stable (Figs. 4 and 5, **paper II**).

Plant seed germination is induced by light, especially red light (Borthwick et al., 1952), which is sensed by a class of photoreceptor proteins named phytochromes (Shinomura et al., 1994). For somatic embryos of both pines and Norway spruce, studies have shown that red light treatment has a positive impact on germination frequency and the elongation length of both the root and hypocotyl (Kvaalen & Appelgren, 1999; Merkle et al., 2005). In **paper II**, the fraction of NH_4^+ uptake was stable at around 30 % over the studied germination period under red light. In a pilot study in which germinants were transferred to white light for three weeks (after the initial red light treatment of three weeks), preliminary results showed that the fractions of NO_3^--N, NH_4^+-N, and Gln-N uptake were 33 %, 24 %, and 43 %, respectively. This suggests that light stimulation enhances the uptake of inorganic N, possibly because increased photosynthetic activity generates more C-backbones for respiration and AA production.

More studies regarding NH_4^+ and NO_3^- uptake during the whole germination phase might resolve this matter and give a deeper knowledge on N nutrition in Norway spruce during the SE process.

4.4.2 The organic N source - Gln

In PEMs (**paper I)** and germinants (**paper II)**, Gln was taken up to a greater extent relative to what was supplemented in the growth media (Fig. 4, **paper I**, and Fig. 2, **paper II**). Comparing the discrepancy in the amount of provided inorganic N and Gln compared to the amounts of inorganic N and Gln taken up, Gln could be considered to be a preferred N source and a way for the plant tissue to save energy (see further discussion below in section 4.5).

The addition of Gln in the growth media could have a repressing influence on NO_3^- uptake, as mentioned generally above in section 1.4.2. Based on the experiments in **paper I**, N repression is unlikely because the observed growth on the proliferation medium amended only with inorganic N sources was poor.

For the germinating somatic embryos (**paper II**), Gln repression of NO_3^- uptake cannot be ruled out based on the results from the study. However, in a recently published study using 12-week-old germinated somatic embryos of hybrid white pine, it was found the root uptake of NO_3^- and NH_4^+ was more rapid if the embryos germinated on media supplied with only organic N compared to if inorganic N also had been supplied to the germination media (Llebrés et al., 2018).

In **paper I**, PEMs grown on medium without exogenously supplied Gln were incapable of maintaining AA metabolism, as shown by the 15-fold lower concentration of total free AA. This suggests that instead of being a negative regulator of inorganic N utilization, Gln is vital in this type of culture system.

4.5 Assimilation of N and C

With respect to N assimilation into Gln, from a plant's point of view it is energetically beneficial to assimilate NH_4^+ as compared to NO_3^-. Assimilation of NO_3^- to NH_4^+ via NR and NiR, and subsequently to Gln via GS/GOGAT, theoretically requires energy corresponding to 12 ATP (Briskin & Bloom, 2010).

The medium composition is the basis for SE growth performance, and in **papers I** and **II** the most abundant N-source in the media was NO_3^-. The results however, show that NO_3^- was the least abundant N-source after assimilation. This finding emphasises why it is important to know the actual uptake in relation to the media composition.

Given the substantial assimilation of NH_4^+-N into Gln and other AAs in both **papers I** and **II**, the data indicate that the GS/GOGAT pathway is functional and does not appear to be down-regulated despite a high concentration of Gln both externally in the media and internally in the tissue. Additionally, in **paper III**, ^{13}C-sucrose was found in Glu, i.e. via glycolysis and the citric acid cycle generating α-ketoglutarate for use as a substrate for Glu, and this is another piece of evidence suggesting that the GS/GOGAT pathway is operating in PEMs of Norway spruce, at least under the conditions studied.

As a final observation, in **paper II** the observation of N-rich AA build-up, i.e. Gln and Arg, adds further support for the GS/GOGAT pathway being active and efficient even during the germination phase of Norway spruce SE.

4.6 Gln alleviates metabolic stress in PEMs

In **paper I**, a remarkable observation for PEMs grown on medium amended with Gln was the high concentration of Ala. Furthermore, out of the total N assimilated into Ala, Gln N contributed about 66 %. Elevated Ala levels in a

conifer SE context have, to my knowledge, only been reported previously by Joy et al. (1997), but these were not further discussed by the authors. High levels of Ala have also been reported for plants grown under anaerobic stress (Good & Crosby, 1989; Miyashita et al., 2007; Limami et al., 2008; Narsai et al., 2011). Hence, the findings in **paper I** suggest that PEMs in this type of *in vitro* cultivation system are under stress and that the Gln supplied in the medium in some way alleviates the symptoms of this stress. In **paper I**, it is speculated that Ala is (i) a storage arrangement for both assimilated C and N during low-oxygen stress, (ii) a regulatory pathway avoiding the production of acetaldehyde, which is very toxic for plants, or (iii) a by-product from the so-called GABA shunt, which short-cuts the citric acid cycle, i.e. it eliminates the oxidation steps of α-ketoglutarate to succinate.

As an alternative connection between AA metabolism and the citric acid cycle, the GABA shunt operates via three enzymes – glutamate decarboxylase (GAD), GABA transaminase (GABA-T), and succinic semialdehyde dehydrogenase (SSADH). GABA is synthesised by GAD using Glu as the substrate, followed by GABA and pyruvate generating Ala and succinic semialdehyde via GABA-T. In the final reaction, succinic semialdehyde is used to create NADH and succinate via SSADH (Shelp et al., 1999; Michaeli & Fromm, 2015). The findings in **paper III** showed that PEMs sucrose-C metabolism was enhanced in PEMs grown on medium amended with Gln as compared to PEMs grown on without Gln supplied in the medium. Thus, the work presented in this thesis suggests that the AA metabolism in PEMs is dependent on two stress-related metabolic pathways – the AlaAT pathway and the GABA shunt – and that both pathways require supplied Gln for generating Glu as a substrate and N donor. Additionally, C-backbones for AA metabolism and respiration are generated from sucrose supplied in the medium via glycolysis and the citric acid cycle (illustrated in Fig 8).

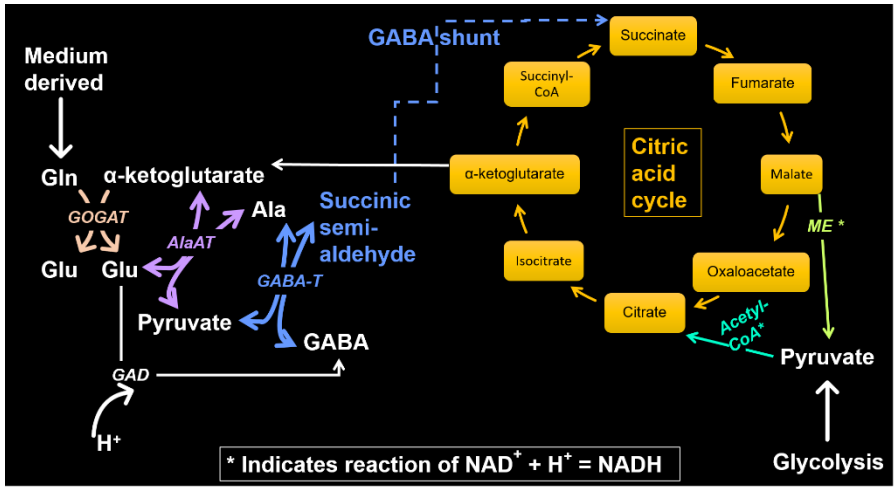

Fig 8. Schematic overview of the suggested connection between C and N metabolism in Norway spruce SE cell culture. PEMs seems to have a constrained C metabolism and by the addition of Gln in the medium Glu can be generated via GOGAT and used as a N donor and substrate in two stress-related metabolic pathways – the AlaAT pathway and the GABA shunt. Furthermore, C-backbones e.g. α-ketoglutarate, for AA metabolism and respiration are generated via glycolysis and the citric acid cycle from sucrose supplied in the medium. Glutamate-2-oxoglutarate aminotransferase, GOGAT; alanine aminotransferase, AlaAT; GABA transaminase, GABA-T; glutamate decarboxylase, GAD; malic enzyme, ME; nicotinamide adenine dinucleotide, NADH.

5 Conclusions

In this thesis, I have presented and discussed results regarding N nutrition in Norway spruce SE, with a focus on the role of exogenously supplied Gln for SE proliferation and germination. The main conclusion is that Gln is a crucial N source, both for PEMs during proliferation and for somatic embryos during germination.

All N sources supplied in the medium were taken up by PEMs, with a preference for Gln as compared to inorganic N. Furthermore, all exogenously supplied N sources were assimilated into the free AA pool, implying that the enzymes involved in NO_3^- assimilation, i.e. NR and NiR, as well as the enzymes GS and GOGAT, were active. However, the assimilation of NO_3^- appeared to be limited, indicated by low NO_3^- uptake and assimilation in relation to the amount of NO_3^- provided in the growth medium.

PEMs could not maintain a functional AA metabolism when only inorganic N was supplied in the growth medium, indicated by low total free AA concentration and slow growth. Hence, in the presence of exogenously supplied Gln in the medium, the metabolic constraints on AA metabolism were relieved, resulting in higher total free AA concentration and increased growth.

Gln addition also enabled PEMs to better utilise sucrose C in metabolism, reflected by the increase of citric acid cycle metabolites and respiration in PEMs grown on medium supplied with Gln.

Two low oxygen stress-related pathways were active in the PEM cells, the AlaAT pathway and the GABA shunt, both being dependent on Gln. These pathways could, directly or indirectly, be the underlying mechanisms alleviating the metabolic constraints of PEMs.

For germinating somatic embryos, as expected, N stored in the mature somatic embryo is not adequate to support germination. As shown by the results presented here, substantial amounts of N originated from the germination medium, and exogenous N was needed to a significant extent. In addition, Gln N contributed to more than 50 % of the total N assimilation after 24 days of

germination. The majority of assimilated N was found in N-rich AAs, e.g. Arg, possibly for N storage. Speculatively, this stored N could be used for growth after a shift to conditions that allows for a net gain from photosynthesis and thus increased amounts of C-backbones for N assimilation.

Taken together, the results from the work presented in this thesis provide an increased understanding of N metabolism and its contribution to growth during the phases of proliferation and germination of Norway spruce SE.

6 Future Perspectives

The stress constraints that the PEMs were experiencing during cultivation on solidified medium could possibly be reduced using alternative culture conditions such as liquid cultures. Hence, future studies should investigate to what extent the use of petri dishes in SE causes the stress that was observed.

Continued studies of N nutrition of germinating somatic embryos in photosynthetic light will give further insights into different aspects of N metabolism during germination. For example, studies investigating the assimilation of N into N-rich AAs under red light conditions and examining how those N-rich AA pools shift after transfer to photosynthetic light would contribute to increasing our knowledge of AA metabolism during germination of Norway spruce somatic embryos.

The NH_4^+:NO_3^- ratio has previously been studied in Norway spruce during initiation and proliferation and was shown to be optimal around 1:5 in the presence of Gln (Bozhkov et al., 1993). Based on the findings in this thesis, there is a need to investigate media compositions for both proliferation of Norway spruce PEMs and germination of somatic embryos. In **papers I** and **II** the NH_4^+:NO_3^- ratio was 0.28 and 0.22, respectively. Because NO_3^- was found to be the least assimilated N source in this culture system, it would seem reasonable to investigate whether it is possible to reduce the concentration of NO_3^- while maintaining an appropriate NH_4^+:NO_3^- balance. Even though the medium contains more total N than "needed", because the relative uptake of NO_3^- was low but was provided in large amounts and the relative uptake of NH_4^+ was high but was provided in small amounts, there is a possibility that the PEMs and germinants were at risk of being NH_4^+ limited, and consequently also N limited. Therefore, it is likely that there is both an excess of added NO_3^- and an imbalance of NO_3^- and NH_4^+ in the growth media used for conifer SE. This suggests that adjustment of the inorganic N provided in the medium could improve the potential biomass and development in this type of cultivation system.

Additionally, future work should investigate the C:N balance in the SE system, aiming to understand how this balance affects SE growth and development because the current debate implies that plants in natural settings can sense their N status and can regulate their N uptake accordingly. However, at this time the sensing mechanism and how it might be controlled remains unclear.

There is a balance between how much time and money should be invested in achieving a maximum result. Past efforts to investigate and optimise basic growth parameters can be replaced by different omics-techniques exploring global changes within the plant tissue to obtain further information about the SE process. Nevertheless, growth medium composition is the basis for SE culture performance, and an appropriate nutrient composition is needed to understand the metabolic processes that are active during Norway spruce SE. This will support the forest industry's need for future large-scale operations for Norway spruce SE production, thus fortifying Norway spruce plant propagation for more growth in the forests.

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Popular science summary

Globally, forest industries are facing a growing demand for higher production yields and better quality materials from the forests. New areas for forest products, including bioenergy, are developing together with strengthening importance for forest conservation and biodiversity. Forestry and forest management must advance to meet these demands.

After a forest felling, forest regeneration is generally started by planting seedlings. The seedlings are primarily produced from seed orchard elite seeds coming from crosses of selected plus trees from the breeding program. Unfortunately, over the last decade Norway spruce seed orchards in Sweden have been suffering from pathogen and pest problems that have led to a reduction in the number of seeds. Consequently, to maintain the seedling production for reforestation, there is a need for more effective techniques. Somatic embryogenesis is a technique that can be implemented for large-scale production of conifer species, including Norway spruce. Therefore, my work has been directed to contributing to and supporting the production of Norway spruce trees.

Embryogenesis - start of a new plant life

One plant comes from one seed, or rather, a seed embryo. A seed embryo consists of different types of cells that, when the embryo grows, develop into a plant consisting of different organs with specific functions. My research uses somatic embryogenesis as an experimental tool for producing embryos that allow us to study embryo development and germination in the Norway spruce. The focus of my first line of research is on the earliest stages, the embryogenic cells, which are the cells that exist before the embryo is fully developed. My second line of research focuses on the stage where the mature somatic embryo starts to germinate into a plantlet with fully developed shoot and root structures. I have studied how nitrogen is taken up and transformed into free amino acids, the building blocks of DNA, proteins, chlorophyll, etc. The amino acids are the pathway of the nitrogen into the cells and are necessary for the embryo and the

later development of the plant, regardless of how (e.g. from the field or from a nutritional solution) or in what form the nitrogen has been taken up (e.g., as ammonium, nitrate, or as individual amino acids such as glutamine).

The role of glutamine during the process of somatic embryo development

In the first part of my studies, it was shown that the growth of the embryogenic cells increased if the amino acid glutamine was added to the standard nutritional solution with ammonium and nitrate. This work suggests that nitrogen uptake is regulated, with a strong preference for glutamine, and I found that 64 % of the nitrogen in the free pool of amino acids came from the glutamine added to the nutritional solution. I also found that exogenously supplied glutamine increased the cell culture's ability to respire sucrose-derived carbon. Cell cultures grown with only ammonium and nitrate as nitrogen sources had significantly lower concentrations of free amino acids, and the cells appeared to have died, possibly because the cells were unable to maintain their metabolism. Cells grown with added glutamine appeared healthy, and this observation suggests an underlying metabolic stress in this somatic embryogenesis system that glutamine relieved.

In the second part of my work, the overall goal was to increase our knowledge of the carbon and nitrogen budget during the germination of a mature somatic embryo, with a more detailed look at nitrogen uptake and transformation into free amino acids. I found that carbon and nitrogen from the nutritional solution were the main sources contributing to development and growth and that the embryo's own storage reserves were not adequate. The nitrogen taken up and used to produce amino acids showed a preferred consumption of glutamine and ammonium nitrogen compared to whatever nitrogen sources were provided in the growth medium. This emphasises the importance of more basic knowledge on how the germinating somatic embryo uses nutrients for growth.

The contribution of my work has been a better understanding of the role of glutamine during the process of somatic embryogenesis. More so, my results have implications for the advancement of industrial applications for more effective Norway spruce plant production through somatic embryogenesis at a time when current seed supplies are threatened.

Populärvetenskaplig sammanfattning

Skogsindustrin står inför en globalt stigande efterfråga på ökad produktion och bättre kvalité av skogsråvaror. Efterfrågan på förnyelsebara råvaror blir allt större, och utöver de traditionella produkterna utvecklas nya användningsområden för skogsråvara, så som bioenergi. Samtidigt har medvetenheten för skogens roll i landskapets ekologi och bevarande av biologisk mångfald ökat. För att kunna möta de olika mål och intressen av skogen och skogsråvaran måste skogsbruket utvecklas och förnyas.

Efter en skogsavverkning återplanteras marken vanligtvis med plantor producerade främst av förädlat frö från fröplantager. Produktionen av granfrö i Sverige har dessvärre under det senaste decenniet drabbats av skadesvampar- och insektsangrepp vilket lett till minskad frötillgång. Följaktligen finns det ett behov av effektivare metoder för att säkerställa produktion av granplantor. En sådan metod är somatisk embryogenes (en laboratoriemetod där en eller en grupp av celler, de somatiska cellerna, kan multipliceras och utvecklas till ett obegränsat antal embryon genom att växa på en näringslösning) som kan användas för storskalig produktion av barrträd, inklusive gran. I min avhandling har jag studerat upptag och inkorporering av kväve under processen somatisk embryogenes hos gran med syftet att öka kunskapen om aminosyran, glutamin och dess betydelse för processen, och stödja produktionen av granplantor genom somatisk embryogenes.

Embryogenes - början på ett nytt plantliv

Inuti ett granfrö finns fröembryot som kan gro och bli en planta. Ett fröembryo består av ett flertal sorters celler som utvecklas till de olika delarna i en planta. Alla celler, utom könscellerna, kallas somatiska celler, och embryogenes är utveckling från en cell till ett embryo.

I min forskning använde jag somatisk embryogenes som ett verktyg för att producera embryon och i dem studera granens embryoutveckling och groning. Första delen av min avhandling fokuserar på de tidiga embryogena cellerna -

cellerna före embryot är helt utvecklat. Den andra delen i avhandlingen fokuserar på det mogna embryot som börjar gro till en planta med en rot-och skottdel. I de två utvecklingsstadierna studerade jag hur kväve tas upp och omvandlas till fria aminosyror, byggstenarna till DNA, proteiner, klorofyll etc. Aminosyror är kvävets väg in i cellen och är livsnödvändiga för embryots utveckling till en planta. Den tredje delen i avhandlingen syftade till att öka kunskapen om kol- och kvävenyttjande under groningsprocessen av ett moget embryo till en planta, med speciellt fokus på kväveupptag och dess omvandling till fria aminosyror.

Glutaminets roll för utvecklingen av granplantor från somatiska embryon

När standardnäringslösningen, innehållande kväve i form av ämnena ammonium och nitrat, kompletterades med aminosyran glutamin ökade tillväxten av de embryogena celler. Sextiosju procent av kvävet i cellens aminosyror kom från det tillsatta glutaminet. Det tyder på att kväveupptaget regleras av cellerna och att glutamin föredras över andra kvävekällor. Tillsatt glutamin ökade även cellernas förmåga att omvandla kol från tillsatt sackaros (socker) till andra kolämnen, som är cellernas näring under arbeta. De cellkulturer odlade utan tillsatt glutamin hade lägre koncentrationer av fria aminosyror, och cellerna föreföll i stor utsträckning dö, till skillnad från de embryogena celler odlade med tillsatt glutamin. Det kan eventuellt förklaras med en låg ämnesomsättning (metabolism) i de celler som inte fick extra glutamin, vilket indikerar att odling av celler via somatisk embryogenes har en underliggande metaboliskstress som minskas genom tillsatts av glutamin.

Kol och kväve från näringslösningen var de främsta näringskällorna för embryots utveckling och tillväxt, och embryots egna näringsreserver inte var tillräckliga. Glutamin- och ammoniumkväve var de kvävekällor som föredrogs framför nitratkväve, vilket är den kvävekällan som ursprungligen utgjort störst andel i groningsmediet. Följaktningsvis krävs det djupare kunskap om hur näringsämnen nyttjas av somatiska embryon under groning till planta för att optimera groningen.

Mitt doktorsarbete har bidragit till ökad förståelse av glutaminets roll vid den somatiska embryogenesprocessen av gran. Resultaten från studierna kan användas för att fortsätta utvecklingen av en industriell tekniklösning med somatisk embryogenes för en effektivare granplantproduktion i en tid när nuvarande fröförsörjning hotas.

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