Nitrogen utilization during germination of somatic embryos of Norway spruce: revealing the importance of supplied glutamine for nitrogen metabolism

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Abstract

Key message This paper shows that germinating Norway spruce somatic embryos are dependent on the carbon and nitrogen supplied in the medium, and that supplied glutamine accounts for 50 % of assimilated nitrogen during germination.

Abstract The female megagametophyte, which provides the zygotic embryo with nitrogen (N), carbon (C) and energy during germination, is not present in Norway spruce (*Picea abies*) mature somatic embryos. Therefore, somatic embryos presumably rely on nutrients supplied in the germination medium in addition to their storage compounds accumulated during maturation. However, to what extent stored versus supplied compounds contribute to a somatic embryo germination is unclear. In this 24-day study, we addressed the above question by monitoring the biomass changes and the N and C budget during somatic embryo germination, under low-intensity red light. We found that the C and N storage reserves, accumulated during the maturation phase, were not sufficient to support the growth of the germinating somatic embryos, rather they were dependent on the medium components. In addition, in a previous study it has been found that glutamine (Gln) supplied in the medium was crucial for maintaining the primary amino acid (AA) metabolism and growth of the proliferating embryogenic cultures of Norway spruce (Carlsson et al., PLoS One 12(8):e0181785, 2017). Therefore, we hypothesised that Gln would be required as a significant source of N also during somatic embryo germination. By tracing the uptake of isotopically labelled N-sources from the medium and further into primary N assimilation, we found that Gln was the preferred source of N for the germinating somatic embryos, accounting for 50% of assimilated N. As the amounts of both arginine (Arg) and Gln were increased in the germinating somatic embryos, it also suggested that germination in low-intensity red light promoted N storage, similar to what has been observed in the zygotic embryo maturation in conifers (King, Gifford, Plant Physiol 113:1125–1135, 1997).

Keywords Amino acids · Glutamine · Nitrogen assimilation · Somatic embryogenesis

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Introduction

Conifer somatic embryos are generated in a process in which plant growth regulators are used to induce somatic embryos from somatic cells (Elhiti et al. 2013; Pullman and Bucalo 2014; Winkelmann 2016). A major difference between

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zygotic embryogenesis (ZE) and somatic embryogenesis (SE) is the absence of a megagametophyte in the latter. Consequently, somatic embryos rely on reserves accumulated during embryo maturation and/or media-supplied nutrients (Hakman 1993; Goldberg et al. 1994). The main objectives in this study were (1) to determine the relative contribution of nitrogen (N) and carbon (C) uptake versus the endogenous stocks (present at the end of maturation) to Norway spruce (*Picea abies*) somatic embryos during germination and (2) to follow the assimilation of N supplied in the medium.

In pine (*Pinus spp.*) seeds, storage reserves are mainly contained within the megagametophyte (Stone and Gifford 1997; Trontin et al. 2016). Seed storage proteins have a high N content and are broken down to amino acids (AAs) (Lammer and Gifford 1989; King and Gifford 1997). Storage carbohydrates and lipids have a high C content and are transformed to soluble carbohydrates (Ching 1966; Carrier et al. 1997). AAs and soluble carbohydrates released from the storage proteins are used for the synthesis of metabolites, building biomass and provide energy during germination. N is a building block of several biomolecules involved in photosynthesis, e.g. chlorophyll and key enzymes in the Calvin cycle, and is thus vital for the assimilation of C into plant metabolism. Similarly, C is an integral part of N metabolism, serving as an energy source and providing C-backbones to N assimilation and AA biosynthesis (reviewed by Pratelli and Pilot 2014).

Several phases of the SE-process take place in darkness under heterotrophic conditions. Thus, SE tissue culture systems are dependent on exogenously added sugars for C and energy. Several different sugars are commonly used in plant culture media of which sucrose is the most widely used (Yaseen et al. 2012). In plants, sucrose can be hydrolysed by two different enzymes, invertase or sucrose synthase. Invertase and sucrose synthase hydrolyse sucrose into glucose and fructose, and into fructose and uridine diphosphate glucose, respectively. The products are metabolised further by enzymes in the glycolysis pathway, providing substrates for the citric acid cycle and for biosynthesis of, e.g. AAs and lipids (Huppe and Turpin 1994; Nunes-Nesi et al. 2010; Krasavina et al. 2014).

Incorporation of N into AAs is a prerequisite for synthesising proteins, nucleotides and other metabolites needed for plant development and growth (Nunes-Nesi et al. 2010). N is assimilated via two pathways, depending on which form of N acquired. Nitrate (NO_3^-) taken up from the growth substrate is reduced to ammonium (NH_4^+) by the enzymes nitrate reductase and nitrite reductase (Lam et al. 1996; Krapp 2015). NH_4^+ , acquired either by direct uptake, as an assimilation product of NO_3^- , or as a photorespiration product, is incorporated into Gln and glutamate (Glu) via the enzymatic pathway of glutamine synthetase/glutamate-2-oxoglutarate aminotransferase (GS/GOGAT) (Lea and Ireland

1999; Cánovas et al. 2007; Forde and Lea 2007). Sources of inorganic N (NH_4^+ and NO_3^-) and organic N (mainly AAs) are commonly provided in in vitro culture media in various mixtures (Bozhkov et al. 1993; George and de Klerk 2008; Pullman and Bucalo 2014). The reason for adding single or mixed AAs to the culture media is the positive impact of organic N on overall tissue culture performance (Kirby 1982; Pinto et al. 1993; Khlifi and Tremblay 1995; Ogita et al. 2001; Vasudevan et al. 2004; Hamasaki et al. 2005).

In the context of conifers, e.g. silver fir (*Abies alba*) (Hristoforoglu et al. 1995), white spruce (*Picea glauca*) (Bartlett et al. 1997) and Norway spruce (Bozhkov et al. 1993), studies have shown that supplementing the culture medium with organic N, e.g. Gln or casein hydrolysate, enhances SE initiation frequency and proliferation growth, as compared with medium supplemented with solely inorganic N. In a recent study, it has been shown that growing proliferating pro-embryogenic masses (PEMs) of Norway spruce on semi-solid medium with NH_4^+ and NO_3^- in combination with Gln, had a positive effect on PEM culture growth as compared with the growth on solely inorganic N (Carlsson et al. 2017). The same study also showed that the amount of Gln-N taken up was higher than the relative amount supplied in the medium and that Gln-N was assimilated into AA metabolism to a higher extent than inorganic N, contributing to more than half of the N in the total free AA (TFAA) pool of proliferating PEMs.

Many studies have investigated how the culture conditions during the maturation phase influence a somatic embryo's capability to germinate, including factors such as, osmoticum (Bozhkov and von Arnold 1998; Hazubska-Przybył and Wawrzyniak 2017), abscisic acid (Bozhkov and von Arnold 1998; Montalbán et al. 2010; Alvarez et al. 2013), redox agents (Stasolla et al. 2004; Pullman et al. 2015), gelling agents (Klimaszewska et al. 2000; Morel et al. 2014a; Lelu-Walter et al. 2018) and desiccation treatments (Hazubska-Przybył et al. 2015; Carneros et al. 2017). In terms of N metabolism, studies have suggested that a reduced accumulation of storage protein during maturation can have a negative impact on the quality of the mature somatic embryos, as compared with zygotic embryos (Klimaszewska et al. 2004, 2016; Morel et al. 2014b).

Increased rates of germination and survival after transfer from in vitro to ex vitro conditions are desirable for many plant applications. For Norway spruce SE, it would benefit the currently most acute bottleneck of SE, both in terms of knowledge and success as a commercial cost-effective complement to the existing plant propagation methods. The existing literature addressing N and C during conifer somatic embryo germination is mostly comprised of omics data (e.g. metabolomics, proteomics and transcriptomics) (Robinson et al. 2009; Canales et al. 2014; Dobrowolska et al. 2017; Klubicová et al. 2017). However, in a recent study, improved

root growth of pine somatic embryos germinated on medium supplemented with solely organic N was reported (Llebrés et al. 2017). Nevertheless, fundamental information regarding quantitative data with regards to N and C metabolism is lacking and further information is required for a better understanding of the somatic germination process to facilitate improvements in yield for commercial applications.

The overall aim of the project was to expand our knowledge of the C and N budget during the Norway spruce SE germination phase, with focus on N uptake and assimilation. The main goals of this research project were (i) to investigate the utilization of C and N stored in the mature somatic embryo versus the utilization of C and N supplied in the medium and (ii) to characterise N metabolism during the early stage of germination, investigating the relative use of different N sources supplied in the germination medium. Given the importance of Gln for PEMs (Carlsson et al. 2017), we hypothesised that Gln supplied in the medium would be a major contributor of N during somatic embryo germination.

Materials and methods

Embryogenic line and maturation of somatic embryos

Mature Norway spruce somatic embryos originating from the embryogenic cell line 11:12:02 were used for germination. The cell line was initiated at SweTree Technologies, Sweden, in 2011. Embryogenic cultures were sub-cultured biweekly on a semi-solid ½-LP medium supplemented with 9.0 µM 2,4-dichlorophenoxyacetic acid, 4 µM N6-benzyladenine, 3.1 mM Gln and 1% sucrose (w/v) (von Arnold and Eriksson 1981). To initiate maturation, the clumps of embryogenic cultures were transferred to DKM maturation medium lacking plant growth regulators for one week, followed by DKM medium supplemented with 29.0 µM abscisic acid and 3.1 mM Gln (Krogstrup 1986) and a modified synchronization DKM medium supplemented with 0.25 M myo-inositol (Egertsdotter and Clapham 2011) for six and two weeks, respectively. Mature somatic embryos with four or more developed cotyledons were harvested from the cultures and placed in a 5 cm (diameter) Petri dish. For 3 weeks, desiccation treatment was carried out by placing the 5 cm Petri dish (without the lid) inside a 9 cm Petri dish, holding 1 ml of sterile water, with the lid on and sealed with PARAFILM® M (Bemis Company, Inc.). The mature somatic embryos used in the experiments were obtained from five separate maturation batches. Samples of the desiccated embryo batches were collected to check if they were similar in terms of dry biomass (DW), C and N concentrations or C and N contents (n=5, and for each replicate 23 embryos

were pooled). Statistical analysis showed that the DW, C and N content of the maturation batches were not significantly different (Table S1). However, the C and N concentrations of the day 1–6 batches were significantly different from those of days 12–24.

Experimental design and sample collection

After desiccation, 23 mature somatic embryos were placed in a 9 cm Petri dish with 25 mL germination medium (Table S2). The composition in terms on N supplied in the germination medium is specified in Table 1. The Petri dishes were placed under 24 h of red light (5 µmol m⁻² s⁻¹; Philips TLD Red 18W). The rationale for growing the germinants in low-intensity red light was: (1) to limit photosynthesis to a very low level, ensuring that C was mainly acquired from sucrose supplied in the medium and (2) to promote root development (Kvaalen and Appelgren 1999; Merkle et al. 2005).

The germination medium was divided into five separate aliquots. Four aliquots were separately supplied with one of the four ¹⁵N isotopically labelled compounds, ¹⁵NH₄NO₃ (≥98% ¹⁵N), NH₄¹⁵NO₃ (≥98% ¹⁵N), ¹⁵amide-Gln (≥98% ¹⁵N) and ¹⁵amine-Gln (≥98% ¹⁵N). Gln amine and amide N were separately labelled, making it possible to evaluate if amine and amide N were utilized differently. The amount of ¹⁵N-labelled compound added to the medium represented 22% of NO₃⁻-N (hence, a multiplication factor of 4.5 was used in the targeted AA data analysis calculations) and 100% of NH₄⁺-N, amine-Gln-N and amide-Gln-N. A fifth aliquot did not contain any labelled N compound and was used to determine the natural abundance of ¹⁵N. However, due to a technical limitation of the ¹⁵N IRMS analysis, a subset of germinants had to be grown on medium with a lower fraction of isotopic label for day 6 and 24 of germination (1% ¹⁵N of respective N source). Due to insufficient amount of sample for day 1 and a shortage of mature somatic embryos for experimental use, day 1 and 12 were omitted from the IRMS analysis. The germination of somatic embryos was observed and documented before sampling using a digital camera (D5000, Nikon).

Samples were collected after 1, 3, 6, 12 and 24 days of germination. The experiment required approximately 4500 mature somatic embryos in total. Hence, to make the start-up

Table 1 N composition of the germination medium

	mM N	mg N L ⁻¹	mg N plate ⁻¹	% of total N
NH ₄ ⁺	2.1	29.9	0.7	12
NO ₃ ⁻	9.7	135.7	3.4	54
Gln	6.2	86.2	2.2	34
Total	18.0	251.8	6.3	

and sampling manageable, the work of placing the somatic embryos on the germination media was carried out over a number of days. At each sampling, all germinated somatic embryos were counted in each Petri dish, pooled into one biological replicate and weighed (fresh weight; FW). The somatic seedlings collected at 24 days were cut at the shoot–root junction and the shoots and roots were collected separately. Unless stated differently, five biological replicates were analysed for each treatment and at each of the five time points. All samples were frozen in liquid N₂ and freeze-dried for 72 h (Scanvac Coolsafe freeze dryer, Labo-Gene™, Denmark). Sample DW was measured and the samples were ground for 3 min using a bead mill (MM400, Retsch GmbH, Germany) set to a frequency of 25 Hz s⁻¹, with three 3 mm tungsten beads.

Analysis of tissue C, N and ¹⁵N

The tissue concentrations of C and N (w/w; presented as C% or N%) of the germinants grown on the unlabelled germination medium, was determined using an elemental analyser (Flash EA 2000, Thermo Fisher Scientific, Bremen, Germany). The ¹⁵N content of the germinants, grown on ¹⁵N-labelled germination media, was determined using an isotope ratio mass spectrometer (IRMS; DeltaV, Thermo Fisher Scientific, Bremen, Germany) interfaced to the element analyser (Flash EA 2000). Sample weight used for elemental analysis was 2 mg (± 10%) DW.

Analysis of AA concentrations

Two mg of freeze-dried sample was extracted in 0.01 M HCl and AA concentrations were measured according to the UPLC-AccQTag method (UPLC amino acid analysis system solution, <http://www.waters.com>).

Targeted AA analysis

The targeted AA analysis [original technique described by Gullberg et al. (2004)] was performed as described in Carlsson et al. (2017) with the following changes; (1) the internal standard (norvaline) was maintained at 0.5 pmol on the column for the calibration curve and (2) a 1 µL aliquot of the sample was injected for the LC–MS analysis. The LC–MS analysis was controlled by the MassHunter™ software package, v B.05.01 and data processing was performed using the MassHunter™ Profinder software, v B.08.00 (Agilent Technologies Inc., Santa Clara, CA, USA). The concentration of N (µmol g⁻¹ DW) assimilated from the ¹⁵N-labelled germination media into AAs in the TFAA pool was calculated according to Carlsson et al. (2017).

Calculations and statistical analysis

All calculations of means and standard error of mean (SEM) were performed using the Microsoft Excel software (Microsoft Corporation, USA). Statistical differences were tested by ANOVA analysis and Tukey HSD test using JMP Pro software (SAS Institute Inc, USA), a *P* value of < 0.05 was defined as statistically significant.

Results

Somatic embryo biomass changes during germination

The average biomass (FW and DW, and the FW/DW ratio) of a germinating embryo at the different time points is shown in Table 2. Germinant fresh biomass increased significantly throughout the sampling series, from 1.6 ± 0.03 mg at day 1 to 13.2 ± 0.24 mg at day 24. Germinant dry biomass did not increase significantly between day 1 (0.49 ± 0.01 mg) and day 3 (0.52 ± 0.01 mg), whereas at day 6, the dry biomass had almost doubled (0.99 ± 0.02 mg). Between day 6 and 24 the germinants increased their dry biomass further, with a final biomass of 1.69 (± 0.03) mg at day 24, 3.4-fold more than the starting weight. FW/DW ratio was 3.2 ± 0.08 at day 1 and thereafter it increased to 4.8 ± 0.17 at day 3, 7.5 ± 0.19 at day 6 and reached 9.0 ± 0.08 at day 12, then the ratio declined to 8.0 ± 0.06 at day 24.

Germinant C and N status

Germinant C concentration decreased significantly between day 1, 3 and 5, from 57% at day 1 to 45% at day 12 and remained unchanged between day 12 and 24 (Fig. 1a). Germinant N concentration did not change significantly over days 1 to 6 at 4.9–5.0%, whereas at day 12 and 24 a significant increase to 5.7% and 5.8% respectively was recorded (Fig. 1b). To examine the cumulative content of C and N taken up from the medium, a statistical analysis was carried out, comparing the C and N content in the germinating embryos versus the initial value obtained from the desiccated embryos before placing them on germination medium (Table S1, average for all batches). C content during germination followed a similar pattern to that of DW biomass development, being unchanged for the first three days in comparison with the starting C content, followed by a significant increase of 88% at day 6 (Fig. 1c). The C content was then unchanged between day 6 and 12, whereas between day 12 and 24, the C content was significantly increased by an additional 120%. Taken together, the C content in the somatic embryos increased by 3.1-fold during the germination experiment compared with the C content of a somatic

Table 2 Biomass (i.e. FW, DW and FW/DW ratio) of the somatic embryos at day 1, 3, 6, 12 and 24 of germination (mg per somatic embryo $\pm$ SEM; $n \geq 49$)

	day 1	day 3	day 6	day 12	day 24
FW	1.6 ± 0.03^a	2.5 ± 0.06^b	7.4 ± 0.10^c	9.8 ± 0.22^d	13.2 ± 0.24^e
DW	0.49 ± 0.01^a	0.52 ± 0.01^a	0.99 ± 0.02^b	1.14 ± 0.03^c	1.69 ± 0.03^d
FW/DW ratio	3.2 ± 0.08^a	4.8 ± 0.17^b	7.5 ± 0.19^c	9.0 ± 0.08^c	8.0 ± 0.06^d

Different letters indicate significant differences among means ($P < 0.05$)

embryo. Similarly, the N content did not increase significantly in comparison with the starting N content during the first 3 days. At day 6, the N content had increased significantly by 76% and continued to rise, increasing 3.6-fold over the 24-day germination (Fig. 1d).

N uptake in germinating embryos

To investigate N uptake, a reciprocal ^{15}N -labelling experiment was conducted, whereby ^{15}N provided by the different N sources in the growth medium and absorbed by the embryo, was analysed using EA-IRMS. The total N concentration in the medium was 18 mM, with individual N sources corresponding to 9.7 mM NO_3^- -N, 2.1 mM NH_4^+ -N and 6.2 mM Gln-N (Table 1). Since 23 embryos were placed on 25 ml of medium, each somatic embryo had access to 273.7 μg N, consisting of 147.5 μg NO_3^- -N, 32.5 μg NH_4^+ -N and 93.7 μg Gln-N, representing 54%, 12% and 34% of total N (Table 3). After 3 days of germination, each germinant contained on average 28.9 μg N, of which 27% originated from the medium. The respective N sources (NO_3^- -N, NH_4^+ -N and Gln-N) contributed to 2.3 ± 0.4 , 1.9 ± 0.4 and 3.7 ± 0.7 μg N per embryo, corresponding to 29%, 24% and 47% of the total N taken up. After 6 days, 52% of the total N in the germinant originated from the medium, corresponding to 2.7 ± 0.5 μg NO_3^- -N, 2.8 ± 0.6 μg NH_4^+ -N and 6.8 ± 1.4 μg Gln-N. The fractions of NO_3^- -N, NH_4^+ -N and Gln-N contribution were 22%, 23% and 55% of total N taken up. At day 24 of germination, the N content in an average germinant was 110.5 μg N, of which 90% was taken up from the medium. The contribution of the three N sources was 30 ± 6.5 μg NO_3^- -N, 19.5 ± 3.6 μg NH_4^+ -N and 50.5 ± 9.4 μg Gln-N, representing 30%, 19% and 51% of total N taken up.

N assimilation into the TFAA pool

To further investigate the N metabolism in germinating somatic embryos we also assessed the TFAA pool and its composition. The TFAA pool concentration increased significantly throughout the germination phase, from 118 ± 10 $\mu\text{mol g}^{-1}$ DW at day 1 to 1172 ± 72 $\mu\text{mol g}^{-1}$ DW at day 24, an almost 10-fold increase (Fig. 2a). With respect to the TFAA profiles at the different time points, Gln and Arg were the only AAs at a concentration higher than 5% of TFAA at all five measured time points (Fig. 2b). Gln was the overall most abundant AA, ranging from 33% at day 1 to 69% at day 24 of the TFAA pool, followed by Arg that fluctuated from 29% at day 1 to 16% at day 3, to 12% at day 6, to 14% at day 12 and reached 17% at day 24 of the TFAA pool. In addition, the asparagine (Asn) concentration was higher than 5% of the TFAA pool at all time points except day at 3. The sum fraction of AAs with an individual concentration lower than 5% of the TFAA pool decreased from 25% at day 1 to only 5% at day 24.

Assimilation of the N sources

Targeted AA profiling using ^{15}N -labelled N sources was carried out to assess the relative contribution of each N source to N metabolism, i.e. to what extent each N source taken up was assimilated into AAs. For the results presented in this section, Gln is separated from the remaining TFAA pool. The reasons for separating Gln from the other AAs when analysing N assimilation are: (1) When ^{15}N -Gln is included in the media, it is not possible to distinguish between taken up, non-metabolised ^{15}N -Gln present in the germinants and ^{15}N -Gln generated from internal metabolism (even though the N is derived from Gln supplied in the medium). (2) The tissue concentration of Gln in the germinants was high, accounting

Fig. 1 C and N concentration and content in the somatic embryos at day 1, 3, 6, 12 and 24 of germination. C% (a) and N% (b) (w/w ± SEM). C content (c) and N content (d) during the germination period of 24 days (mg per embryo ± SEM; $n=5$). Different let-

ters indicate significant differences among means ($P<0.05$). *Indicate starting C and N content of a desiccated mature somatic embryo before placing it on the germination medium

Table 3 Amount and percental composition of available N in the form of NO_3^- , NH_4^+ and Gln added in the germination medium per somatic embryo (first column to the left) and the amount and propor-

tion of N taken up as NO_3^- , NH_4^+ or Gln per somatic embryo at day 3, 6 and 24 of germination ($\mu\text{g N}$ per somatic embryo ± SEM; $n=5$)

	Available in medium $\mu\text{g N}$	Day 3 $\mu\text{g N}$ per somatic embryo	Day 6	Day 24
NO_3^-	147.5 (54%)	2.3 ± 0.4 (29%)	2.7 ± 0.5 (22%)	30.0 ± 6.5 (30%)
NH_4^+	32.5 (12%)	1.9 ± 0.4 (24%)	2.8 ± 0.6 (23%)	19.5 ± 3.6 (19%)
Gln	93.7 (34%)	3.7 ± 0.7 (47%)	6.8 ± 1.4 (55%)	50.5 ± 9.4 (51%)

for up to 69% of the TFAA pool. Hence, by presenting the incorporation of N into Gln separately, a wider data interpretation was possible. Moreover, no major differences in the incorporation of Gln amide N and amine N into the TFAA pool were observed, therefore the sum of amide and amine N is presented as Gln-N hereafter.

The contribution of each N source assimilated into the TFAA pool (except Gln) is shown in Table 4. The corresponding data showing the incorporation of the different

N sources into Gln is shown in Table 5. The relative contribution of the different N sources to the TFAA pool was relatively stable during the germination period (Table 4), with NO_3^- -N, NH_4^+ -N and Gln-N representing on average 22, 25 and 53% of the assimilated N, respectively.

The fractions of N derived from the three different N sources found in Gln (Table 5) followed a similar pattern to that of the TFAA pool (Gln excluded; Table 4). The only difference was a slightly higher fraction of NH_4^+ -N assimilated

Fig. 2 The TFAA pool concentration (**a**) and profile (**b**) in Norway spruce somatic embryos at day 1, 3, 6, 12 and 24 of germination ($\mu\text{mol g}^{-1}$ DW $\pm$ SEM; $n = 25$). Different letters indicate significant differences among means ($P < 0.05$)

Table 4 Concentration and percentage of N from each N source, NO_3^- , NH_4^+ or Gln, assimilated into the TFAA pool (excluding any N assimilated into Gln) in somatic embryos at day 1, 3, 6, 12 and 24 of germination ($\mu\text{mol N g}^{-1}$ DW $\pm$ SEM; $n = 5$)

	Day 1	Day 3	Day 6	Day 12	Day 24
$\mu\text{mol N g}^{-1}$ DW					
NO_3^-	3.4 ± 0.5 (24%)	10.9 ± 1.2 (22%)	27 ± 2 (24%)	70 ± 3 (22%)	111 ± 7 (20%)
NH_4^+	3.4 ± 0.6 (23%)	11.1 ± 0.7 (23%)	28 ± 3 (25%)	81 ± 5 (25%)	148 ± 10 (27%)
Gln	7.9 ± 1.8 (54%)	26.3 ± 3.7 (55%)	56 ± 5 (50%)	176 ± 13 (53%)	281 ± 17 (53%)

Table 5 Concentration and percentage of N from each N source, NO_3^- , NH_4^+ or Gln, found in or assimilated into Gln in somatic embryos at day 1, 3, 6, 12 and 24 of germination ($\mu\text{mol N g}^{-1}$ DW $\pm$ SEM; $n = 5$)

	Day 1	Day 3	Day 6	Day 12	Day 24
$\mu\text{mol N g}^{-1}$ DW					
NO_3^-	3.3 ± 0.3 (26%)	9.5 ± 0.8 (22%)	30 ± 5 (24%)	74 ± 9 (21%)	143 ± 17 (20%)
NH_4^+	3.3 ± 0.6 (27%)	12.1 ± 1.0 (27%)	41 ± 5 (32%)	113 ± 9 (32%)	190 ± 5 (27%)
Gln	5.9 ± 1.6 (47%)	22.5 ± 3.3 (51%)	56 ± 4 (44%)	162 ± 18 (46%)	368 ± 13 (52%)

into Gln between days 1–12 (27–32% for Gln as compared with 23–25% for the TFAA pool) and a corresponding a lower Gln-N fraction in Gln during day 1 to day 12. However, at day 24, the N source patterns of the TFAA pool and Gln were similar. When analysing the incorporation of ^{15}N into individual amino acids, we found that Arg was far more ^{15}N -enriched than all other AAs except Gln (Table S3). The interpretation of ^{15}N incorporation into Gln is more complex due to the reasons already mentioned. However, in terms of NO_3^- and NH_4^+ derived N, approximately 50% more N was assimilated into Gln than into Arg.

Discussion

This work aimed at increasing our understanding of Norway spruce somatic embryo germination, by assessing the importance of C and N supplied in the medium, with the focus on the relative contribution of the provided N sources. To investigate this, mature somatic embryos were monitored for biomass changes, C- and N budget, and N assimilation over the first 24 days of germination in low-intensity red light. We found, as expected, that C and N supplied in the medium was of great importance, as illustrated by the germinants 3.1 to 3.6-fold increase in these elements over the 24-day germination period. With respect to N source and N assimilation, Gln accounted for 50% or more of total N, supporting the proposed hypothesis that it was the preferred source of N. NO_3^- on the other hand, being the most abundant medium N source was the least utilized.

Biomass changes, C and N uptake during the first 24 days of germination

For the first 3 days of germination no major differences were observed in the analysed parameters. At this point, the germinant was probably re-hydrating (Table 2; FW/DW ratio) and initializing metabolic pathways involved in germination. At day 6, the C concentration had decreased from 57% to 49% (Fig. 1a) and at the same time the total C content had increased by 88% (Fig. 1c). This suggested that (1) stored C was metabolised and (2) C was taken up from the medium. The results thus suggest that a substantial part of the C stored in the germinants is in the form of lipids, since the germinants had a relatively high C concentration. In an earlier study, lipid bodies have also been found in somatic embryos and were considered as an indicator of a viable embryo (Carrier et al. 1997). Between day 6 and 12, the C content remained unchanged whereas the C concentration continued to decrease, suggesting that lipid reserves transformed into other C-compounds with a relatively higher oxygen content. Alternatively, the lipids may also have been respired and the C content was maintained by the uptake of

sucrose C. The unchanged C content could be due to several factors. Around day 6–12 many morphological changes occurred; the germinants developed into seedling-like structures with a pronounced root and shoot and at the same time it can be assumed that cell-expansion also took place. These processes are likely associated with respiration costs, something that could explain the unchanged C content. Finally, between day 12–24, C concentration did not change, and C content increased by an additional 120%, suggesting that the storage lipids were consumed, and that medium supplied sucrose was the driving factor for growth during that time-frame.

The increase of germinant N content followed closely the same pattern as that of C, with the exception that N content increased consistently after day 6, without stagnating at day 12. After 3 days, 27% of the total N content in the germinating somatic embryos originated from the medium, which increased to 52% and 90% on days 6 and 24 respectively. It is therefore clear that the somatic germinants were highly dependent on medium supplied N for their growth. With respect to the uptake preference, we found that somatic germinants appeared to prefer Gln, followed by NH_4^+ and NO_3^- . Gln-N accounted for at least 50% of total N uptake, consistent with previous findings on N uptake in PEMs of Norway spruce (Carlsson et al. 2017). However, even though conifers have been found to take up AAs in quantities comparable to NH_4^+ and NO_3^- (Metcalf et al. 2011; Gruffman et al. 2013, 2014), most plant literature addresses mineral N and the preference for either NH_4^+ or NO_3^- . Hence, the ecological relevance of plant AA uptake is under debate, but these results challenge this dogma, at least in terms of plant in vitro culture. Excluding Gln, the results presented here suggest that the somatic germinants preferred NH_4^+ , corroborating previous reports that conifer species have an uptake preference for NH_4^+ as compared with NO_3^- (Öhlund and Näsholm 2001; Miller and Hawkins 2007).

N assimilation into the TFAA pool

In this study, ^{15}N -labelled NO_3^- -N, NH_4^+ -N and Gln-N was traced into primary N assimilation (TFAAs) during the first 24 days of Norway spruce somatic embryo germination. After 3 days on germination medium, N originating from all N sources occurred in all AAs analysed. Gln and the inorganic N sources contributed approximately equally to assimilated N found in the TFAA pool (Table 4), indicating that the Gln supplied in the medium was a significant source of N for somatic embryo N metabolism during germination. However, the relative amount of inorganic N assimilated into TFAA did not reflect the amount of N taken up from the medium, i.e. NO_3^- -N accounted for 30% of the total N uptake at day 24 of germination (Table 3), whereas NO_3^- -N accounted for only 20% of the TFAA pool

at day 24 (Table 4). A possible explanation for this difference is that a part of the NO_3^- taken up is not assimilated into TFAA but instead is stored as NO_3^- , presumably in vacuoles (Andrews et al. 2013). Moreover, as NO_3^- and NO_2^- reduction enzymes are light regulated (Kaiser et al. 2002; Krapp 2015) and NO_3^- assimilation is partly dependent on photosynthesis through ferredoxin (Joy and Hageman 1966; Hanke et al. 2004), the utilization of NO_3^- may have been negatively affected by the non-photosynthetic lighting conditions used in this study. The assimilation of NO_3^- can also be repressed when the concentration of Gln is high as, for example, in fungi, due to a process called ‘nitrogen metabolite repression’ (Crawford and Arst 1993; Gent and Forde 2017). Contrary to NO_3^- , NH_4^+ was found to account for a larger fraction of assimilated N as compared with its contribution to total N uptake, i.e. 27% of the TFAA pool N at day 24 (Table 4) versus 19% of total N uptake at day 24 (Table 3), suggesting that NH_4^+ assimilation was less restricted than that of NO_3^- . Furthermore, the TFAA pool increase during germination also demonstrates an active N metabolism, synthesising new AAs that could be used for protein production and subsequently for the growth, which in turn suggests no apparent limitation of metabolism/assimilation via the GS/GOGAT pathway.

Utilization of exogenously added N

The high uptake and assimilation of Gln suggest that it is a preferred N source for germination, the question is why? In Carlsson et al. (2017) it was evident that SE cell culture was relying on medium supplied with Gln as a N donor to make large amounts of Ala. In this study however, high tissue levels of Ala were not present. Perhaps the most logical explanation in the context of somatic embryo germination is that NO_3^- was a non-preferential N-source as opposed to Gln being a preferred one and the relatively low utilization of NO_3^- would force the germinants to search for other sources of N. Moreover, compared with mineral N that must be assimilated in a process that requires energy (Bloom et al. 1992; Zerihun et al. 1998), Gln is an already assimilated N source and it therefore saves energy and C (Franklin et al. 2017). The germination medium used for the experiments was, in addition to 3.1 mM Gln, also supplied with NH_4^+ and NO_3^- at a ratio of 0.22 with respect to N, a composition that has been found to be optimal for the initiation and maintenance of Norway spruce SE culture (Bozhkov et al. 1993). Even though it was developed for initiation and SE culture, this N composition is also suitable for germination. Compared with other plant media, e.g. Murashige and Skoog (1962), with a NH_4^+ to NO_3^- ratio of 0.5, the relative amount of NH_4^+ in the germination medium is low. As the utilization of NO_3^- was limited, it could suggest that it would be beneficial to increase the fraction of NH_4^+ in

the germination media. However, the rationale for using a low concentration of NH_4^+ is to minimise the risk of NH_4^+ toxicity and avoid acidification of the culture media caused by the NH_4^+ uptake (Kaul and Hoffman 1993). It is thus not certain that NH_4^+ at a higher concentration in the germination medium would improve N utilization.

AA metabolism during germination

In this study, the germinants were placed in a N-rich environment, possibly leading to excessive N uptake. Given the substantial assimilation of NO_3^- and NH_4^+ N into Gln and other AAs, our data did not suggest that uptake and assimilation of mineral N were highly inhibited despite a high internal concentration of tissue N, resulting in a build-up of the N-rich AAs Gln and Arg (Fig. 2b). Hence, the N in the cells of germinating Norway spruce somatic embryos could be incorporated into the AAs Gln and Arg as a way of storing it, similarly to what has been reported for maturing seeds of other conifer species (King and Gifford 1997; reviewed by; Cánovas et al. 2007). This is also in agreement with the studies on seed germination of white spruce (Gifford and Tolley 1989) and loblolly pine (*Pinus taeda*) (King and Gifford 1997), which showed that hydrolyzation and mobilization of storage reserves cause a concentration increase in the TFAA pool, with Glc, Gln and Arg being the most abundant (King and Gifford 1997). The accumulation of AAs with a possible function in N storage presented in this work, might explain why red light treatment had a positive impact on the somatic embryo development and overall germination success in Norway spruce (Kvaalen and Appelgren 1999) and pine species (Merkle et al. 2005). However, further studies of this process under white light are required to confirm this hypothesis.

Summary

This study showed that C and N stored during the maturation phase was not sufficient for the somatic embryo during germination, thus making it dependent on the accessible resources in the medium. The utilization of different N sources, together with N available in the medium, suggested that the uptake was controlled, preferring Gln and NH_4^+ over NO_3^- . The finding that Gln-N accounted for 50% or more of N taken up and assimilated is new considering that the majority of the relevant publications have focused on NH_4^+ and NO_3^- utilization. The results presented here justify Gln and perhaps also other forms of organic N to be regarded as major sources of N in the context of plant in vitro culture. However, to validate this claim, experiments on additional plant species are required. The low uptake and assimilation of NO_3^- could also imply that the fraction of NO_3^- in

the medium was higher than necessary. On the other hand, this study was only covering half the germination time and theoretically, at the end of the second germination phase N or specific N source depletion is possible. This suggests that there are factors in the somatic embryo germination process to investigate further, e.g. the uptake and assimilation of different N sources under white light, a knowledge needed to balance the medium composition with the requirements of germinating somatic embryos.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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