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The effect of arginine on root system development in Norway spruce (*Picea abies* L. Karst) somatic embryos

Effekt av arginin på rotutveckling av somatiska embryon i gran (*Picea abies* L. Karst)

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Abstract

The demands for higher production yields and better quality materials from the forests are increasing globally. Tree breeding programs are directed to meet the future demands on forests. In order to capture the full benefits from the breeding programs, clonal propagation is necessary. For most conifer species, somatic embryogenesis (SE) is the only available option for large scale clonal propagation of Elite clonal material. For Norway spruce (*Picea abies* L. Karst.), SE procedures suitable for laboratory scale propagation have been available since the mid-1980s. The SE technique has a great potential for mass propagation of conifer species; however the implementation in forestry for plant production has so far been hampered by the labor intensive procedures making the technique costly. SE involves a series of developmental processes, starting with induction of embryogenic cultures from an initial explant, multiplication of somatic embryos in the proliferation phase, early embryo differentiation, maturation and germination.

The aim of this study was to investigate and characterize how organic nitrogen affects the first emergence and formation of the root system in maturated and germinated Norway spruce somatic embryos and plants. The present study suggests that organic nitrogen (arginine) interacts with glutamine and positively affects embryo germination and root development. It was also observed that the amino acid D-serine inhibits embryo germination at the concentrations tested here, and had no visible positive effect on root development during germination. Image analysis of the development of root systems, in planted SE plants, showed no significant difference between plants treated with 5 mg ammonium nitrate or arginine 5 mg. However, analyses of the amino acid content revealed that plants treated with 10 and 5 mg arginine had higher amino acids content than plants treated with 10 and 5 mg ammonium nitrate, respectively, which might indicate that arginine was preferable to ammonium nitrate as nitrogen source.

Sammanfattning (Swedish)

Kraven på skogsindustrin för högre produktionsvolymer och råvarukvalitet ökar globalt. Det skogliga förädlingsarbetet är därmed inriktat på att möta framtidens krav. För att ta bäst ta till vara på förbättringarna från förädlingsprogrammet är dock klonförökning nödvändig. För de flesta barrträdsarter är somatisk embryogenes (SE) det enda möjliga alternativet för storskalig produktion av klonat elitmaterial. Laborativa SE-metoder för gran (*Picea abies* L.Karst.) etablerades i mitten av 1980-talet. Det är i princip möjligt att använda SE-tekniken för massproduktion av granplantor, men den praktiska implementeringen inom skogsbruket har dröjt på grund av arbetsintensiva processer vilket gör SE-plantan dyr att framställa. SE-processen börjar med start av embryogena kulturer och följs därefter av massförökning av omogna somatiska embryon. De somatiska embryona genomgår därefter en mognadsfas innan de gror och utvecklas till plantor.

Syftet med den här studien var att undersöka och beskriva hur organiskt kväve påverkar utvecklingen av rotsystemet från mogna somatiska granembryon under groningen och planterad gran SE-plantor. Resultaten från studien indikerade att organiskt kväve (arginin) interagerar med glutamin och påverkar embryots groning samt dess rotutveckling positivt. Det observerades även att aminosyran D-serin hämmar embryo groning vid de koncentrationer som testades i studien och det fanns ingen synlig positiv effekt på rotutveckling under groning. Bildanalys av rotsystemen från de odlade SE-plantorna visade en signifikant skillnad mellan plantor behandlade med 5 mg arginin eller 10 mg arginin, 10 mg ammoniumnitrat och kontrollen utan något tillsatt kväve. Det upptäcktes ingen signifikant skillnad mellan plantorna behandlade med 5 mg arginin eller 5 mg ammoniumnitrat. Emellertid upptäcktes från analysen av aminosyra innehållet att plantorna som behandlats med 10 mg och 5 mg arginin hade högre aminosyra-innehåll än plantorna behandlade med 10 mg och 5 mg

ammoniumnitrat, vilket skulle kunna indikera att SE plantor föredrog arginin som kvävekälla framför ammoniumnitrat.

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1. Introduction

Norway spruce (*Picea abies* L. Karst.) has significant social and economic value in Sweden, Poland and Finland. Norway spruce forests have existed naturally for over two thousand years providing important raw material for fuel, timber, fiber, pulp and paper industries (Östlund et al. 1997). Sustainable forest management evolved during the 20th century to deal with the increasing demand for forest products in the threat of diminishing forest covers (Östlund et al. 1997, Cubbage 2003, Szymański 2007). The concept of sustainable forest management is that economic, environmental and social values of the forest should be equally valued and the forest should be managed in such a way these values will be preserved for future generations (The Swedish Environmental Code 2000, Cubbage, 2003). Forest industries are now moving into an era where the demand for forest products is increasing, and consequently the demand for high yield, elite trees with higher timber quality, and characteristics like pest-, cold- and drought resistance will increase (Tzfira et al. 1998, Cubbage 2003, The Swedish Government's 'Oil Commission' 2006). Efficient forest tree production will require trees with shorter re-generation time and efficient large scale propagation strategies in order to meet the existing demands for the future (Intergovernmental Panel on Climate Change 2000, The Swedish Government's 'Oil Commission' 2006).

Norway spruce plants for reforestation are primarily produced from seeds in nurseries and to a much lesser extent from stem cuttings (Wennström et al. 2008). However, in the future the forest companies are going to need efficient techniques for large scale clonal propagation of trees with desired qualities to fully capture the values from the tree breeding programs. Somatic embryogenesis (SE) is a well-established *in vitro* technique which produces plants from non-zygotic cells from the hypocotyl region of the seed embryo by the same developmental pathways as the seed embryo development (zygotic embryogenesis) (von

Arnold et al. 2002). Somatic embryogenesis involves a series of developmental processes starting with induction of embryogenic cultures, multiplication of somatic embryos at the proliferation phase, early embryo differentiation, maturation and germination (von Arnold et al. 2002, Pullman et al. 2003, George et al. 2008). Early stage somatic embryos are initiated from zygotic embryos in the presence of auxin and cytokinin and multiply to form pro-embryogenic masses (PEMs) on the same composition medium (Fig. 1) (Filonova et al. 2000). PEM cultures are then transferred to medium without plant growth regulators for a period of seven days to stimulate early embryo differentiation from PEMs. Next, the cultures are transferred to an embryo- maturation medium supplemented with ABA to promote maturation. After maturation, mature somatic embryos are desiccated for a period of 21 days before being transferred to germination medium where they develop into plants (Fig. 2) (Filonova et al. 2000, von Arnold et al. 2002). When the plants have reached a desirable size (typically a root length of 3-4 centimeters) they are planted in peat-based compost under greenhouse conditions with 24 hours light at 100-200 μmol $m^{-2}s^{-1}$, temperature of 20^{o}C and 75-80 % humidity (Högberg et al. 2001).

The SE developmental process is supported by different nitrogen (N) sources supplemented in the growth media (i.e. glutamine, ammonium nitrate and potassium nitrate). In higher plants, nitrogen metabolism occurs via the GS/GOGAT (glutamine synthetase/glutamate-2-oxoglutarate aminotransferase) pathway with ammonium as the primary source of nitrogen (Lee and Ireland 1999). Ammonium is taken up by the root into the GS/GOGAT pathway. In the GS/GOGAT pathway the two enzymes glutamine synthetase and glutamate synthase transfer the nitrogen to the production of the amino acids glutamine and glutamate. Both glutamine and glutamate are important nitrogen supporters for further production of other amino acids (i.e. arginine or proline) or nitrogenous compounds (proteins) used for plant

growth (Fig. 3) (Slocum 2005, Cánovas et al. 2007). Arginine is one example of an amino acid that is synthesized from glutamate. Different arginase- and urease enzymes are involved in a two step reaction leading to arginine production. In the first step, ornithine is produced from glutamate and in the final step, arginine is produced from ornithine (Verma and Zhang 1999, Slocum 2005). Recycling of amino acids and nitrogenous compounds is another important feature in plant nitrogen metabolism (Forde and Lea 2007).

Two forms of the enzyme glutamine synthetase have been detected in angiosperms, GS1 – found in the cytosol; and GS2 – found in the plastids. However, in conifers only one of the enzymes (GS1) has been discovered. In Pine, the GS1 enzyme exists in two isoforms, 1a and 1b, which are suggested to have duplicated roles as both GS1 and GS2 (Avila et al. 2001, Cánovas et al. 2007). Glutamine synthetase catalyzes the formation of glutamine form glutamic acid and ammonium (Forde and Lea 2007).

One of the limiting factors for forest growth in Sweden is nitrogen. The total atmospheric nitrogen deposition in forests in Sweden ranges from about 15-20 kg N/ m^2/ yr in the south-west to 3-2 kg N/ m^2/ yr in the northern (Lövblad 2000). The forest companies are therefore applying fertilizers containing nitrogen (i.e. nitrate and/or ammonium) to compensate the potential growth loss due to limiting availability of nitrogen in the soils (Östlund et al. 1997).

The results from research studies on the effect of different sources of nitrogen on growth and development of conifer seedlings show that ammonium rather than nitrate is the preferred nitrogen source for conifers (Buchmann et al. 1995, Öhlund and Näsholm 2001, Heiskanen 2005). Moreover, it has been reported for Scots pine (*Pinus sylvestris* L.) seedlings that the nitrogen uptake was higher from the amino acid glutamine than from nitrate (Persson et al. 2006). A recent study with *Arabidopsis thaliana*, showed that different nitrogen sources such as nitrate, ammonium and glutamine affected the partitioning of plant biomass (Cambui et al.

2011). In the study, all plants were comparable in size irrespective of nitrogen source, but plants growing with glutamine had a significantly larger root mass fraction than those growing only with inorganic nitrogen. They also tested to supply the medium with nitrate and arginine and it was then observed that the absorption of arginine nitrogen in the roots was twice as high compared to the shoots. Hence, nitrogen from arginine had a similar distribution pattern in the roots as nitrogen from glutamine (Cambui et al. 2011). It has been shown that arginine can be used as an organic nitrogen source and equally support growth in place of inorganic sources (Öhlund and Näsholm 2001). Furthermore, greenhouse grown seedlings supplemented with arginine showed enhanced growth and higher needle nitrogen content compared with seedlings supplemented with a commercial fertilizer (Öhlund and Näsholm 2002). In a recent study, arginine enhanced root to shoot ratio and induce growth of root system in Norway spruce seedlings compared to seedlings given an inorganic nitrogen source (Gruffman et al. 2012).

Almost all amino acids found in nature exist in two reflecting versions of each other, L- (levorotatory) and D- (dextrorotary) amino acids. The L version of an amino acid is the one that is common in nature and can be utilized by plants; whereas the D version is not that common and plants do not have the required enzymes for metabolizing the amino acid into other forms of compounds which the plant can use (Forsum et al. 2008, Näsholm et al. 2010). Nevertheless, it has been observed that D-serine improved the amount of root branching in *Arabidopsis thaliana* at very low concentrations (T Näsholm, pers. comm.).

Somatic embryogenesis offers a model system for studying different stages of embryo development with the advantage that large numbers of embryos of the same genotypes can be obtained. A notable characteristic of somatic embryo germination and early plant

development is the slower development of the first root system compared to seedlings, which have been seen in different species; white spruce (Hakman and von Arnold 1988), North American ginseng (Zhou and Brown 2006), and radiate pine (Montalbán et al. 2010). Norway spruce somatic embryo plants that had developed lateral roots in vitro had better height growth than those lacking lateral roots after they were planted ex vitro in a greenhouse (Högberg et al. 2003). This partly reflects the relative immaturity or less vigorous development of somatic embryos compared to seedlings of similar size developing under the influence of the megagametophyte. Somatic embryos can be used as a model system for studying the earliest stages of germination, which for zygotic embryos take place inside the seeds.

1.1 Aim

The aim of this study was to investigate and characterize how organic nitrogen affects the first emergence and formation of the root system in Norway spruce (*Picea abies* L. Karst) mature somatic embryos during germination and plant formation.

2. Materials and Methods

2.1 Plant material and growth conditions

Embryogenic cultures of cell lines; 09:54:09, 09:71:01, 09:73:05 and 09:77:03 were multiplied on solidified ½ LP medium (von Arnold & Erikson 1981) supplemented with 9 µM 2,4-D and 4.4 µM BAP. To stimulate early embryo differentiation from PEMs, the cultures were transferred to solidified DKM pre-maturation medium without plant growth regulators (PGRs) in the dark at 20 ˚C. After one week, the embryogenic cultures were transferred to solidified DKM maturation medium supplemented with 29 µM ABA (maturation medium) to promote late embryo development and embryo maturation.

Cultures were subcultured every third weeks to fresh medium of the same composition. After six weeks on maturation medium, the cultures were transferred to solidified maturation medium supplemented with 0.25M myo-inositol for another three weeks (Egertsdotter & Clapham 2011).

2.2 Desiccation and germination of mature embryos

Mature somatic embryos from cell lines 09:71:01 and 09:77:03 were isolated from the embryogenic cultures and placed into an empty Petri dish (6 cm), which was transferred and covered inside a 9 cm Petri dish containing 1 ml of sterile water for three weeks. After desiccation, the embryos were transferred to solidified DKM medium without ammonium nitrate (Appendix 1) in the dark for two weeks to promote root development. Subsequently, plants with developed roots were transferred to ¼ Schenk and Hildebrandt (SH) medium (Duchefa, Sweden) and placed in 24 h light with a gradual transition period to illumination 50 µmol $m^{-2}s^{-1}$, for another two weeks.

Mature somatic embryos from cell lines 09:54:09 and 09:73:05, with and without desiccation treatment, were placed to solidified DKM medium without ammonium nitrate (Appendix 1) supplemented with different arginine concentration (Tab. 1) and placed in the dark. Developing plants were transferred to 24 h light with a gradual transition period into illumination 50 μmol $m^{-2}s^{-1}$, for another two weeks and then transferred to new medium after three weeks. Pictures were taken second and fourth week of treatment using a Nikon d5000 digital camera (Nikon Corporation, Sweden). Settings for camera and distance (from camera to plate) and light were the same every photo call.

Desiccated mature somatic embryos from cell lines 09:71:01 and 09:77:03 were placed directly to ¼ SH medium supplemented with various different amounts of D-serine (Table 2) and placed in the dark. Developing plants were transferred to new medium and placed in 24 h light with a gradual transition period into illumination 50 μmol $m^{-2}s^{-1}$ after two weeks of treatment. Pictures were taken after the second, third and fourth week of treatment using a Nikon d5000 digital camera (Nikon Corporation, Sweden). Settings for camera and distance (from camera to plate) and light were the same every photo call.

2.3 Development of somatic embryo (SE) plants

Before planting the germinated embryos in pots with experimental substrate, they were photographed. Also, the embryo shoots and roots were measured. The experimental substrate was prepared manually as a 70/30 % volume substrate mixture of natural peat (Weibulls Naturell, Econova Garden, Sweden) and perlite (Bröderna Nelson, Nelson Garden, Sweden). 4 gram of dolomitic limestone per liter substrate was also added to the substrate to increase the pH. Control substrate was not sprayed at all, whereas the experimental substrates were sprayed with arginine (arGrow® Complete, SweTree Technologies, Sweden) or ammonium nitrate (RIKA - Weibulls växtnäring, Econova Garden, Sweden) mixtures (Tab. 3) before

being manually packed into 20 ml pots (PlantStart Pluggbox, Nelson Garden, Sweden) (method by SweTree Technologies). The SE plants were covered with a hood (PlantStart Pluggbox, Nelson Garden, Sweden) for the first two weeks inside the greenhouse (Swedish University of Agricultural Sciences, Umeå) to create a gradual transition to the *ex vitro* greenhouse conditions at 24 h light, illumination 100-200 μmol $m^{-2}s^{-1}$, 75% humidity and 20-22 ^{o}C. SE plants were watered manually every day to prevent the soil from drying out. The plants were harvested after seven weeks. The whole package with peat and plant was picked up from the planting pots (Fig. 4). Peat particles were gently removed by washing the plant in water baths three times. Thereafter, the plants were placed in sealed plastic bags and left over night at 4^{o}C followed by analysis.

2.4 Root analysis

The whole plant was placed in a tray with water covering the roots and thereafter scanned using EPSON perfection V700 Photo, dual lens system, scanner (SEIKO EPSON Corporation, Sweden). The root system (Fig. 5), with starting point shoot/root junction, was analyzed using *WinRHIZO Pro* image analysis software system (Regent Instruments Inc, Canada), with morphology measurement and no filters (Himmelbauer et al. 2004).

2.5 Analysis of amino acids content

Amino acids were extracted from the roots and analyzed by the AccQTag method (Waters, Milford, USA) and analyzed by the ACQUITY UPLC® (Ultra Performance Liquid Chromatography) Amino Acid Analysis System at Umeå Plant Science Centre, Sweden. Shoot and root was separated at the shoot/root junction using a scalpel and the roots were placed in scint-pots followed by freeze-drying the pots in liquid nitrogen. Two beads per sample were placed in each pot followed by grinding, using a beadmill. 1 ml of 10mM HCl was added to 30 mg of root sample in an Eppendorf tube, for extraction of amino acids. Two technical replicates of each sample were made. The samples were first shaken at room

temperature and then left to shake at 4°C for one hour. The samples were thereafter centrifuged at 14000 rpm for 10 min, at 4°C.

Analyzes of amino acids was performed according to by AccQ•Tag™ Ultra, Waters Corporation. The samples were differentiated using AccQ•Tag™ Ultra Derivatization Kit and analyzed on the ACQUITY UPLC® (Ultra Performance Liquid Chromatography) System with AccQ•Tag™ Ultra column (2.1 x 100 mm, 1.7 µm) and with a TUV-detector, at a flow of 0.6 ml per minute. The contents of all amino acids and ammonium were measured.

2.6 Statistical analysis

One-way ANOVA analysis was made using the statistical program JMP (SAS Institute Inc., USA). Analysis was performed for parameters total root length (cm), projected area of total root system (cm^2), number of root tips from *WinRHIZO Pro* image analysis software system (Regent Instruments Inc) and root weight (mg). Comparisons among all five treatments were performed by the Tukey-Kramer HSD test. The significant level was set at $P \leq 0.05$.

3. Results

3.1 Root germination of mature somatic embryos

Cell line 09:54:09

Embryos without and with standard desiccation treatment after the various arginine treatments are shown in Figure 6. Those embryos which did not undergo standard desiccation treatment were already after two weeks showing signs of strange and weak germination development. Embryos without desiccation had a swollen and crooked phenotype compared to the embryos with desiccation.

After four weeks all non-desiccated embryos had a continuous swollen and crooked phenotype and a red-yellow colour, exept for the positive control and the two additive controls, which had green shoots. The positive control with desiccated embryos also showed a swollen and crooked phenotype with green colour after four weeks from germination. Embryos in both the additive controls had started producing roots after four weeks and had green shoots; whereas the embryos for the negative control, arginine 0.3 mM and 1 mM, arginine 0.3 mM and 1 mM with casein hydrolysate treatments had a red-yellow colour. The only root development observed for embryos without desiccation were those treated with the additive 0.3 mM control and arginine 1 mM supplemented with casein hydrolysate, 15 and 3 percent, respectively (Tab. 4); while root development for embryos with desiccation was much greater. Highest were treatments arginine (0.3 and 1 mM) supplemented with casein hydrolysate and both additive controls with root development around 70 percentage (Tab. 4).

Cell line 09:73:05

Embryos without and with standard desiccation treatment and the different arginine treatments are shown in Figure 7. The positive control and the two additive control embryos

without desiccation treatment had green shoots after two weeks of germination. Embryos from all the other treatments without desiccation had shoots that were more yellow than green. For the embryos with desiccation the colour is green, exept for the negative control which had more red colour.

After four weeks, only the positive control and both additive controls, with and without desiccation, were still green. Whereas, embryos treated with the negetative control, arginine 0.3 mM and 1 mM, arginine 0.3 mM and 1 mM with casein hydrolysate treatments had a red-yellow colour. Furthermore, embryos without desication which had a visible root development were the positive control, both additve controls and those treated with arginine (0.3 and 1 mM) supplemented with casein hydrolysate (Tab. 5). Compared with the desiccated embryos, many of those embryos had started developing a root regardless of treatment (Tab. 5). Those with the highest percentages with roots were both additive controls (97 percentage for 0.3 mM and 91 percentage for 1 mM) and embryos treated with arginine (0.3 and 1 mM) supplemented with casein hydrolysate (91 and 85 percentage, respectively). Those with the lowest percentages were the negative control and arginine 0.3 mM treated embryos, 41 and 3 percentage respectively.

3.2 D-serine effect on root germination of mature somatic embryos

Cell line 09:71:01

After two weeks, embryos growing on the control medium had started to develop roots in 9 out of 15 embryos but none of the embryos treated with D-serine had started to develop roots (Fig. 8). The week after, there were still no roots emerging from the embryos treated with D-serine. However, the embryos treated with 0.3mM had green shoots and were in good physical shape. After four weeks there were still no roots developed from the D-serine treated

embryos. The treated embryos were in bad physical condition, the embryos given 3mM of D-serine being most affected and the 0.3mM treated embryos the least affected.

Cell line 09:77:03

After two weeks, embryos growing on the control medium and treatment 0.3mM D-serine have started getting emerged roots (Fig. 9). Whereas, the embryos treated with 1- and 3mM D-serine had no root germination. The third and fourth week of treatment are looking similar. The control embryos were continuing to grow in size and root length. While, embryos treated with 0.3mM D-serine were green and continuing growing in size but there were no further root germination. The 1mM D-serine treated embryos were also green in colour but they did not have root germination. Moreover, the 3mM D-serine treated embryos looked to be in bad physical condition with pale colour and poor hypocotyls and size growth.

3.3 Root development after planted in greenhouse

After seven weeks of treatment, the weight (mg) of harvested plant from the two cell lines, 09:71:01 and 09:77:03, was compared against the starting plant length (cm). The arginine 5 mg treatment showed the highest plant weight when the starting plant length was 2 and 3 cm for cell lines 09:71:01 and 09:77:03, respectively. The highest similarity was observed in the control treatment for both cell lines, where the weight value was around 100 mg. The four other treatments showed a wider weight distribution in the two cell lines (Fig. 10).

3.4 WinRHIZO Pro analysis

Cell line 09:71:01

In cell line 09:71:01 (Fig. 11), there were significant differences ($P \leq 0.05$) between treatments; arginine 5 mg against control, arginine 10 mg and RIKA 10 mg, for all the measured parameters (total root length (cm), projected root area (cm^2), number of root tips and root weight (mg)). However, there was no significant difference between the arginine 5

mg treatment and the RIKA 5 mg treatment. The lowest length, projected area, tips and weight value were shown in the arginine 10 mg treatment.

Cell line 09:77:03

Cell line 09:77:03 showed no significant difference between the five different treatments irrespective of measured parameter: total root length, projected root area, number of root tips or root weight (Fig. 12). Even though there were no significant differences for this cell-line, the arginine 5 mg treatment had the longest root length, biggest projected root area and highest root weight. The number of root tips is similar for both the arginine 5 mg and RIKA 5 mg treatments. Lowest value was found in the control treatment.

3.5 Amino acid content

With the analysis of amino acid content, 23 amino acids and ammonium were measured. The calculated amount of each amino acid and ammonium (µmol/ g fresh weight root) is shown in appendix 2. Comparison of the treatments for each amino acid and ammonium shows that treatments with addition of 10 mg nitrogen have the highest amino acid content regardless of which amino acid and cell line.

The total amino acid and ammonium content for both cell lines showed that the arginine 10 mg treatment had the highest content of amino acid and ammonium, whereas the control treatment had the lowest content of amino acid and ammonium (Fig. 13). Since only two technical replicates were analyzed no statistical analysis could be preformed.

3.6 Plant mortality

The total number of plants at the beginning (in different root length classes) and the number of those that died are listed in Table 6. Percentage of dead plants after seven weeks in each starting root class (Fig. 14) showed no difference in survival according to whether the plant

started with a short root or a long root. Furthermore, during the seven weeks in the greenhouse, plant mortality rate for the cell lines was observed (Fig. 15).

Cell line 09:71:01

Plants from cell line 09:71:01 started with only two different root classes (0.5-1 and 1.5-2 cm) and no visual difference in plant death between the two classes was confirmed (Fig. 14a). Cell line 09:71:01 had the lowest plant mortality rate in both the control and the arginine 5 mg treatment. The highest mortality rates were with the two treatments with 10 mg of nitrogen (Fig. 15a).

Cell line 09:77:03

Cell line 09:77:03 had four different starting root classes (0.5-1, 1.5-2, 2.5-3 and 3.5> cm) and no observed difference in plant death between the root length classes was shown (Fig. 14b). The lowest plant mortality rate for cell line 09:77:03 was in the control treatment and the highest mortality rates were in the two treatments with 10 mg of nitrogen (Fig. 15b).

4. Discussion

4.1 Root development during germination of mature somatic embryos

Overall results

The best root germination were observed in cell lines 09:54:09 and 09:73:05 for desiccated embryos when cultured on DKM rooting medium without ammonium nitrate supplemented with 0.3 mM and 1 mM arginine (Fig. 6, 7). After the second and fourth week of treatment, lowest root germination was observed for embryos without desiccation for both cell lines (Fig. 6, 7). Furthermore, after two weeks on germination medium, embryos without desiccation from both cell lines had developed a strange phenotype with swollen and crooked hypocotyls. (Surprisingly, also embryos growing on DKM rooting medium without ammonium nitrate, regardless if they had been desiccated or not, developed the same abberant phenotype.) A similar phenotype has been described for *Picea glauca* somatic embryo plants cultured on 15 and 30 µM ABA instead of 7.6 µM together at various concentrations of sucrose (Hakman and von Arnold 1988).

Nitrogen sources

In this study, root germination was improved relative to the control by addition of 0.3 or 1 mM arginine in the germination medium, and also using a germination medium with arginine and casein hydrolysate as the only nitrogen sources in the germination medium. However, arginine and casein hydrolysate as sole nitrogen sources resulted in red-yellowish plants instead of the green plants that were observed with 0.3 or 1 mM arginine added to the standard germination medium; the difference in medium composition is that the additive germination medium also has glutamine as another main source of nitrogen. This indicates that arginine together with casein hydrolysate as sole nitrogen sources can stimulate root germination but that they are not adequate for normal embryo germination. In some plant

species, glutamine and casein hydrolysate in the germination medium are necessary for the development of plants (reviewed by von Arnold et al. 2002). In alfalfa (*Medicago sativa*), it was shown that glutamine alone could function as a replacement for ammonium and stimulate production of somatic embryos, however arginine alone could not stimulate production of somatic embryos (Stuart and Strickland 1984).

Stress symptoms

Embryos from the two tested cell lines cultured on medium without nitrogen were generally smaller in size than the embryos cultured with nitrogen. These embryos also became pale and yellowish whereas the positive control and additative controls cultured on media with nitrogen became green. It has been reported that plants suffering from nitrogen deficiency become pale green and in really severe cases of nitrogen deficiency plants will be yellowish, or even white (Berry 2010). After four weeks, germinated embryos cultured on standard DKM rooting germination medium without any nitrogen sources (used as the negative control), were red and yellow. In contrast, the embryos cultured on standard DKM rooting germination medium without ammonium nitrate (used as the additive controls) and embryos cultured on standard DKM rooting germination medium, without ammonium nitrate, supplemented with 0.3 or 1 mM arginine (used as the additive controls) and embryos cultured on standard DKM rooting germination medium, without ammonium nitrate, supplemented with 0.3 or 1 mM arginine (used as the additative controls) were green and had developed into plants with shoots and roots. The red colour developed in the embryos cultured on medium without nitrogen could indicate that these embryos were stressed owing to the lack of nitrogen. Previously, it has also been reported that a reddish colour appear on the underside of leaves and midribs of deciduous plants suffering from nitrogen loss (Berry 2010).

Desiccation effects

In this study there was a clear difference in root germination depending on whether embryos had been treated with a standard desiccation treatment or not. In the fourth week on germination medium, root germination was overall lower in both cell lines for embryos without desiccation than for the desiccated embryos (Tabs. 4 and 5). The adoption of desiccation has been seen in several species, i.e. *Picea* and *Larix*, to enhance embryo transition and germination (Attree et al. 1993, Stasolla et al. 2002). It has also been reported that conifer mature somatic embryos require desiccation treatment to germinate and develop into plants (Stasolla et al. 2002, von Arnold et al. 2002). During the earlier maturation phase, embryos accumulate storage products which then decrease throughout the desiccation period (Stasolla et al. 2002). In the present study, thicker plants (without roots) were observed when embryos were germinating without desiccation. This may then be caused by a continued accumulation of storage products since the embryos were placed directly on germination medium without a period of desiccation. Furthermore, previous findings show that the number of embryos which germinate and produce root hairs are 90 % after desiccation treatment with a progressively reduced relative humidity from 90 down to 31 % compared to non-desiccated embryos which only show 10 % of root hair formation (Gorbatenko and Hakman 2001), further supporting our findings that desiccation treatment stimulates germination and root formation in Norway spruce somatic embryos.

4.2 D-serine effect on root development during germination of mature somatic embryos

D-serine has previously been observed to stimulate root branching in *Arabidopsis thaliana* (Näsholm pers. comm.). In Norway spruce, D-serine at concentrations of 0.3, 1 and 3 mM had instead a negative effect on root formation germination in cell lines, 09:71:01 and 09:77:03 (Figs. 8, 9). The most obvious effect on germination was at 3 mM D-serine which resulted in pale green embryos and no clear transition into a plant with a shoot and root. Also at the two

lower concentrations (0.3 and 1 mM) of D-serine, the embryos did not germinate as well as in the controls. The treated embryos had started to germinate into green plants with shoots but the roots had still not emerged after four weeks of treatment. The phytotoxic effects of D-serine is well established and has been utilized in selection of *Arabidopsis thaliana* plants carrying the D amino acid metabolizing enzymes *dao1* encoding the D-amino acid oxidase (Erikson et al. 2004) and *dsdA* encoding D-serine ammonium lyase (Erikson et al. 2005). In both studies detectable inhibition of wild type plants was observed when they were growing on medium supplied with 0.1 mM D- serine whereas mutated plants survived and grew selection medium ranging from 0.01 to 3 mM of D-serine (Erikson et al. 2004, Erikson et al. 2005). It is likely that the concentration tested in this study is phytotoxic for Norway spruce at germination phase.

4.3 Nitrogen effects on root development

Stimulatory effects on root development from organic nitrogen

In the present study, cell line 09:71:01 plants treated with 5 mg of arginine were significantly better ($P \leq 0.05$) for all the measured root variables; total root length, projected area of total root system, number of root tips and total weight of root system, than the control cultured without nitrogen, and both of the two 10 mg nitrogen treatments, ammonium nitrate and arginine, but not plants treated with 5 mg ammonium nitrate (Fig.11). In Norway spruce seedlings, arginine has been shown to increase the root to shoot ratio and induce the formation of a larger root system than seedlings treated with an inorganic nitrogen source (Gruffman et al. 2012). In another greenhouse study, the growth rate and retention of nitrogen in Scots pine (*Pinus sylvestris* L.) seedlings supplemented with 3 mM of arginine or 3 mM ammonium nitrate in the commercial fertilizer, Superba S (Hygro Agil), were compared. The seedlings supplemented with 3 mM of arginine had a better growth and higher concentration of nitrogen in their needles compared to seedlings supplemented with ammonium nitrate. The retention

was also shown to be highest for the arginine treated seedlings (Öhlund and Näsholm 2002). Organic nitrogen has also been shown to be preferred over inorganic nitrogen for root growth and development in *Arabidopsis thaliana* in an experiment where plants were grown on solid medium with single or mixed nitrogen sources of nitrate, ammonium and glutamine. After three weeks, it was shown that the plants that had developed the largest root systems had absorbed the main portion of nitrogen in the roots from glutamine. In a supplementary uptake experiment, plants were treated with nitrogen supplied as 3 mM nitrate and 30 µM of labelled L-arginine. Twice as much nitrogen was then observed in the roots as in the shoots, again suggesting that nitrogen distribution derived from an organic nitrogen source favours the roots (Cambui et al. 2011).

Inhibitory effects on root development from organic nitrogen and other components in the experimental treatment

In the present study, plants from cell line 09:71:01 treated with 10 mg arginine showed the lowest values for all the measured root variables; total root length, projected area of total root system, number of root tips and total weight of root system (Fig.11). In the experiments, arginine was added as part of a complete mixed fertilizer (arGrow® Complete) that contains additional nutrients, including chloride (SweTree Technologies, Sweden). It is suspected that chloride affects spruce plant growth negatively, so that arginine chloride is being replaced by arginine nitrate in the forthcoming new version of arGrow (Arginine Group, Umeå, pers. comm.). Chloride could thus have influenced plant root development negatively in the present study. High sodium and chloride concentrations in soils and waters affect the water potential in plants leading to a disorder in ion balance and toxicity that can result in detrimental effects on plant growth and development. Salinity tolerance is fundamentally different in different species and relatively low concentrations can be lethal in some species (reviewed by Parida &

Das 2005, Zhu 2007). However, in cell line 09:77:03, the control plants without any added nitrogen showed the lowest values for all the measured root variables; total root length, projected area of total root system, number of root tips and total weight of root system (Fig.12). Hence, in this cell line it appears that the absence of nitrogen can have been more important than the absence of chloride.

Comments on plant growth in the green house

Seven weeks of growth of the somatic embryo plants in the greenhouse resulted in a relatively low average plant weight of 100 mg, however with some plants weighing over 400 mg (Fig. 10). Norway spruce seedlings grown for seven weeks in hydroponic conditions with 50 mg l^{-l} nitrogen had an average fresh weight of 355 mg (Vapaavuori et al. 1992). At the start of the greenhouse experiments, many of the germinated embryos did not, however, have an optimal root length (longer than 3 cm) or a well-developed shoot, which could explain the wide-range results in growth (Högberg et al. 2001). Furthermore, the height growth of Norway spruce somatic embryo plants is better if they have well-developed lateral roots when they were planted to ex vitro conditions in a greenhouse (Högberg et al. 2003). None of the germinated embryos that were planted in this experiment had developed lateral roots by the time of planting. This experiment however required the plants to be planted at that specific time point because time was limited.

Plant mortality

In this study the speculation that plants planted with shorter roots (0.5-1 cm) should have higher mortality compared to plants planted with longer roots was not true, since the percentage of dead plants at the time of harvesting was shown to be the same between the root classes (Fig. 14). However, plant mortality was highest for the plants treated with the highest

amount of nitrogen (10 mg) (Fig. 15). This could be explained by the extensive growths of algae and mosses in the planting boxes supplemented with 10 mg of nitrogen, whereas there was no growth of algae or mosses in the control planting boxes without nitrogen. Thus, plants given 10 mg of nitrogen might have been out-competed by the algae and mosses for water and/or nutrients (Ross and Puritch, 1981). Dehydration has also been observed to cause restricted and reduced root growth in Norway spruce SE plants (Becwar et al. 1989). It is also possible that 10 mg of nitrogen from the fertilizer arGrow® Complete, where arginine is present as arginine.HCl, was accompanied by quantities of chloride that might have affected the plant root system negatively (Arginine Group, Umeå, pers.comm.). The overall poor growth of the plants in the greenhouse can be explained by technical issues with the greenhouse at the time for the experiments resulting in e.g. overheating and poor watering.

4.4 Amino acid content

The contents of amino acid and ammonium in the fresh roots were analyzed but with no statistical analysis owing to the absence of sufficient replications (Appendix 2). As expected, the control plants growing without supplemented nitrogen had the lowest amino acid and ammonium content. Nitrogen is an important molecule in the GS/GOGAT pathway for production of glutamine and glutamate, which are important nitrogen sources for the synthesis of other amino acids (e.g. arginine or proline) (Slocum 2005, Cánovas et al. 2007).

Plants treated with 10 mg arginine had higher amino acid and ammonium contents than plants treated with 10 mg RIKA (Fig. 13). In a recent uptake study with *Arabidopsis thaliana* cultivated on agar plates, it was shown that from a combination of glutamine and nitrate, the main portion of nitrogen in the roots had been derived from glutamine. It was also observed that when *Arabidopsis thaliana* plants were grown with nitrate supplemented with arginine, most of the nitrogen in the roots was derived from the arginine (Cambui et al. 2011). This

result supports the theory that plants prefer to take up organic nitrogen rather than inorganic nitrogen.

5. Conclusions

In this study, arginine supplemented together with glutamine was shown to stimulate root emergence and germination in somatic embryos during the germination phase. Arginine supplemented together with casein hydrolysate stimulated root formation during germination but was not adequate for normal embryo germination and the embryos became red-yellowish instead of green. D-serine inhibited root germination and did not have any visible positive effect on root formation.

In the greenhouse, plants treated with 5 mg arginine seemed to utilize arginine better for the development of their root system (root length, projected area of total root system, number of root tips and root system weight) than plants treated with no nitrogen, or 10 mg of nitrogen. Furthermore, plants treated with 10 mg arginine had a higher amino acid and ammonium content than the other plants in the study. This may indicate that arginine was preferred over ammonium nitrate as the nitrogen source, although the mortality rate was highest in the arginine treated plants amongst all of the treatments. However, the mortality could have been caused by chloride toxicity, or water-, sun-, and/or nutrient competition with the growing algae and mosses. The present study was not large enough to allow for statistical analyses of the differences in root growth between plants supplemented with 5 mg arginine or 5 mg RIKA. Future studies will show if (a) arginine is the preferred nitrogen source over ammonium nitrate, and (b) if arginine and organic nitrogen sources can better support the development of the root system in SE Norway spruce plants.

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7. Tables

Table 1. Composition of germination media used to study the effect of arginine on root development during germination of non-desiccated and desiccated mature somatic embryos.

Treatment	Concentration arginine / (mM)	Total concentration nitrogen / (mM)
Positive control	0	30.12
Negative control	0	0.009
Additive control	0.3;1	30.42 ; 31.12
(a) arginine as the only nitrogen sources	0.3;1	0.309 ; 1.009
(b) addition of casein hydrolysate according to original recipe, otherwise as in (a)	0.3;1	4.23 ; 4.93

Table 2. Composition of germination media for studying the effect of D-serine on root development during germination of desiccated mature somatic embryos.

Treatment	Concentration of D-serine (mM)
Control	0
D-serine	0.3
D-serine	1
D-serine	3

Table 3. Composition of substrates pre-treated with different nitrogen sources.

Treatment	Amount of nitrogen (mg)/plant pot
arGrow® (arginine)	5
arGrow® (arginine)	10
RIKA (ammonium nitrate)	5
RIKA (ammonium nitrate)	10
Control	0

Table 4. Percentage of germinated somatic embryos in cell line 09:54:09 with and without standard desiccation treatment after four weeks on germination medium containing different nitrogen sources.

09:54:09		
Treatment	**Root development after desiccation (%)**	**Root development after no desiccation (%)**
Positive control	15	0
Negative control	3	0
Additive control 0.3 mM	68	15
Additive control 1 mM	74	0
Arginine 0.3 mM	0	0
Arginine 1 mM	0	0
Arginine 0.3 mM + casein hydrolysate	68	0
Arginine 1 mM + casein hydrolysate	71	3

Table 5. Percentage of germinated somatic embryos with and without standard desiccation treatment after four weeks on experimental germination medium in cell line 09:73:05.

09:73:05		
Treatment	**Root development after desiccation (%)**	**Root development after no desiccation (%)**
Positive control	59	47
Negative control	41	0
Additive control 0.3 mM	97	9
Additive control 1 mM	91	15
Arginine 0.3 mM	3	0
Arginine 1 mM	62	0
Arginine 0.3 mM + casein hydrolysate	91	26
Arginine 1 mM + casein hydrolysate	85	24

Table 6. Overview of total number of plants planted per cell line, nitrogen treatment, starting root length and number lost plants per size class of root lengths.

09:71:01		Root class: interval for measured root lengths/ cm		09:77:03		Root class: interval for measured root lengths/ cm			
		0.5-1	1.5-2			0.5-1	1.5-2	2.5-3	3.5 ≥
Arg 5mg	Planted	10	11	**Arg 5mg**	Planted	4	21	2	1
	Dead	0	6		Dead	0	11	1	1
Arg 10mg	Planted	10	11	**Arg 10mg**	Planted	5	18	5	0
	Dead	6	4		Dead	1	13	4	0
RIKA 5mg	Planted	10	11	**RIKA 5mg**	Planted	5	21	1	1
	Dead	3	4		Dead	2	8	1	0
RIKA 10mg	Planted	8	13	**RIKA 10mg**	Planted	2	21	4	1
	Dead	3	7		Dead	1	11	2	
Control	Planted	11	10	**Control**	Planted	7	17	3	1
	Dead	3	3		Dead	1	3	1	0

8. Figures

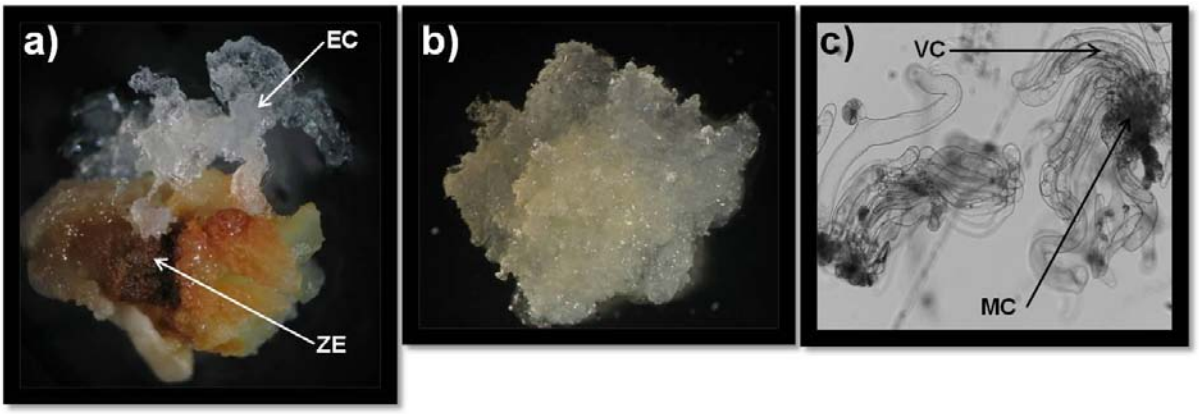


Figure 1. Initiation of somatic embryogenesis; a) Early somatic embryos (pro-embryogentic masses; PEMs) initiated from a zygotic embryo (ZE), b) embryogenic culture (EC) composed of proliferating PEMs, c) early somatic embryos composed of vacuolated suspensor cells (VC) and meristematic cells (MC) in the embryonic head-region.

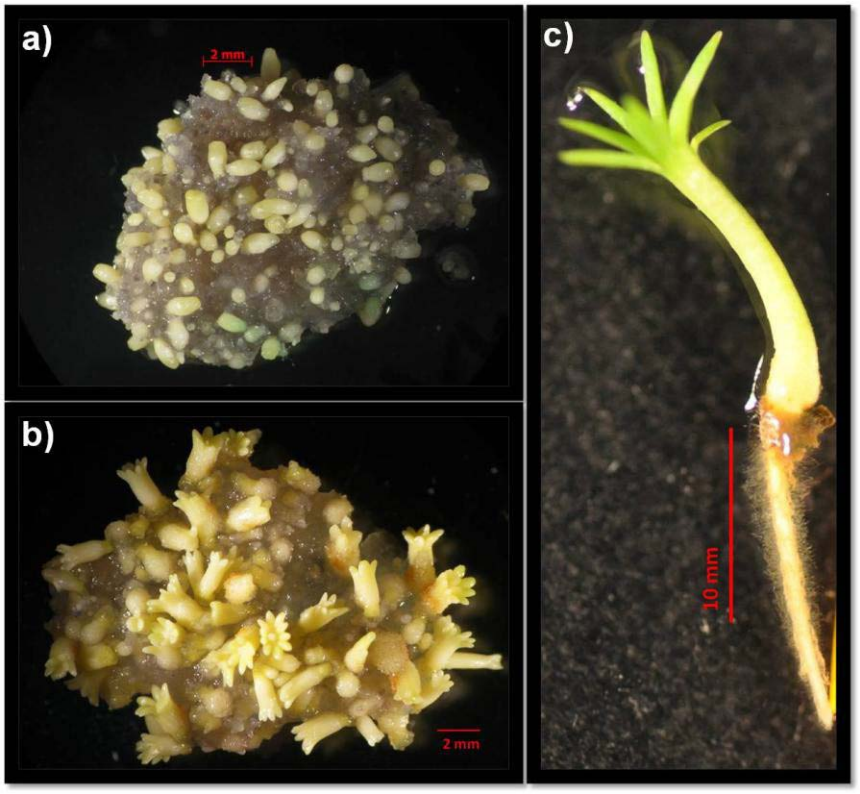


Figure 2. Maturation of somatic embryos; a) maturing somatic embryos interspersed with PEMs; b) fully mature somatic embryos with split cotyledons and c) germinated mature embryo.

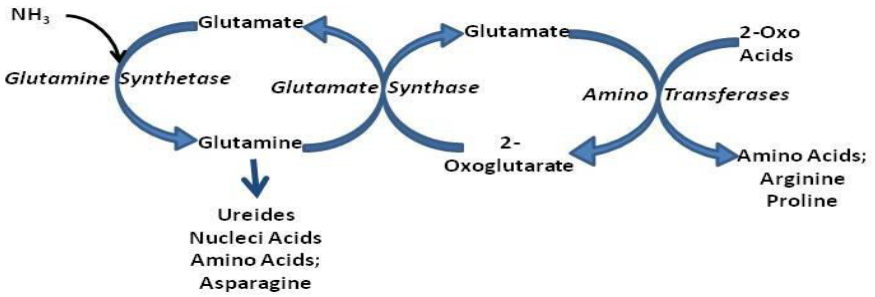
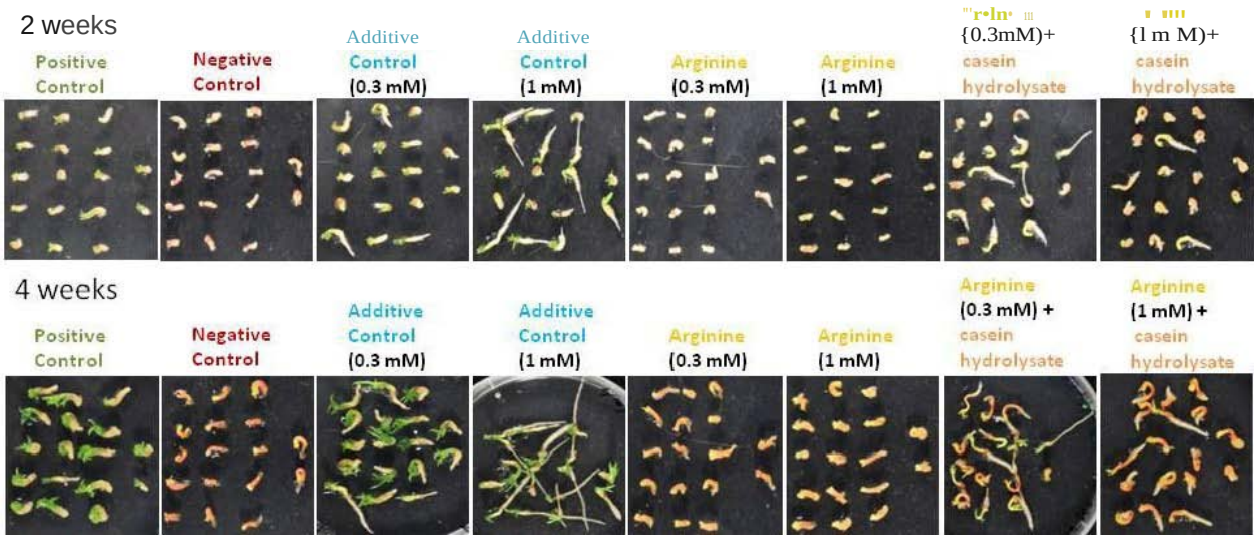


Figure 3. The GS/GOGAT pathway with ammonium as the primary nitrogen source (modified from Forde and Lea 2007).

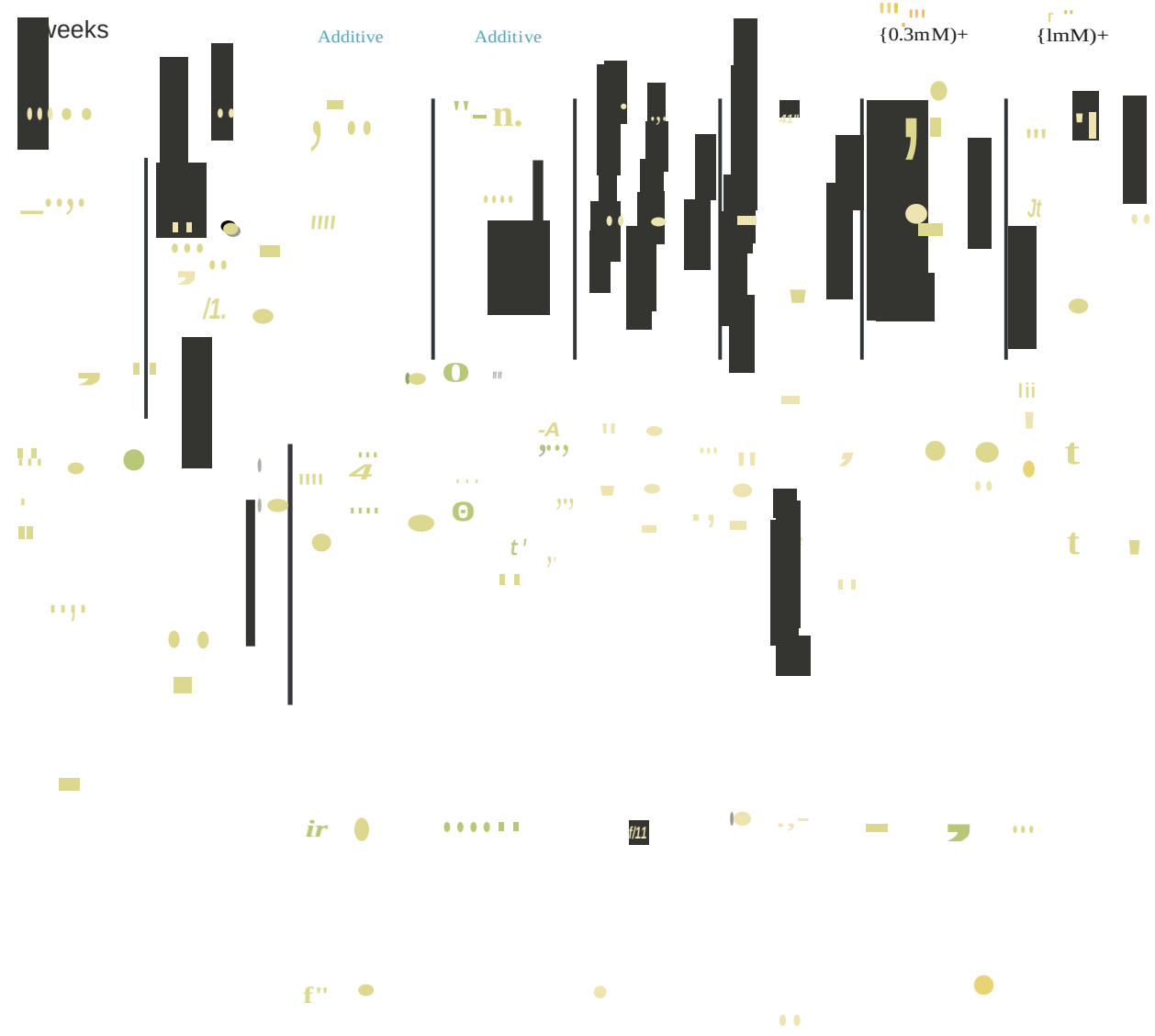
Figure 4. A somatic embryo Norway spruce plant with peat and perlite after being picked up from the planting pot after seven weeks in the greenhouse. Red scale bar; 1cm.

Figure 5. Red area around root system (with starting point shoot/root junction) was analyzed with *WinRHIZO Pro* image analysis system. Scale bar (cm).

09:54:09 – With desiccation treatment



09:54:09 – Without desiccation treatment



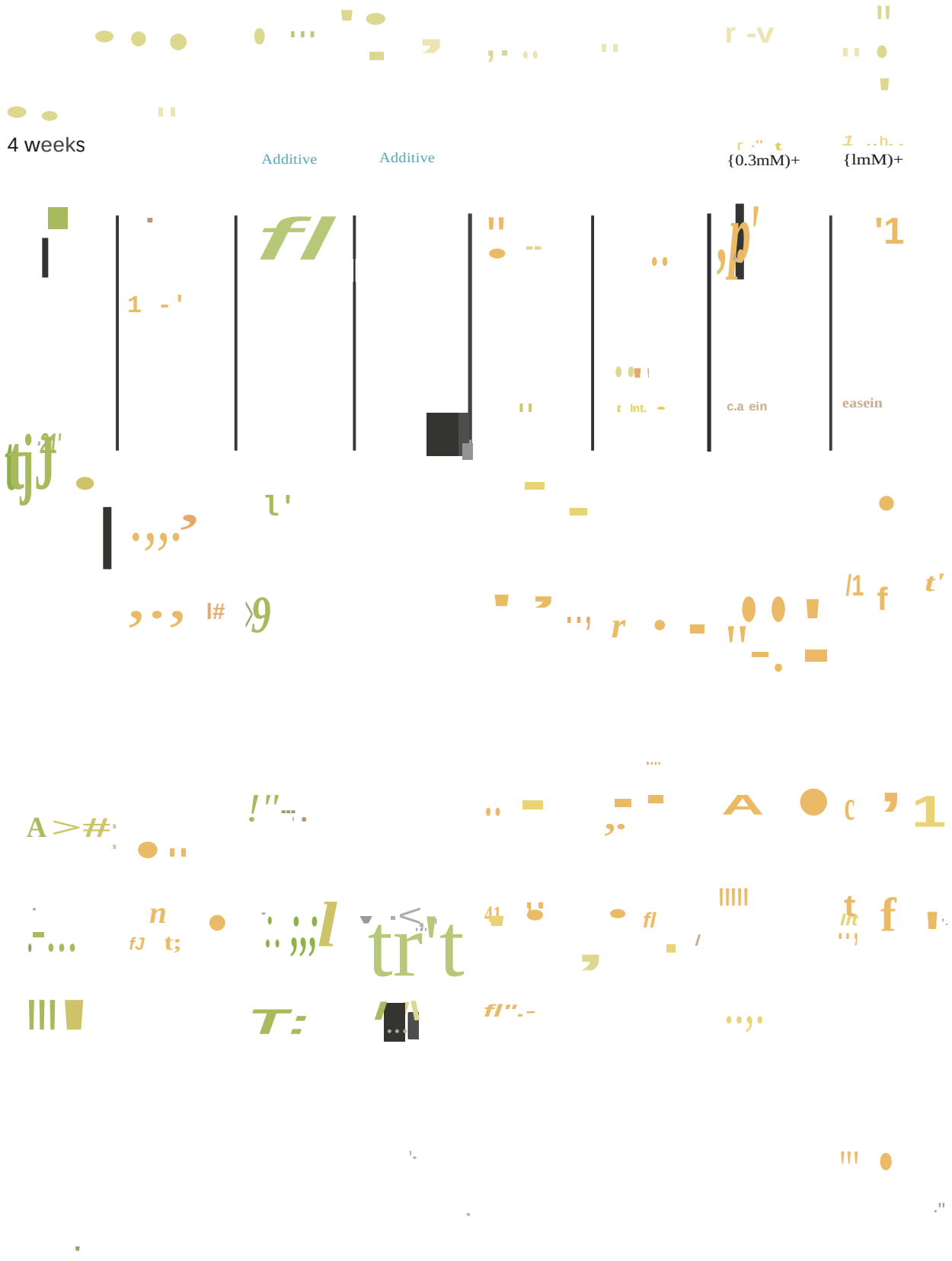


Figure 6. Cell line 09:54:09 without and with standard desiccation treatment after culture on the different arginine treatments for two and four weeks.

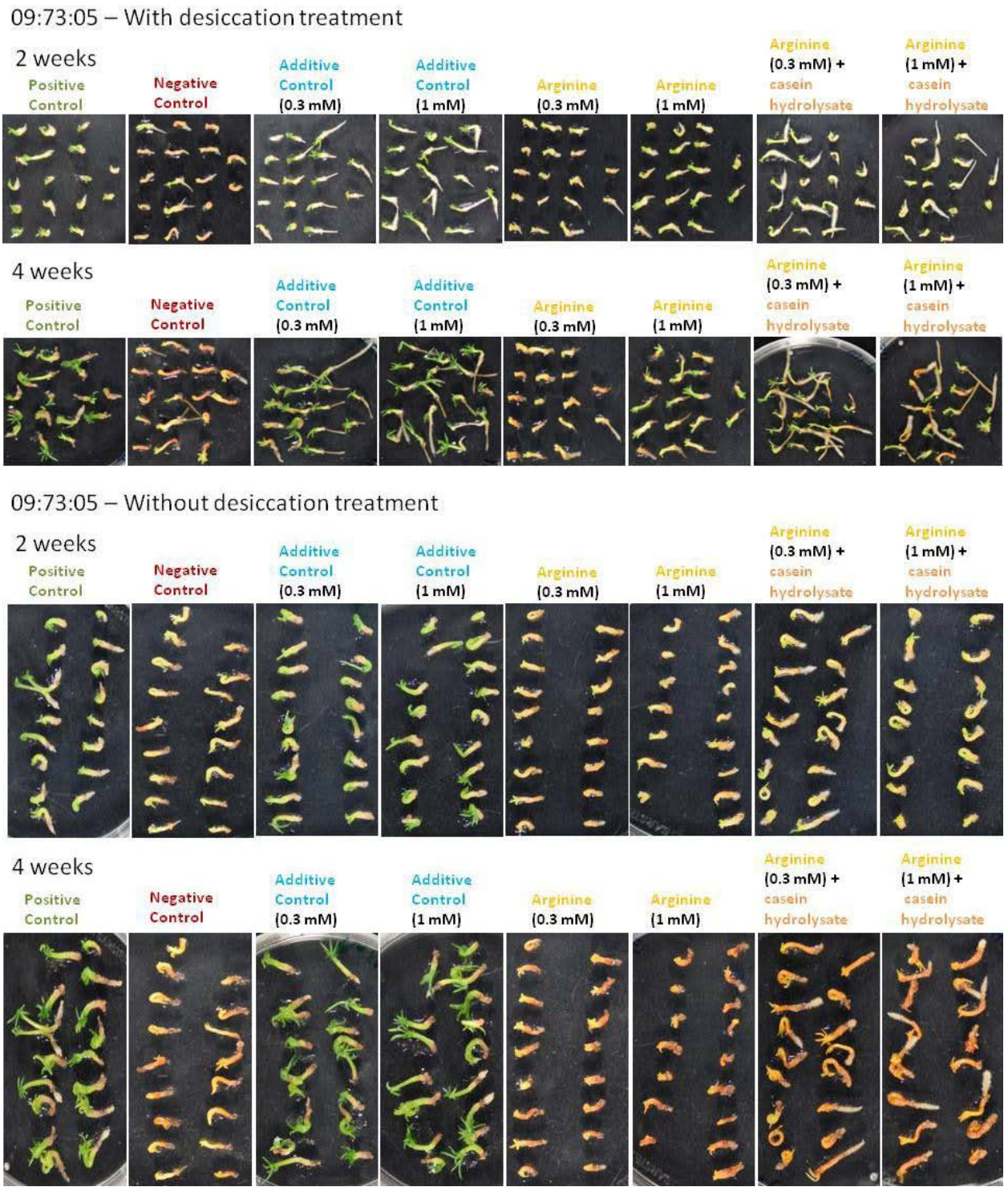


Figure 7. Cell line 09:73:05 without and with standard desiccation treatment after culture on the different arginine treatments for two and four weeks.

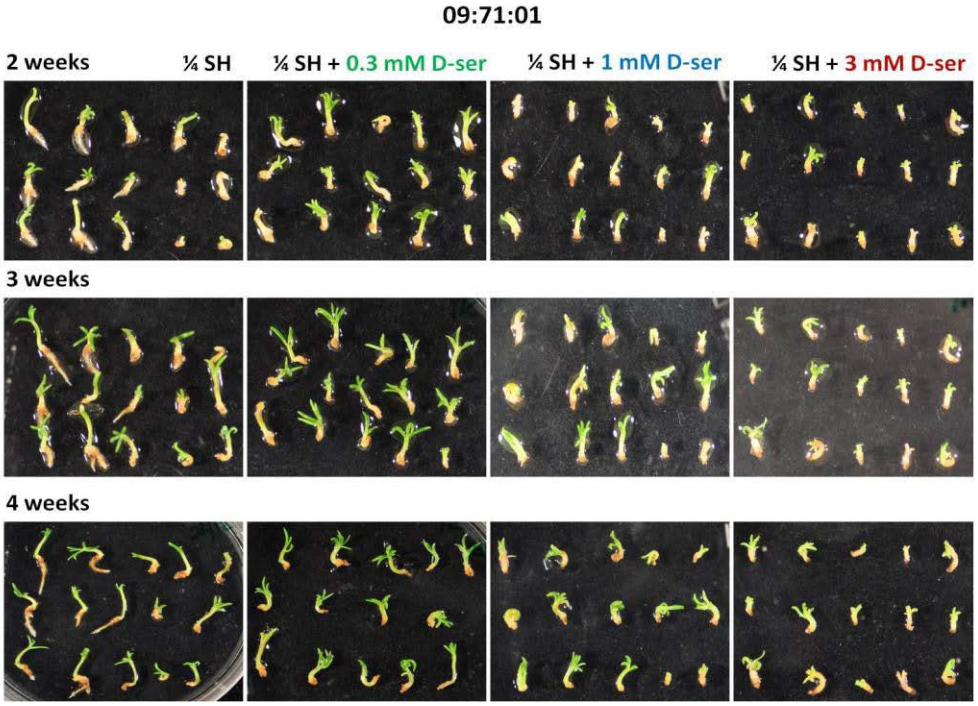


Figure 8. Embryos from cell line 09:71:01 after 2, 3 and 4 weeks of the four different treatments.

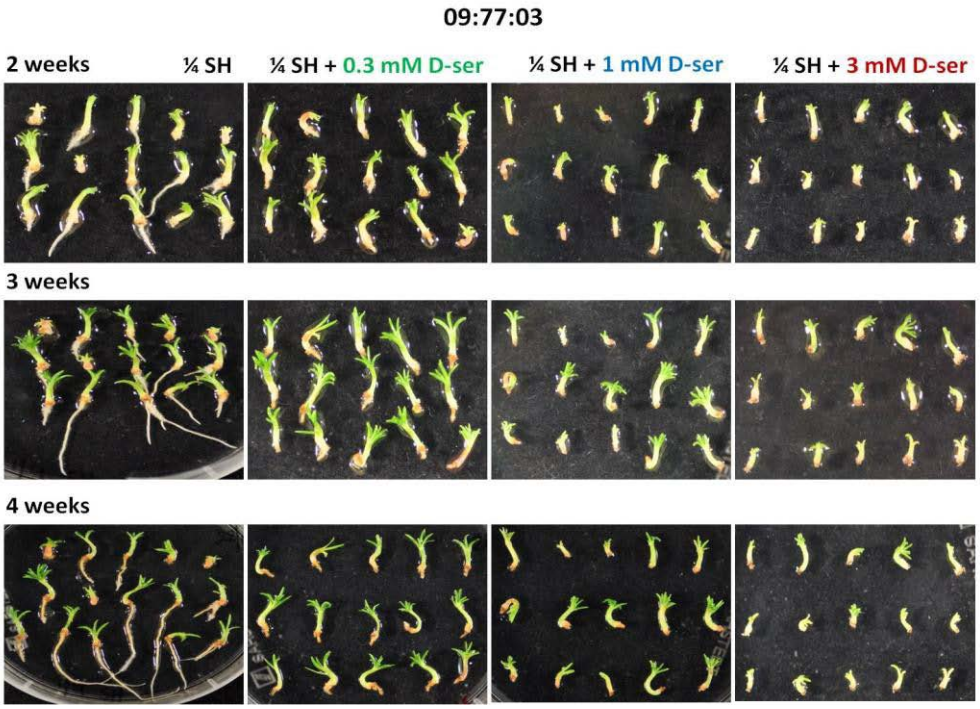


Figure 9. Embryos from cell line 09:77:03 after 2, 3 and 4 weeks of the four different treatments.

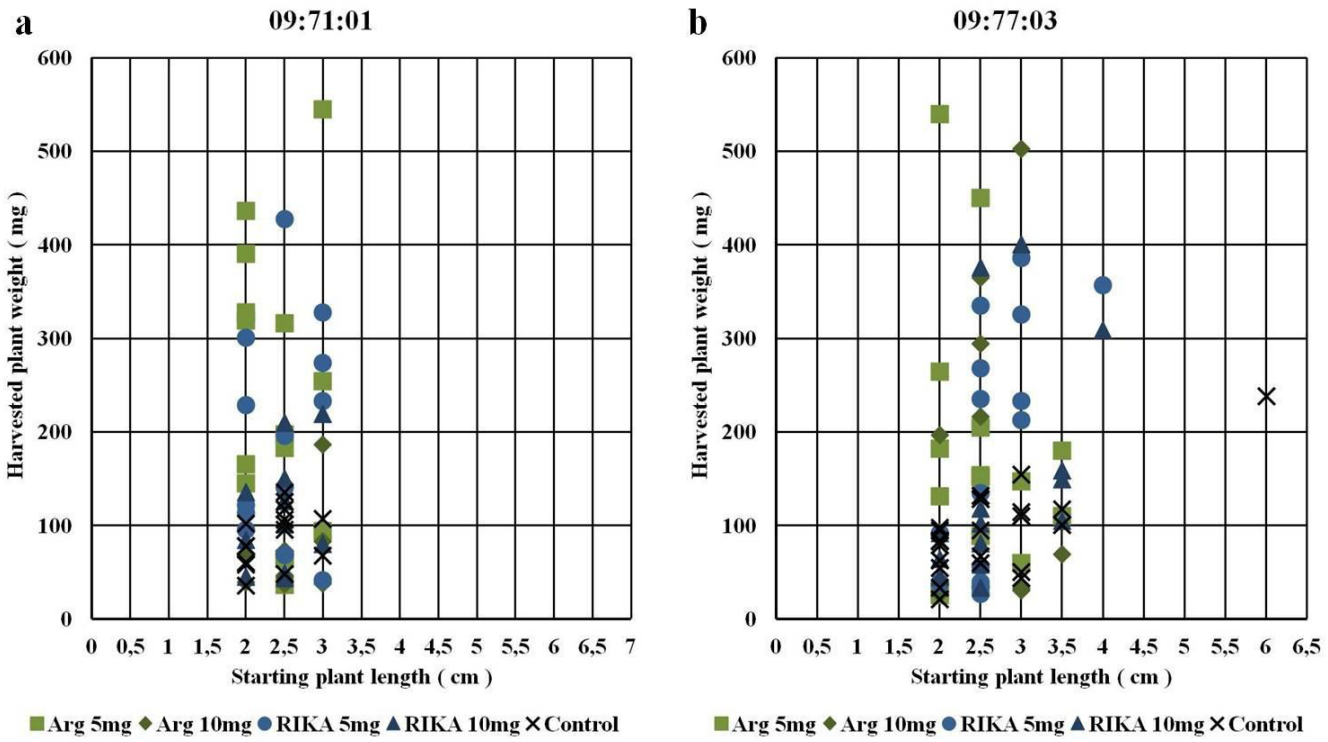


Figure10. Correlation between initial plant length at the start of the experiment and harvested plant weight after seven weeks of treatment. The plants were cultured in substrates supplemented with 5 mg arginine, 10 mg arginine, 5 mg RIKA, 10 mg RIKA or no nitrogen (control). All plants that survived during the treatment period were analysed. a) In cell line 09:71:01, the following number of plants were analysed in each of the above treatments respectively; 15, 11, 14, 11 and 15; b) in cell line 09:77:03 the following number of plants were analysed in each of the above treatments respectively; 13, 10, 17, 14 and 22.

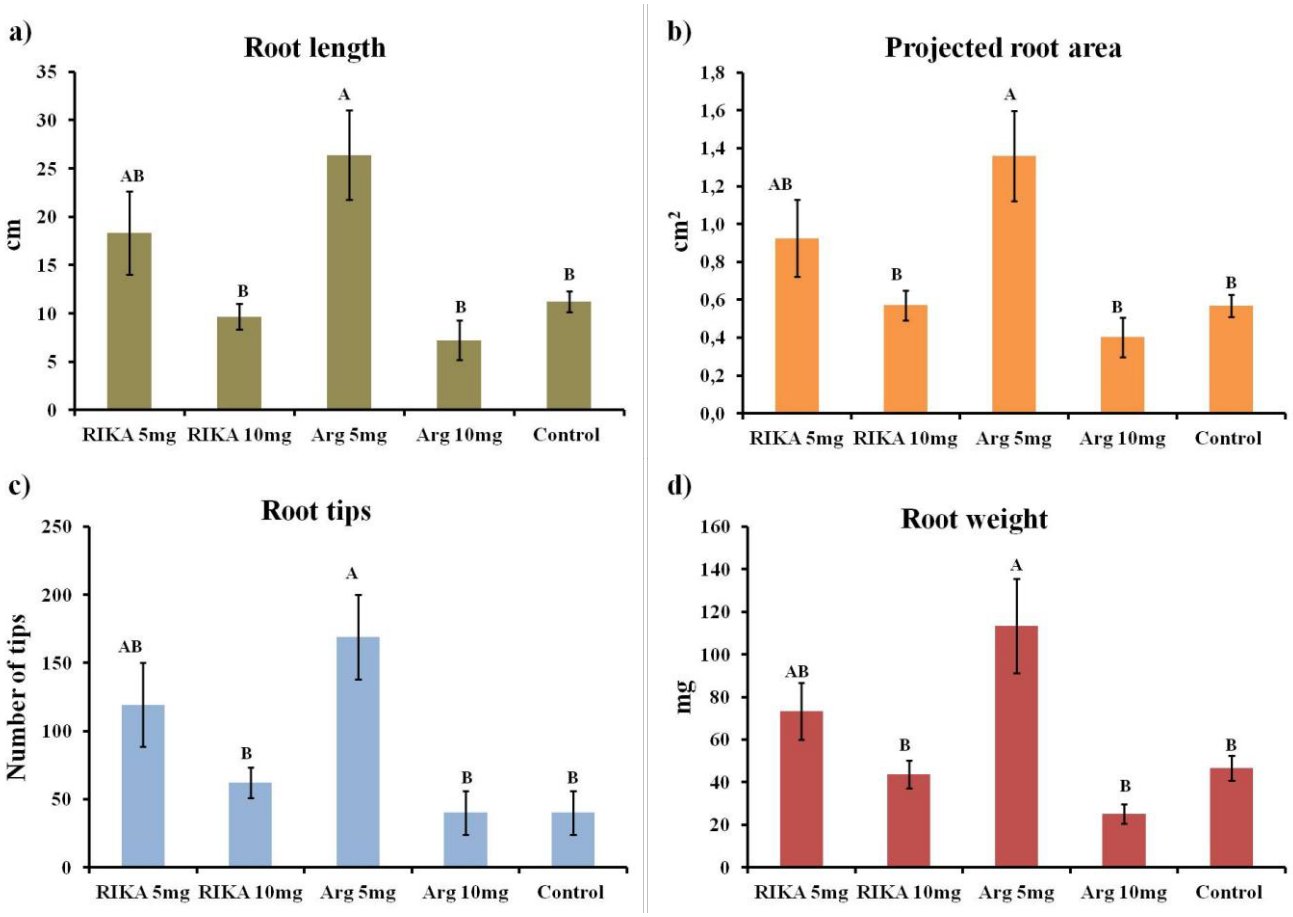


Figure 11. Results from the *WinRHIZO Pro* image analysis system from one-way analysis of root development in cell line 09:71:01. a) Total root length (cm), b) projected root area (cm^2) and c) number of root tips and d) root weight (mg). Statistical analyses of the results from all treatments were performed with the Tukey-Kramer HSD test. Bars with different letters are significantly different ($P \leq 0.05$).

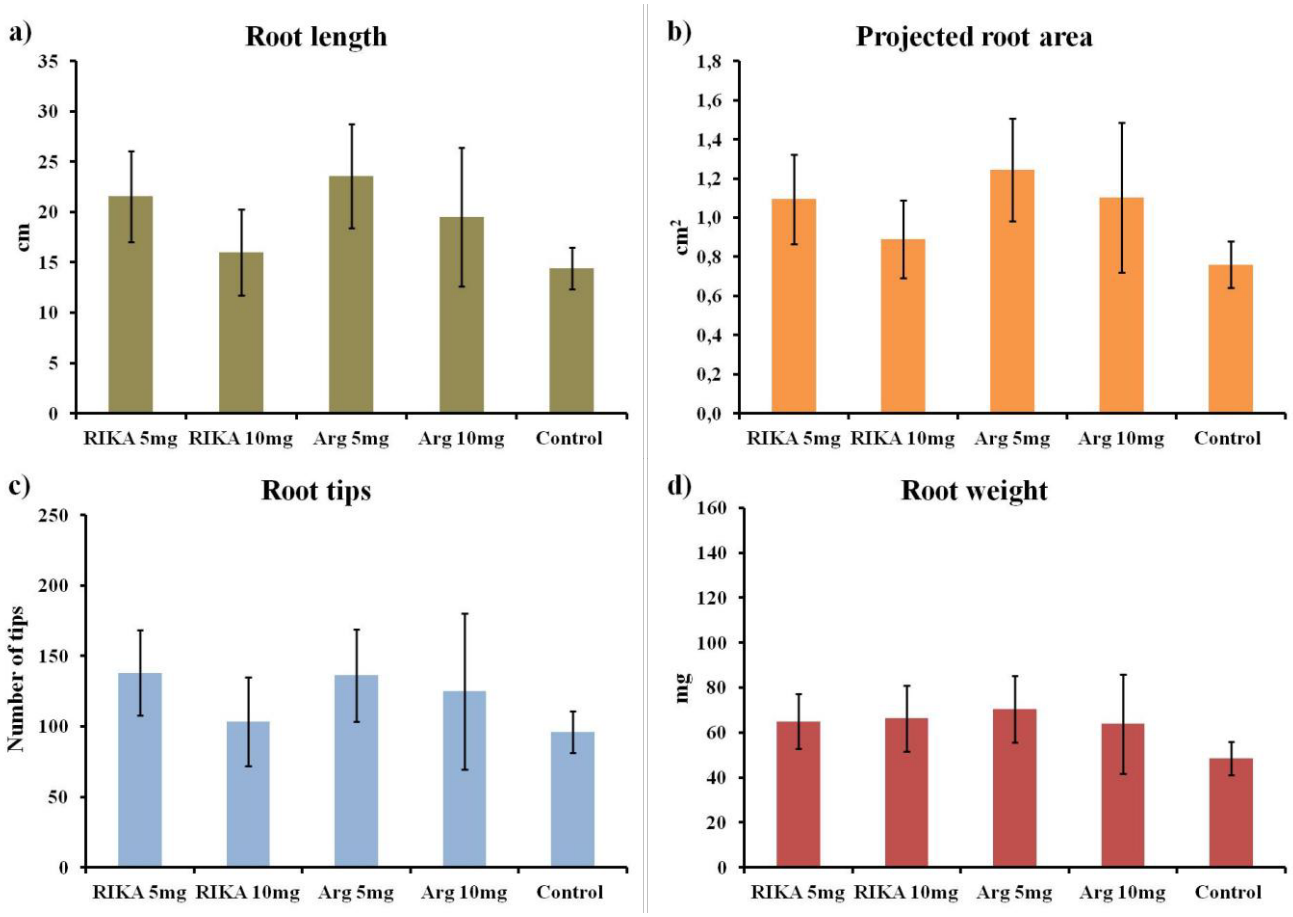


Figure 12. Results from the *WinRHIZO Pro* image analysis system from one-way analysis of root development in cell line 09:77:03. a) Total root length (cm), b) projected root area (cm^2) and c) number of root tips and d) root weight (mg). Statistical analyses of the results from all treatments were performed with the Tukey-Kramer HSD test. Bars with different letters are significantly different ($P \leq 0.05$).

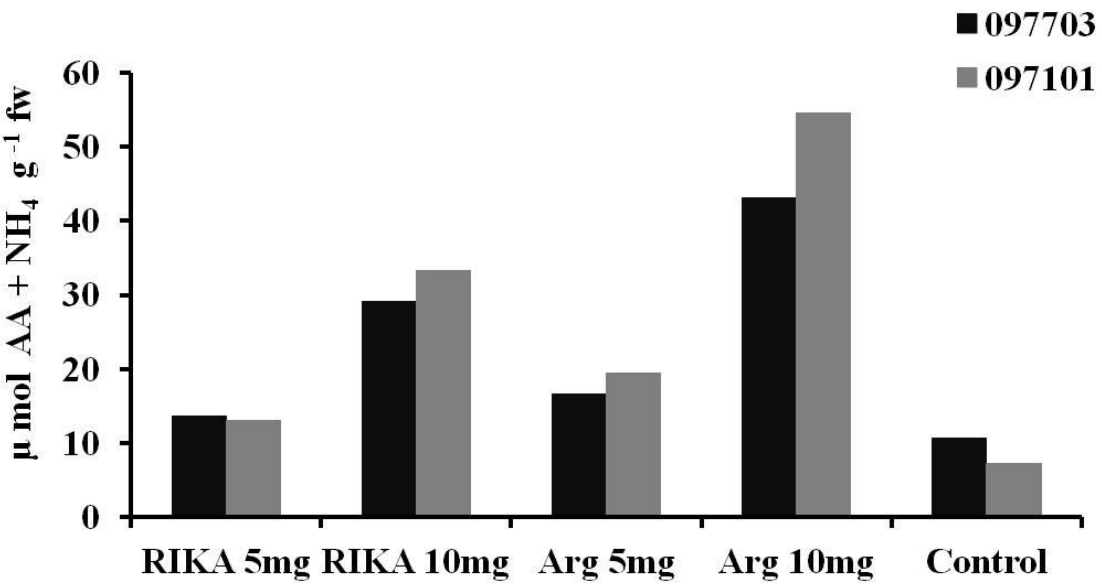


Figure 13. Total amount of amino acids and ammonium (µmol/ g fresh weight root) after roots cultured for seven weeks with different nitrogen sources in the greenhouse.

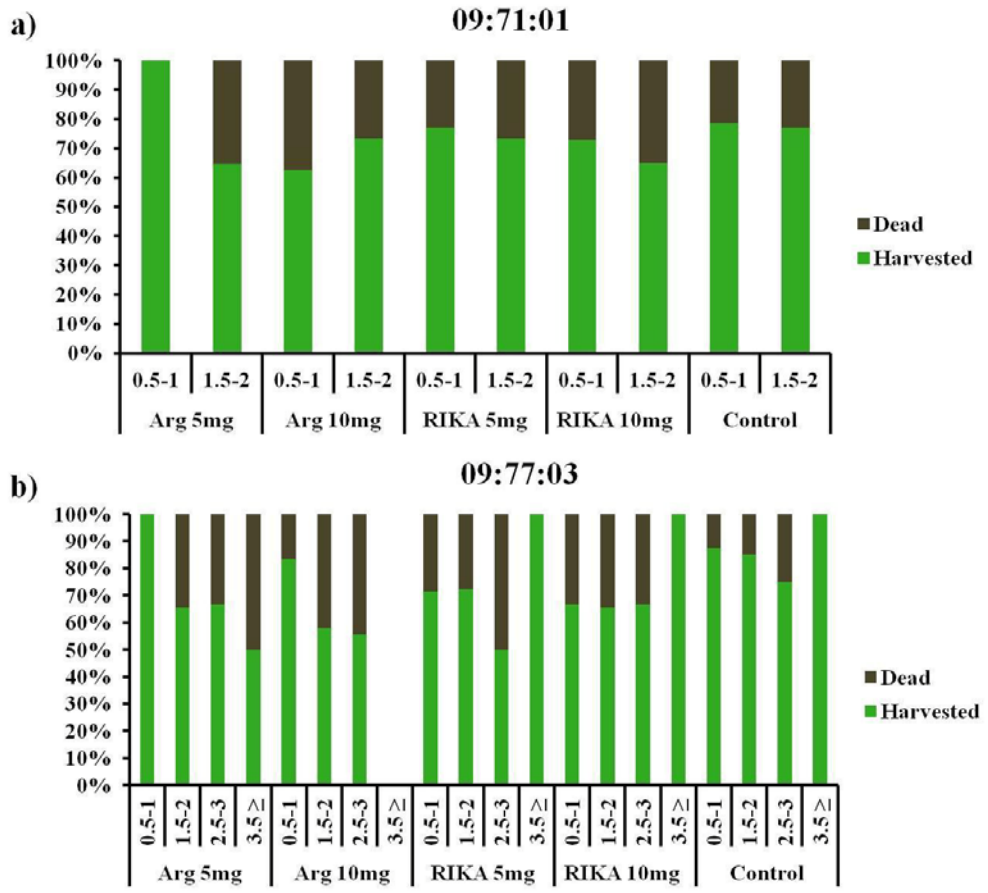


Figure 14. Percentage of dead plants at the time of plant harvesting in each starting plant root class and treatment. a) Plants with starting root length classes 01 0.5-1, 1.5-2 cm in cell line 09:71. b) Plants with starting root length classes 0.5-1, 1.5-2, 2.5-3, 3.5 ≥ cm in cell line 09:77:03.

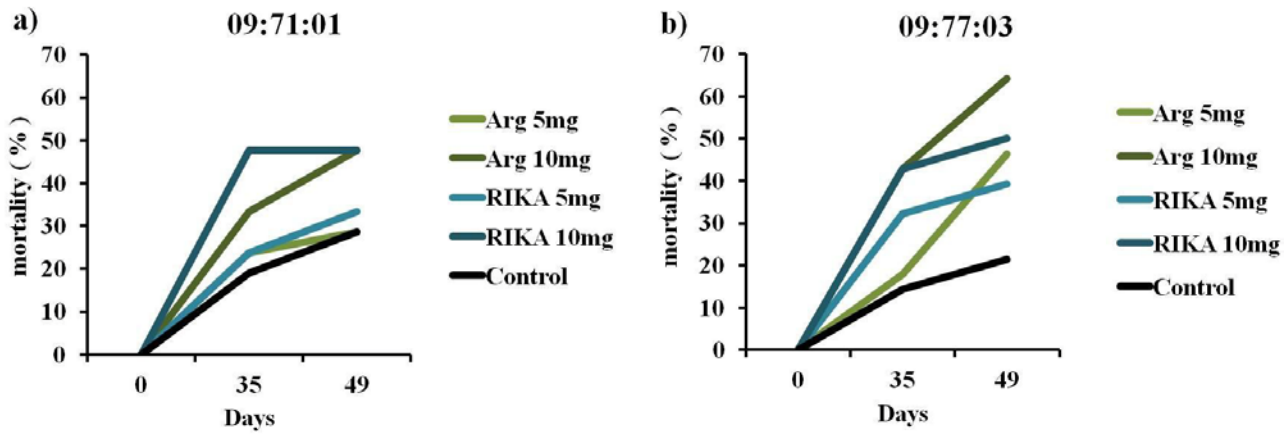


Figure 15. Rate of plant mortality during the seven week greenhouse phase of the experiments. a) 09:71:01, b) 09:77:03.

9. Appendix

Appendix 1. Preparation protocol of 1 liter DKM root media without ammonium nitrate for mature somatic embryos.

DKM root	**1 l**		
MQ H_2O	200 ml		
Macro elements		**Stocks:**	**Nitrogen**
A.) KNO_3	24.6 ml	95 g/l	23.11 mM
(OR KCl	1.72 g)		
C.) $MgSo_4 .7H_2O$	10.2 ml	37 g /l	
D.) KH_2PO_4	2.5 ml	34 g/l	
E.) CaCl . 2 H_2O	5.0 ml	44 g/l	
DKM Vitamines	0.5 ml	**Stocks:**	**Nitrogen**
Pyridoxine-HCl (B6)		0.5 mg/ml	1.22 µM
Nicotinic acid (B9)		0.5 mg/ml	2.03 µM
Thiamin (B3)		1 mg/ml	5.93 µM
DKM Micro	0.5 ml		
Fe-EDTA	5 ml		
		Nitrogen	
Casein hydrolysate	0.5 g	3.93 mM	
Myo-Inositol	0.1 g		
Sucrose	30 g		
MQ H_2O	**Up to 1l**		
Gelrite	3.5 g		
NOTE! After cooling add		**Stocks:**	**Nitrogen**
L-Glutamine	5 ml	45 mg/ml	3.07 mM
L-Arginine	60 µl	70 g N / l	0.3 mM
L-Arginine	200 µl	70 g N / l	1 mM

Appendix 2. Results from analysis of the content of 23 amino acids and ammonium in roots cultured for seven weeks with different nitrogen sources in the greenhouse.

Cell line 09:71:01	NH_4	His	Asn	Arg	Ser	Gln	Gly	Asp	Glu	Thr	Ala	GABA	Pro	Orn	Lys+Cys	Tyr	Met	Val	Nor Val	Ile	Leu	Phe	Trp
RIKA 5mg	2.433	0.057	0.063	4.910	0.267	0.919	0.361	0.486	0.711	0.067	0.285	0.320	0.027	0.111	0.171	0.029	0.003	0.044	1.601	0.031	0.026	0.034	0.022
RIKA 10mg	7.264	0.171	1.786	12.103	0.427	6.635	0.429	0.459	0.603	0.182	0.310	0.535	0.015	0.245	0.204	0.064	0.011	0.091	1.563	0.051	0.050	0.044	0.051
Arg 5mg	4.323	0.113	0.307	6.437	0.417	2.527	0.469	0.466	0.810	0.107	0.544	0.576	0.011	0.179	0.222	0.047	0.016	0.083	1.564	0.057	0.047	0.053	0.036
Arg 10mg	8.612	0.330	5.646	18.818	0.603	15.264	0.557	0.487	0.462	0.309	0.316	0.618	0.021	0.139	0.325	0.096	0.013	0.149	1.515	0.098	0.099	0.045	0.076
Control	0.567	0.031	0.028	3.292	0.130	0.289	0.202	0.140	0.367	0.036	0.150	0.095	0.009	0.090	0.111	0.020	0.010	0.022	1.539	0.029	0.013	0.026	0.029

Cell line 09:77:03	NH_4	His	Asn	Arg	Ser	Gln	Gly	Asp	Glu	Thr	Ala	GABA	Pro	Orn	Lys+Cys	Tyr	Met	Val	Nor Val	Ile	Leu	Phe	Trp
RIKA 5mg	1.192	0.051	0.137	6.153	0.287	1.108	0.322	0.602	0.939	0.066	0.300	0.339	0.078	0.071	0.184	0.032	0.011	0.045	1.564	0.033	0.025	0.082	0.029
RIKA 10mg	6.259	0.125	0.992	11.916	0.514	4.281	0.434	0.431	0.737	0.128	0.425	0.520	0.053	0.244	0.170	0.050	0.014	0.082	1.615	0.053	0.052	0.038	0.035
Arg 5mg	2.137	0.082	0.668	7.022	0.430	1.731	0.413	0.423	0.788	0.090	0.284	0.428	0.083	0.123	0.172	0.037	0.011	0.066	1.527	0.044	0.038	0.036	0.028
Arg 10mg	8.929	0.152	1.789	16.933	0.712	7.577	0.555	1.037	1.269	0.171	0.579	0.710	0.071	0.289	0.345	0.055	0.020	0.103	1.588	0.060	0.061	0.093	0.049
Control	0.482	0.030	0.066	6.628	0.149	0.382	0.189	0.150	0.335	0.039	0.128	0.114	0.019	0.089	0.136	0.019	0.002	0.023	1.551	0.019	0.015	0.014	0.032