Universidade Federal do Rio Grande do Sul

Centro de Biotecnologia

Programa de Pós-Graduação em Biologia Celular e Molecular

Rizogênese Adventícia em *Eucalyptus globulus* Labill e *Arabidopsis thaliana* (L.) Heynh

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Tese submetida ao Programa de Pós-Graduação em
Biologia Celular e Molecular da UFRGS
como requisito parcial para a obtenção
do grau de Doutor em Ciências

Porto Alegre

Outubro, 2007

Esta tese foi realizada no Laboratório de Fisiologia Vegetal – Centro de Biotecnologia e Departamento de Botânica - UFRGS, Brasil, e no Umeå Plant Science Center – UPSC, Umeå, Suécia.

A tese é composta de artigos que serão submetidos a periódicos científicos e de um manuscrito ainda em preparação, apresentados como introdução e capítulos um, dois e três.

AGRADECIMENTOS

Gostaria de iniciar agradecendo minha família... Meu porto seguro, que onde quer que eu esteja está sempre comigo... Agradeço todo amor e paciência... Muito do que foi feito aconteceu com a ajuda de vocês (seja lavando vidros no lab, buscando plantas no horto ou ouvindo minhas reclamações e idéias). Agradeço minha vó Suely pelo amor, alegria e histórias de vida... Muito obrigada por dividirem esse tempo comigo e por me incentivarem a sonhar sempre mais e mais... Amo vocês...

Ao Arthur G. Fett-Neto, meu orientador, agradeço todo o tempo de convivência - entenda-se como aprendizado... Muito obrigada principalmente pela liberdade de trabalho e escolhas... Esses quatro anos de doutorado não foram os mais fáceis (considerando-se que passei 9 anos no laboratório), mas apesar de algumas frustrações estes foram os anos mais positivos para o meu crescimento e rendeu bons frutos... Obrigada...

À grande família do Laboratório de Fisiologia Vegetal... Pelo trabalho em conjunto, pelas conversas e risadas, pelas festas...

À Carol e Márcia, que trabalharam comigo, muitas vezes exaustivamente, sem nunca reclamar... A ajuda, o companheirismo e a amizade de vocês foram fundamentais para que esse trabalho acontecesse...

Aos meus velhos amigos, por estarem sempre ao meu lado... Amo vocês...

Ao Daniel, que apareceu na minha vida em um momento que muita gente não acreditou que ia dar certo e que, no entanto fez com que tudo ficasse mais fácil e leve... Te amo...

À CAPES pela bolsa de doutorado e pela bolsa sanduíche para Suécia...

À Aracruz Celulose S.A. pelas sementes, plantas e espaço...

I would like to thank Catherine Bellini for the opportunity of doing one year of my PhD time at Umeå Plant Science Center under her supervision… It was a great time in the scientific and personal field… Thank you very much.

I very much appreciate all the assistance from Bellini's group: Laurent Gutierrez, Gaia Geiss, Mathieu Castelain, Rozenn Le Hir and Mariusz Kowalkzyc.

I am grateful to all the people at UPSC for the great working environment and help.

I would also like to acknowledge few friends that became my family in northern Sweden: Andreas Sjödin, Delphine Gendré, Mélanie Mariaut, Rozenn Le Hir and Yohann Boutté… Miss you and our Sunday Movie's nights…

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Environmental and Internal Factors Controlling Adventitious Rooting

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Manuscript to be submitted to The Open Forest Science Journal (Bentham Science Publishers)

Abstract

Adventitious root formation is a key step in vegetative propagation and attracts considerable economic interest because many plant species are difficult to root. In this review, the specific features of adventitious rooting and some of the recent advances integrating practical and fundamental approaches to optimize this important developmental process are explored, with emphasis on the control factors involved. Although little is known on the molecular basis of adventitious root formation as compared to lateral root development, both processes seem to share at least some components, particularly auxin signaling and action as an essential step. However, adventitious rooting is strongly influenced by associated stress-related responses such as water stress and wounding response. The phases of root induction, initiation and elongation have specific requirements that must be recognized in order to achieve adequate control of the rooting process. On the basis of physiological, biochemical and molecular investigations using different plant species, it appears that among the most significant factors controlling adventitious rooting response are auxins, mineral nutrients and light. Gene array expression and proteomic studies of adventitious rooting in unrelated species have pointed to the relatively sequential participation of water and wounding stress-related proteins, auxin transport proteins, cell wall turnover and synthesis proteins, cell fate-related transcription factors, cell replication machinery, growth and differentiation-related transcription factors. The combined basic and applied approaches to modulate internal and external factors affecting adventitious rooting may pave the shortest way to improve clonal propagation of economically relevant species.

Key words: adventitious rooting, regulation, plant clonal propagation, gene expression

Introduction

In horticulture, agriculture and forestry, vegetative or clonal propagation is widely used to multiply elite plants obtained in breeding programs or selected from natural populations (Hartmann et al. 1990), producing genetically homogeneous plants in large quantities. Adventitious root formation is a key step in vegetative propagation (De Klerk et al. 1999) and attracts considerable economic interest because many plant species are difficult to root (Davies et al.1993). The inability to form adventitious roots is often the limiting factor for cloning when using conventional cuttings or tissue culture (Haissig et al. 1992), representing losses in the production of new individuals. To improve cloning, large research efforts on physiological, biochemical and molecular studies of rooting have been carried out, aiming at understanding the control points of adventitious root formation. In this review, the specific features of adventitious rooting and some of the advances integrating practical and fundamental approaches to optimize this important developmental process will be explored, with emphasis on the control factors involved.

Origin of adventitious roots

Whereas the primary root is established during embryogenesis, lateral and adventitious roots are formed post embryonically. Typically, lateral roots form from the root pericycle and adventitious roots, from stem tissue. Adventitious roots are less predictable in their cellular site of origin than lateral roots. They can form from the cambium or, in the case of detached stem cuttings, from calli (Ludwig-Müller et al. 2005). The anatomical patterns of adventitious root formation can be divided in direct and indirect. Some species can present both patterns, depending on the physiological conditions or developmental stage. The direct pattern depends on the existence of

competent cells to form roots, which, once induced, initiate cell divisions with polarity, starting the formation of root primordia. These primordia are often associated with vascular tissue. The indirect formation of roots involves an initial non competent stage (in the beginning the cells are incapable to respond to induction signals); after induction, non determined cell divisions take place, usually leading to the development of calli. Competent cells are formed and, upon new induction (that may also be an extended initial induction), divide in polar fashion, establishing the new root primordia (Geneve 1991).

The subsequent steps of root development involve the establishment of a vascular linkage of the stem and the emerging root (Lovell and White 1986). In the direct pattern of root formation, the cells close to the vascular bundle are induced to divide and differentiate into vascular tissue. In the indirect pattern, callus vascularization normally occurs in a basipetal fashion, before primordia differentiation. The final linkage is established from the vascular tissue primordia to the vascular bundle of the callus. The root grows through the stem tissue by compression and/or enzymatic dissolution of cells walls. The emergence of the root can be a limiting factor for the rooting of some species. Potential blockages that can delay the emergence of roots include fibers, esclereids, resin and secretory channels (Lovell and White 1986).

Quantitative analyses of adventitious rooting

The analyses of adventitious rooting involve quantitative parameters that measure cutting population response, individual cutting response intensity and root system architecture. Rooting percentage provides information on the population response to rooting stimuli and is a good parameter to estimate genetic variability related to rooting. Since rooting is a binomial phenomenon, kinetic analyses of the rooting process have been

proposed and applied based on the concept of mean time of germination (for application example and calculation details see Fett-Neto et al. 2001). This measure provides, in one value, information on the kinetic profile of the rooting of cuttings exposed to a certain treatment. Briefly, the mean rooting time (MRT) expresses the number of rooted cuttings within a certain time in relation to the total number of rooted cuttings at the end of the experiment. It can be statistically evaluated based on the overlap of confidence intervals based on the distribution of t (Student test) and is very useful to investigate differences in the efficiency of root induction treatments that display similar final values of rooting percentage.

The intensity of the rooting response can be quantitatively evaluated as root density per cutting, *i.e.* the number of roots per rooted cutting. The number of cell clusters that responded to the rooting stimuli by differentiating a new root primordium at the cutting base is an important factor in the overall rooting architecture. Root extension and branching are other components of the root system architecture that may determine plant survival and fitness during acclimation to field conditions and even to later avoid precocious felling in planted forests. The analysis of root length can be done by measuring all roots, the longest root or the root system area, based on quadrant intercept, both manually and automated. Root branching corresponds to lateral root formation from adventitious roots derived from the stem base (sometimes incorrectly referred to as "primary" adventitious roots). Another method to quantify root branching is to count the number of branched roots per cutting and the number of ramifications per centimeter of root (both adventitious and lateral). These are important parameters that are often strongly related to regenerated plant survival and fitness.

Rooting phases

Physiological, histological and biochemical evidence accumulated over the years indicates that the adventitious rooting process consists of a series of successive but interdependent developmental phases with different requirements (Kevers et al. 1997, Bellamine et al. 1998, De Klerk et al. 1999). It is largely accepted the identification of three physiological phases of the adventitious rooting process: induction – time period covering the first molecular and biochemical events; initiation – when cellular divisions occur, originating the internal root meristems and the constitution of root primordia; formation – the phase covering the growth of the root primordia and their emergence out of the cuttings (Kevers et al. 1997). Often, for simplification purposes, the last two phases are called root formation. The success of adventitious rooting, in terms of rooting system quality and percent of rooting, depends on the optimization of each of these three successive rooting phases, and significant improvements in this process have been obtained using different culture media to promote each of these phases (Bellamine et al. 1998, Schwambach et al. 2005).

Phytohormones and related compounds

Soon after the discovery of indole-3-acetic acid (IAA), its rhizogenic activity was reported. In the same period of time, indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA) were chemically synthesized, and their capacity to induce roots was discovered (De Klerk et al. 1999). IBA is most commonly used to root cuttings from various plant species and in many instances is more efficient than IAA (Epstein and Ludwig-Müller 1993). It has been shown that IBA can be converted to IAA in cuttings, acting as a potential reservoir of IAA; however, endogenous IBA can have auxin activity

on its own (Ludwig-Müller 2007). Auxin stability varies with chemical structure; IAA is the most labile form, IBA has intermediate stability and NAA is the most stable type of auxin. Moreover, IAA and IBA can be conjugated with aminoacids or carbohydrates; conjugates are regarded as inactive forms, less sensitive to auxin oxidative enzymes and upon hydrolysis can release free active auxin (De Klerk et al. 1999). A possible role of endogenous IBA in adventititous rooting may be inferred from the fact that IBA synthetase, that catalyses biosynthesis of this auxin from IAA, has been shown to be induced by drought stress and abscisic acid (ABA) application (Ludwig-Müller 2007). Transient drought stress is a common feature in the preparation of cuttings for rooting. The differences observed between the various auxins may lie in the stability of the compound, the concentration of free auxin that reaches the target cells and cell sensitivity to this phytohormone (Assis et al. 2004). However, other differences such as uptake and transport can also account for the differences in rooting response (Epstein and Ludwig-Müller 1993).

In many species, application of exogenous auxin is required to achieve rooting (Diaz-Sala et al. 1996, Blaskova et al. 1997). However, longer supply of auxin can have inhibitory effects on further development of the root system (Kevers et al. 1997). The distinct phases have different or even opposite hormonal requirements (De Klerk et al. 1999). Studies with *Malus domestica* showed that auxin is necessary during the induction phase, becoming inhibitory in the formation phase (De Klerk et al.1999). Numerous initial attempts to establish a correlation between specific changes in endogenous levels of IAA with the adventitious rooting process were quite discrepant (Gaspar and Hofinger 1988, Kevers et al. 1997). The improvement in techniques of extraction, purification and quantification of auxin made possible a more acccurate description in the changes of

endogenous IAA during the rooting phases. From a number of investigations a general profile emerged: a transient increase in free endogenous IAA level during the induction phase is followed by a decrease until a minimum in the initiation phase and, later, a new increase during the root formation phase (Gaspar and Hofinger 1988, Kevers et al. 1997, Bellamine et al. 1998).

Auxin transport systems along the stems are essential part on adventitious root development process. Cells in vascular tissues, particularly parenchyma cells, have a number of transporter proteins involved in IAA basipetal transport. Such transporters are established early in embryogenesis and play a major role in the polarity of cells, tissues and organs (Berleth et al. 2007). Members of three major protein families have been considered auxin transporters: auxin resistant 1 (AUX1), pin-formed (PIN) and multidrug resistance/*p*-glycoprotein ATP-binding cassette transporters (MDR/PGP) (Bouteé et al. 2007). AUX1 has been associated with auxin influx, whereas PIN1, predominantly localized in the basal membrane of shoot cells, is involved with auxin efflux. PGP proteins are also part of the efflux system and possibly take part in lateral auxin transport. PIN members participate in various growth and developmental processes such as embryogenesis, meristem organization, organogenesis and tropisms. Depending on the cell type, PIN may be localized with different polarity and its position can be modulated by gravity and developmental cues. The retargeting of PIN to different polar domains of cells is partly related to its constitutive clathrin-mediated endocytosis and recycling (Bouteé et al. 2007). An antagonistic regulation of PIN phosphorylation in its middle hydrophilic loop by the phosphatase PP2A and the kinase PINOID (PID) was recently shown to change the polarity of PIN localization (Michniewicz et al. 2007). According to a model proposed by the authors, conditions in which PID kinase is high, would result in predominatly

phosphorylated PIN proteins, leading to their targeting to the apical side of cells, whereas when PP2 phosphatase activity is high, PIN would be mostly dephosphorylated, resulting in preferential basal targeting of the auxin efflux carrier.

The important role of auxin in controling adventitious rooting implies an involvement of the circadian clock in the regulation of this developmental process. A genome wide transcriptional profiling showed that many auxin-induced genes are under clock control. The use of a luciferase based assay showed that endogenous auxin signaling is clock regulated, in such a way that the clock gates plant sensitivity to auxin (Covington and Harmer 2007). This finding will certainly have important implications for auxin induced rooting in cuttings for commercial purposes.

There are several *Arabidopsis* mutants altered in auxin signal transduction, transport and conjugation. Among these, it has been possible to isolate the mutants *superroot1* (*sur1*) (Boerjan et al. 1995) and *superroot2* (*sur2*) (Delarue et al. 1998) that overproduce auxin and therefore spontaneously develop adventitious roots in hypocotyls (Delarue et al. 1998, Sorin et al. 2005). These mutants represent a tool to study in detail auxin biosynthesis and homeostasis in vivo, as well as to shed light in some aspects of the adventitious rooting process. One major drawback of the use of *Arabidopsis* as a model in adventitious rooting analyses is the close interaction between auxin and glucosinolate metabolism, a class of metabolites not widely distributed in plant species, particularly in woody ones.

Cytokinins are involved in the control of the cell cycle, particularly the transition from G1 to S and G2 to mitosis (Werner et al. 2001), which is necessary to recruit new cells to form roots; hence, this group of regulators has a positive effect during root initiation, despite being strongly antagonic to auxins (De Klerk et al. 1999). In fact, kinetin

(KIN) inhibited rhizogenesis when applied during induction phase in *Eucalyptus globulus* (Corrêa et al. 2005). However, the weakly active diphenylurea cytokinin derivatives positively influenced rooting in apple and mung-bean – *Vigna radiata*, mainly due to their weak stability within the tissues, being rapidly degraded and not presenting strong antagonist effects to auxin (Ricci et al. 2003, Ricci et al. 2005). Trans-zeatin riboside present in root xylem sap negatively regulates adventitious root formation in cucumber hypocotyl (Kuroha et al. 2002); when the plants had their main root system removed a rapid decrease in the trans-zeatin riboside level occurred along with an accumulation of auxin and synthesis of ethylene in the basal part of the cut hypocotyl, resulting in the formation of adventitious roots (Kuroha et al. 2002). Transgenic tobacco plants over-expressing a zeatin *O*-glucosyltransferase gene from *Phaseolus lunatus*, showed increased adventitious root in their lower stems, suggesting that the reduction of active cytokinin content by this enzyme leads to a lower cytokinin/auxin ratio (Martin et al. 2001). It seems that cytokinins play a dual role in adventitious rooting, being promoters in some stages and inhibitors in others.

Some results point to the fact that ethylene plays a species-specific role in rhizogenesis. In apple, ACC (aminocyclopropane carboxylic acid), an ethylene precursor, promoted rooting in leaf disks, but was inhibitory in agar-grown cuttings, presumably due to toxic amounts of ethylene accumulated around the basal stem (De Klerk et al. 1999). On the other hand, Arigita et al. (2003) found that ACC promoted rhizogenesis in minicuttings of kiwi (*Actinidia deliciosa*). It has been proposed that ethylene could contribute to rooting emergence by regulating the production of cell wall lytic enzymes. Ethylene seems to play a minor role in the rooting of *Eucalyptus globulus* and *E. saligna* because co-exposure to $AgNO_3$ (an ethylene action inhibitor) and auxin did not affect adventitious root formation

(Fogaça and Fett-Neto 2005). In contrast, adventitious rhizogenesis was strongly promoted in apple microcuttings treated with silver thiosulfate in the auxin exposure phase (DeKlerk et al. 1999). Recently it was found that ethylene modulates cell division at the quiescent center during post embryonic root development in *Arabidopsis* (Ortega-Martínez et al. 2007); it would be interesting to check this role during adventitious root formation.

Recently, a model was proposed to explain the development of lateral roots involving auxins, cytokinins and ethylene (Aloni et al. 2006). It is based on the occurrence of two parallel streams of polar IAA transport, one within a differentiating protoxylem vessel and another in the pericycle. The major pathway of auxin transport is in the pericycle, and its main effect would be the maintenance of the meristematic identity of this cell layer. The auxin stream in the differentiating vessel would induce its differentiation. During the process of differentiation of a protoxylem vessel element, a local increase in ethylene would occur, and the gas released in centrifugal direction could block auxin transport in the adjacent pericycle cells, causing a local increase in IAA concentration immediately above the blockage. This increase would induce root cell division and lateral root initiation. Cytokinin from the root cap moving acropetally towards the shoot may inhibit lateral root development near the root tip. It is possible that at least some of the components proposed in the assembly of this model of lateral root formation are also involved in adventitious root development. The basal accumulation of auxin basipetally transported in shoots, the removal of a main cytokinin source by elimination of the embryonic root system and the production of ethylene induced by tissue severance and/or local auxin accumulation could be responsible for the re-programming of parenchyma cells from which new root poles could differentiate.

In the first studies with gibberellins comparing its morphogenic effects with those of auxin, it became evident that these were often opposite. Exogenous gibberellin inhibits adventitious root formation in many species; however, in some species and under certain environmental conditions, it can enhance root formation (see Hansen 1988 for a review). More recently, it was reported that gibberellin has a negative effect on rooting of microcuttings (Eriksson et al. 2000) or embryos (Carvalho et al. 1998) of woody species favoring the growth of shoots. Gibberellins also inhibited adventitious root formation when applied to wild-type poplar (Busov et al. 2006).

Brassinosteroids were effective in promoting rhizogenesis of cuttings of *Picea abies* (Rao et al. 2002, Ronsch et al. 1993). Brassinosteroids may have a role during the root formation phase, since it has been shown that exogenous application of this phytohormones promoted root elongation in both wild type plants and, more pronouncedly, in brassinosteroid-deficient mutants of *Arabidopsis* (Mussig et al. 2003). The lack of effects of auxin transport inhibitors and gibberellins in brassinosteroids mutants, as well as the absence of changes in ethylene sensitivity or in the expression of auxin and ethylene related genes in brassinosteroid-treated mutants, suggest an independent role for brassinosteroids in the stimulation of root growth. However, general roles for brassinosteroids in adventitious rooting have not been defined.

Abscisic acid has a role in the inhibition of lateral root development, possibly by blocking the development of auxin-induced root primordia. ABA-deficient mutants have larger root systems and ABA application inhibits lateral root development (Malamy 2005, for review see De Smet et al. 2006). The role of ABA in adventitious root development is not clearly established.

Polyamines have been considered biochemical markers of adventitious root development because their content reaches a peak during the final portion of the root induction phase. A series of studies in poplar showed a relationship between rooting and polyamines. Among the evidence provided by these studies are: a) a transient peak in putrescine at the end of the root induction phase was not observed in non-rooting cuttings; b) the putrescine peak was restricted to the basal portion of the cutting where rooting takes place; c) inhibitors of putrescine biosynthesis, such as arginine and ornitine analogs, applied to cuttings before or at the beginning of the root induction phase were capable of inhibiting the rooting response; d) application of putrescine to cuttings at these same times, promoted rooting; e) cyclohexylamine, an inhibitor of putrescine degradation to spermidine, favored rooting in the absence of auxin supply (Kevers et al. 1997). Studies in pear cultivars with contrasting rooting capacity showed that putrescine metabolism in later stages of the rooting process was lacking in difficult-to-root cuttings (Baraldi et al. 1995). Similar results have been reported for the rooting of cuttings of *Berberis buxifolia* (Arena et al. 2003). Rooting in cuttings of *Fraxinus angustifolia* in the absence of exogenous auxin was also correlated with changes in polyamine metabolism, particularly putrescine, as inferred from studies employing inhibitors of putrescine biosynthesis and catabolism. In this species, the root induction phase showed a transient increase in endogenous free IAA and putrescine concentrations, whereas the root expression phase was characterized by increased peroxidase activity and low concentrations of polyamines (Tonon et al. 2001).

Working with cucumber plantlets, Pagnussat et al. (2002, 2003 and 2004) discovered an important role of nitric oxide (NO) in the adventitious rooting process. The NO donors, sodium-nitroprussiate (SNP) and S-nitroso, N-acetyl penicillamine (SNAP) applied to hypocotyl cuttings of cucumber (*Cucumis sativus*) yielded *de novo* root

organogenesis similar to that observed for the application of the auxin. The authors suggested that, although a direct effect of NO could not be discarded, their findings indicated that NO could mediate auxin response during the adventitious rooting process in cucumber. Other pathway components affected by NO were also investigated and a correlation with cGMP (cyclic guanosine monophosphate) and MAP kinases and adventitious rooting was found. Moreover, carbon monoxide was able to promote rooting in mung bean (*Phaseolus radiatus*), probably via the NO pathway (Xu et al. 2006).

Peroxidases, phenol compounds and flavonoids

Peroxidase (E.C. 1.11.1.7) activity has been proposed as a biochemical marker for the successive phases of adventitious rooting in cuttings for many plants (Fett-Neto et al. 1992, Hand 1993, Caboni et al. 1997, Rout et al. 2000, Saxena et al. 2000, Metaxas et al. 2004, Syros et al. 2004, Hatzilazarou et al. 2006, Husen and Pal 2007). Changes in peroxidase activity have often been correlated to an opposite profile of changes in endogenous auxin concentration. During rooting, peroxidase activity is typically lowest in the induction and highest in the initiation phase (Gaspar et al. 1992, Kevers et al. 1997). It has been suggested that some peroxidase isoforms, particularly basic ones, are involved in the regulation of auxin catabolism and in the differentiation of root primordia (Hand 1994). However, the endogenous role of peroxidases *in vivo* is still not completely established (Kevers et al. 1997). A non decarboxylative degradation pathway for IAA has been described and proposed as more relevant than the peroxidase-mediated decarboxylative route in intact plants (Normanly et al. 1995). The role of peroxidase in the catabolism of auxin *in vivo* has been questioned due the lack of significant concentration of the products of IAA oxidation in plant tissue, as well as due to the lack of significant changes in the

content of IAA in transgenic plants of tobacco overexpressing peroxidases (Normanly et al. 1995). Nonetheless, this last observation could be the result of modified expression of peroxidase isoforms with low capacity of auxin oxidation, since these enzymes are encoded by a gene family (Duroux and Wellinder 2003). Peroxidases have been used as biochemical markers of the successive rooting phases of various species in which rooting occurred after the cuttings had experienced a lower level of activity followed by a peak of peroxidase activity when crude extracts were used (Fett-Neto et al. 1992, Gaspar et al. 1992, Hand 1993, Caboni et al. 1997, Rout et al. 2000, Saxena et al. 2000). In spite of the possible limited *in vivo* role for peroxidases in controling auxin catabolism in intact plants, at the severed base of cuttings, in which the enzyme cofactors such as hydrogen peroxide, Mn^{+2} and monophenols, as well as the substrate IAA, become co-localized, it is conceivable that these enzymes may play a role in auxin homeostasis and adventitious root development (Assis et al. 2004).

Phenolic compounds are also known to be involved in rooting, perhaps by acting as antioxidants at the cutting base (De Klerk et al. 1999). In several cases of adventitious rooting, the content of phenolic compounds undergoes a time-course variation which parallels that of free IAA, thus being the reverse of peroxidase activity (Kevers et al. 1997 and references therein). Changes in phenolic compounds might influence the control of peroxidase-IAA-oxidase activity (promotion by monophenols and inhibition by diphenols and polyphenols) and, as a consequence, IAA content (Hand 1994); however, it is not excluded that IAA itself might control the metabolism of phenolics by feedback (Kevers et al. 1997). Flavonoids are a class of phenolic compounds that are potential biochemical markers of rooting (Hand 1994) and are reported to influence auxin transport. A high concentration of flavonoids of the quercetin glycoside type has been related to easy-to-root

phenotype in *Eucalyptus gunnii* (Curir et al. 1990). A study with flavonoid mutants of *Arabidopsis* defective in genes for chalcone synthase and application of exogenous flavonoids (naringenin) to wild type plants showed that these compounds can act as natural inhibitors of basipetal auxin transport (Brown et al. 2001). The rooting of cuttings of *Ilex paraguariensis* was significantly promoted by quercetin application and all flavonoids evaluated (quercetin, naringenin and rutin) improved roots distribution around the stem without impacting the number of roots per rooted cutting derived from 20-year-old plants (Tarragó et al. 2004). Whether the effects of flavonoids on adventitous rooting in these examples are related to auxin transport and/or antioxidant role with auxin protection activity remains to be determined.

Carbohydrates

Sucrose has been used as the C source of choice in plant tissue culture to compensate for low photosynthetic rates due to the small irradiance of growth chambers, being generally used at 3% (w/v) (Gollagunta et al. 2004). Calamar and De Klerk (2002) tested a wide range of sucrose concentrations and found that rooting percentage and root density of apple leaf disks were not affected. On the other side, Corrêa et al. (2005) found that a higher concentration of sucrose in the formation phase allowed better root elongation in *Eucalyptus* microcuttings. In *Hosta tokudama* explants, micropropagated cultures with 5% to 7% sucrose developed greater root dry weight in the rooting phase, when compared to treatments with 1% or 3% sucrose; possibly cellulose and lignin deposition could account for this difference (Gollagunta et al. 2004). These authors also found that the content of endogenous soluble carbohydrates of explants is directly proportional to sucrose concentration in propagation media. These results point to the fact that internal sugar levels

are important for root formation, increasing elongation and/or structural carbohydrates production.

Carbohydrates have important regulatory roles in plants, such as repression of photosynthetic genes (Sheen 1990), interaction with abscisic acid and ethylene signaling (León and Sheen 2003) and differential response in somatic embriogenesis (Pesce and Rugini 2004). This regulation can be extended to rhizogenesis; the hexose/sucrose ratio influences cell division (Borisjiuk et al. 1998), and different types of sugar (or combinations of sugars) have distinct effects on organogenesis, promoting more or less the formation of roots, depending on the studied species (Moncousin et al. 1992, Pawlicki and Welander 1995). Another feature to be observed is the phase dependent effect of carbohydrates; in *Eucalyptus globulus*, glucose had a stronger promotive effect in root induction, whereas sucrose promoted better rooting response when used in formation step (Corrêa et al. 2005).

Mineral nutrition

Nutrition is a key factor determining root morphogenesis (Assis 2001), such as lateral root formation and control of root length and density. Root system architecture in intact plants can be strongly influenced by nutrients such as nitrogen and phosphorus, and this has been recently reviewed (Osmont et al. 2007). Although adventitious rooting and mineral nutrition are intimately related, few studies have attempted to characterize the effects of specific minerals in each phase of the rooting process.

Calcium is involved in cell division, root primordia elongation and auxin transport (Blazich 1988, Bellamine et al. 1998). Bellamine et al. (1998) found that Ca plays a key role in the root formation phase in poplar; perhaps because of its role in the binding of

pectic chains in the cell wall, providing higher stability after cell wall loosening is induced by auxin for cellular elongation. However, Schwambach et al. (2005) observed that a higher Ca concentration in the induction phase resulted in greater root number in *E. globulus* microcuttings; this may reflect differences in Ca storage capacity or Ca requirements for adventitious rooting between tree species.

Root architecture can be potentially modulated by patches of high nitrate, which tend to cause branching and elongation of lateral roots in many plants, although the majority of examined species are herbaceous (Assis et al. 2004). Nitrate has been indentified as a signaling molecule controlling the root branching independently of its effect as nutrient in nitrogen metabolism (Leyser and Fitter 1998, Zhang et al. 1999). Moreover, a potential overlap of nitrate and auxin signal transduction pathways in the control of root architecture has been indicated by the observation that *axr4*, an auxin resistant mutant of *Arabidopsis* with defective root gravitropism and lateral root initiation, also lacks the nitrate induced branching response (Forde and Zhang 1998). In *E. globulus* microcuttings, Schwambach et al. (2005) showed that, when nitrate was the sole N source, rooting percentage improved, and when nitrate was present as the N source in the root formation phase longer roots were produced. In the same species, Bennett et al. (2003) observed that the presence of NH_4^+ caused a decrease in pH and that when nitrate compounds other than ammonium nitrate were used as nitrogen source, the medium pH was more stable and this was associated with increased root production.

Iron deficiency during the root induction phase showed a trend toward higher root number and longer root length in cuttings of *E. globulus* and its deficiency during formation phase resulted in root browning (Schwambach et al. 2005). A reduction of iron content in the induction phase could cause a decrease in the activities of peroxidases

involved in auxin catabolism (Fang and Kao 2000) suggesting that the positive effect is related to enhanced auxin activity. On the other hand, the deficiency of iron during formation phase could have compromised cell wall formation and lignification (Schwambach et al. 2005). Manganese promotes the activity of enzymes that degrade IAA (Campa 1991) such as peroxidases with IAA oxidase activity, possibly playing a similar role to iron as decribed by Schwambach et al. (2005).

Zinc is required in the biosynthesis of tryptophan, an auxin precursor (Blazich 1988, Marchner 1995). Schwambach et al. (2005) suggested that a high zinc concentration in the induction phase influences auxin concentration, thereby favoring the rooting response; indeed this was noted for *E. globulus* cuttings, which displayed higher root number in this situation.

Boron deficiency inhibits cell division and expansion (Lukaszewski and Blevin 1996), as well as root growth in intact plants (Josten and Kutschera 1999). These effects could explain the results obtained by Schwambach et al. (2005) in cuttings of *E. globulus* laking boron in medium that showed poorer rooting than the treatments with 30 and 200 μM B. However, Trindade and Pais (1997) obtained a 10% increase in rooting of the species when boron was removed from the rooting medium. This discrepancy between studies could be due to difference in mineral nutrient status of the donor plant.

The appropriate manipulation of specific mineral nutrition balances considering the rooting phases in microcuttings of the difficult-to-root species *Eucalyptus globulus* yielded significant improvements in the rooting response (Figure 1 and 2). The combination of optimal nutritional balances for rooting optimization produced plants with a robust root system architecture, that proved to be more resilient to face acclimation to *ex vitro* conditions and survive induced water stress after adaptation to growth in soil

(Schwambach et al. 2005) (Figure 3). The significant improvements obtained by mineral nutrient manipulation are relevant for operational commercial production systems based on minicuttings and minihedges, in which phytohormones are generally not employed (Assis et al. 2004).

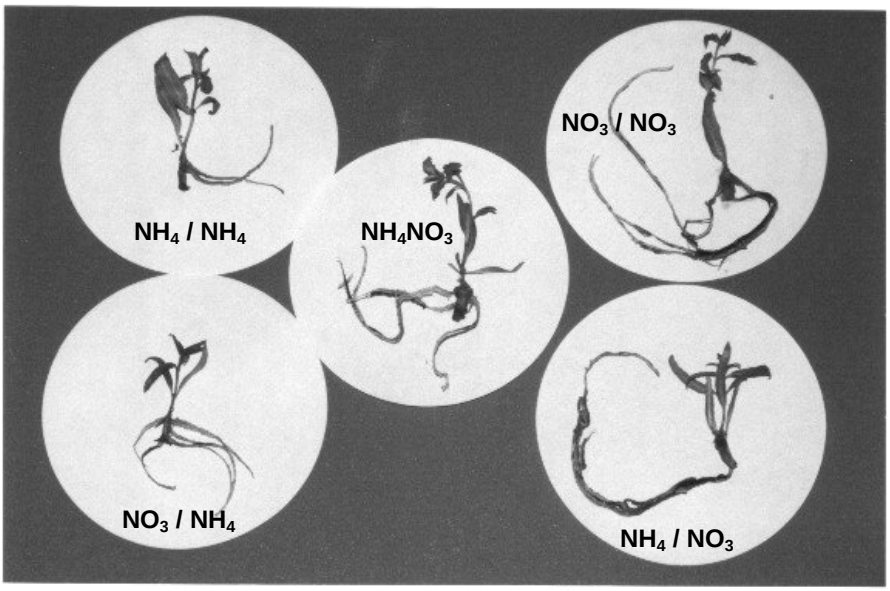

Figure 1: Adventitious rooting in microcuttings of *Eucalyptus globulus* grown in different nitrogen sources in induction and formation phases after 20 days in formation medium (N source in induction / N source in formation; control - NH_4NO_3 in both phases). White discs measure 9 cm of diameter.

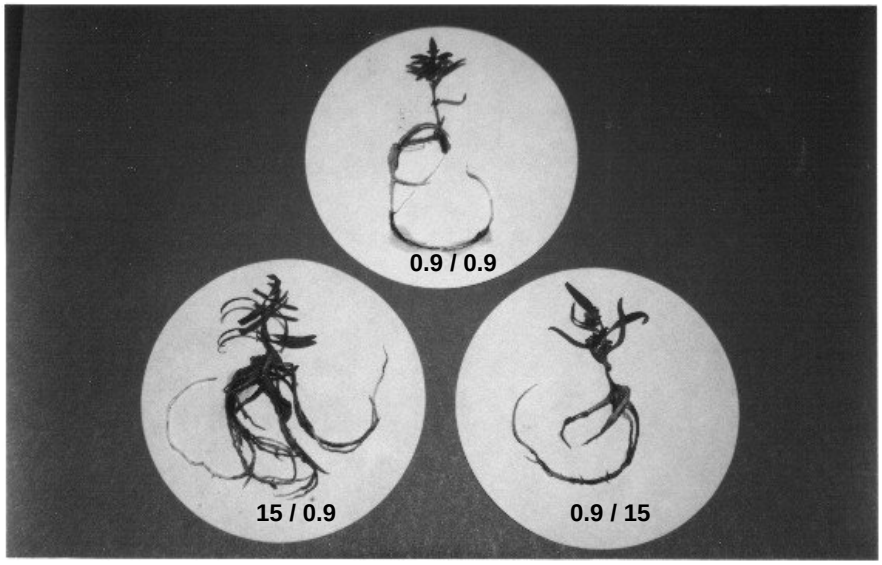

Figure 2: Adventitious rooting in microcuttings of *Eucalyptus globulus* grown in different calcium concentrations in induction and formation phases after 20 days in formation medium (presented as Ca concentration in induction (mM) / Ca concentration in formation phase (mM)); control 0.9/0.9. White discs measure 9 cm of diameter.

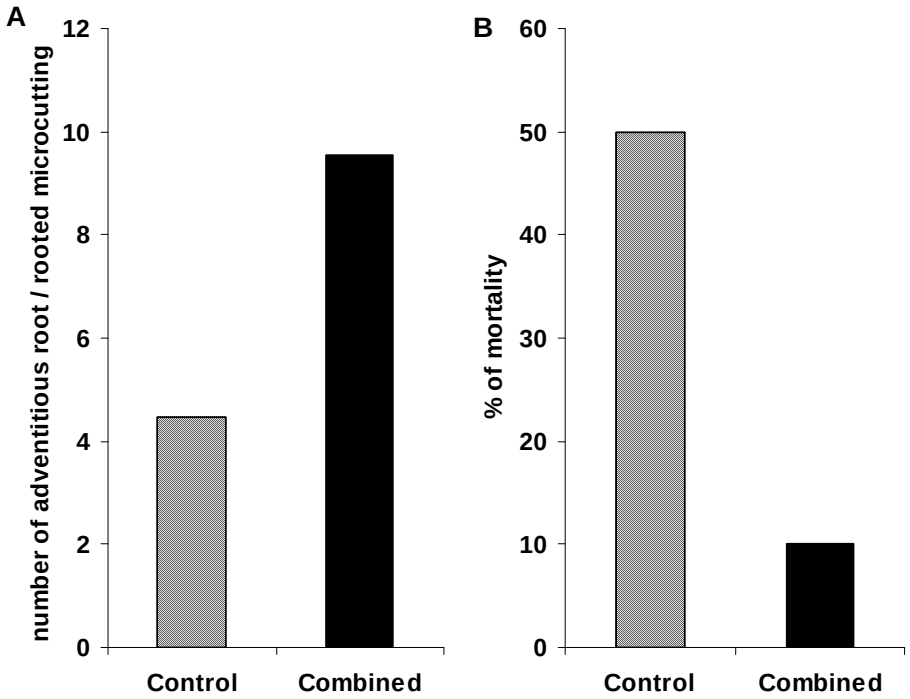

Figure 3: Effects of combined optimal mineral nutritional balances for phase-specific rooting medium compared to control in *Eucalyptus globulus*. A – Root density in microcuttings grown in control or optimized media after 20 days in formation medium. B - Mortality of cuttings rooted in control or combined media, acclimated for 2 months, and then exposed to water stress for 15 days. Redrawn from Schwambach et al. (2005).

Temperature

This environmental factor has influence in several physiological processes, such as water and nutrient uptake, enzyme activity (Sung et al. 2003) and phytohormone regulation (Vidal et al. 2003). Most protocols of adventitious rooting employ a constant controlled temperature in which explants are kept (usually between 20 and 30°C) during the experiments (Wilson 1998). Corrêa and Fett-Neto (2004) exposed donor plants and cuttings of *Eucalyptus saligna* and *Eucalyptus globulus* to different temperatures and the formation of adventitious rooting was affected in species-specific fashion. Moderate heat-shock in the donor plant of *E. saligna* (40°C) improved rooting efficiency, as already seen in *Gladiolus* (Kumar et al. 1999). *E. globulus* was more sensitive to heat stress, possibly

reflecting its geographic origin, and best rooting responses in this species were observed with alternating day/night temperatures (30/20°C) (Corrêa and Fett-Neto 2004). These responses are probably the result of a modulation of auxin metabolism, transport and/or uptake by temperature. Nine temperature-sensitive mutants in *Arabidopsis* have been identified and they were defective in various stages of adventitious root formation from hypocotyl explants (Konishi and Sugiyama 2003) emphasizing the connection between temperature and the different phases of adventitious root formation.

Light

Rhizogenesis can be affected by light availability and a beneficial effect in changing light-dark at precise moments can be obtained (Kevers et al. 1997). Maintenance of explants in the dark proved to have a positive effect on rhizogenesis of stems explants of *Rhododendron* and apple (Economou and Read 1987), whereas continuous illumination prevented or diminished root formation. Many explanations were proposed for rooting inhibition by continuous light involving changes in indole-3-acetic acid (IAA) and content of other rooting-related compounds *(e.g.* phenolics compounds), production of histological or anatomical barriers and changes in peroxidase activity (Economou and Read 1987). More recently, a positive effect of a dark root formation phase was described for *Eucalyptus globulus* microcuttings, which was no longer observed upon the application of exogenous auxin (Fett-Neto et al. 2001). In general, depletion of photosynthesis does not seem to be critical for rooting, as long as an adequate carbon source is available (Corrêa et al. 2005).

Generally, low irradiances seem to favor stem cutting rooting, what could be seen in assays with natural shading in mother-plants of *Rhododendron* (Johnson and Roberts

1971) and, more recently, for *Eucalyptus globulus* (Wilson 1998). Nevertheless, these results must be considered with caution before being adopted as a rule, because some non-woody species follow the inverse pattern - for more details, see Hansen (1987). There seems to be an optimum of light irradiance for each species; in *Persea americana* microcuttings 35 and 60 $\mu mol.m^{-2}.s^{-1}$ allowed highest rooting percentage than 85 $\mu mol.m^{-2}.s^{-1}$ (De La Viña et al. 2001). Fogaça and Fett-Neto (2005) submitted microcuttings of *Eucalyptus saligna* to 0, 30 and 100 $\mu mol/m^2.s$ with and without IBA supplementation; when not supplemented with IBA, the highest irradiance caused an inhibition in *E. saligna* rooting percentage; however this light condition allowed the development of longer roots. It has been suggested that this could be explained by photoinduced changes in endogenous content of regulatory substances, such as cytokinins and phenolics compounds, which may act as inhibitors of adventitious rooting, as well as by differences in carbon allocation (Assis et al. 2004).

Recently, Sorin et al. (2005) investigated a potential interaction between light and auxin in the regulation of adventitious root formation studying *Arabidopsis* mutants altered in their capacity to form adventitious root namely *superroot2* and *argonaute1*. They used an allelic series of *ago1* and the double mutant *ago1sur2* showing that the defect in adventitious root formation in *ago1* mutants is correlated with an alteration of auxin homeostasis in the apical part of the seedling and a hypersensitivity to light. The authors concluded that the *AGO1* gene could regulate the expression of genes acting at the cross talk of auxin and light signaling pathways.

Photoperiod seems to affect the rooting response of each species differently. Some species show long-day stimulation response (*Rhododendron*, *Vigna radiata*) and others

have the opposite behavior (*Ilex crenata*, *Pinus silvestris*). These distinct patterns can also be seen within a plant genus, such as *Salix* (Hansen 1987).

Supplementing white light with near ultraviolet or red light promoted rooting in explants of some tree species, and illuminating stock plants with far red – red light sequence (for 2 weeks both) gave better results in rooting explants than using the reverse sequence (Economou and Read 1987). Red light supplementation improved the rooting response in *Vitis* and *Prunus persica* (hybrid GF 655/2) (Chée and Pool 1989, Baraldi et al. 1998), whereas yellow and blue irradiance have inhibited adventitious root formation (Fuernkranz et al. 1990). However, a different *P. persica* hybrid (GF 677) showed reduced rooting under red light and improved rooting after short periods (2 to 4 days) of exposure to darkness, yellow or blue light followed by white light (Antonopolou et al. 2004). Rooting of *Eucalyptus grandis ex vitro* was promoted by stockplant exposure to lower red to far red light ratios; rooting was associated with low pre-severance starch and water soluble sugar concentrations and higher total water soluble carbohydrates per cutting (Hoad and Leakey 1996). Therefore, although results confirm morphological and physiological effects of light quality on rooting, the responses seem to vary considerably with species (Antonopolou et al. 2004).

Another feature to be considered is that different kinds of white lamps (in *in vitro* chambers) can influence rooting of explants due to differential quality of light, for example, incandescent light corresponding to enriched red wavelength (Magalhães Júnior and Peters 1991).

Light availability has been shown to affect auxin transport. Under daylight auxin transport in stems occurs mainly through the central cylinder, whereas in the shade the preferential pathway for auxin transport is the lateral route, through external cell layers,

resulting in reduced cell expansion in leaves and enhanced elongation in stem; these responses are presumably mediated by the phytochrome (Morelli and Ruberti 2002). These effects may influence the availability of auxin that flows basipetally from the shoot to drive root differentiation in cutting bases.

Infection by *Agrobacterium rhizogenes*

A. rhizogenes is a soil bacterium which causes the emission of profuse adventitious roots, causing the so-called "hairy root disease". Their well-known promotive effect on rhizogenesis is due to the integration and subsequent expression of the T-DNA from the Ri (Root inducing) plasmid in the plant genome. Four loci involved in the root formation were identified in the T-DNA, and called *rol* (root loci) A, B, C, D (Kevers et al. 1997, Damiano and Monticelli 1998). Several authors reported successful rooting using *Agrobacterium rhizogenes* mediated transformation in fruit trees, such as almond, apple, plum, walnut (Damiano and Monticelli 1998, Falasca et al. 2000), and in other woody plants such as the genus *Pinus* and *Eucalyptus* (MacRae and Van Staden 1993). Falasca et al. (2000) infected microcuttings of a recalcitrant walnut (*Juglans regia*) by *A. rhizogenes*. It enhanced the rhizogenic effect of exogenously applied auxin, however PCRs (polymerase chain reactions) using root material showed that about half of the new formed roots did not have the bacterial genes *rol*B or *vir*, the latter necessary for infection. Consequently, the authors argued that the stimulatory effect, besides a possible increase in auxin signal transduction or sensitivity, could be due to pathogenic stress itself, since compounds exudated by wounded cells, known as Wound Related Compounds (WRCs) can increase rhizogenic response (De Klerk et al. 1999). In difficult-to-root *Eucalyptus* species and *Sequoia sempervirens*, the infection with *A. rhizogenes* improved rooting (MacRae and Van Staden

1993, Mihaljevic et al. 1999). r*ol*B was also introduced in *Populus* and apple cuttings by infection with *A. tumefaciens*, inducing rhizogenesis (Zhu et al. 2001, Dai et al. 2004).

Transcriptome and Proteome Analyses

Gene expression during radical meristem formation in woody plants is still little understood. Studies related to physiological and biochemical processes (pos-translational) identified factors associated with rooting, although the regulatory aspects were not fully elucidated. It has long been suggested that this studies were strategically used to redirect adventitious rooting research to unveil details of genetic changes associated with the process (Haissig et al. 1992). There are few studies that evaluate expression profiles during adventitious rooting. More recently, genes involved in this process are being characterized in different plants with a variety of techniques, such as: mutants of *Nicotiana tabacum* and histochemical staining (Lund et al. 1997), Malus *domestica* and RDA – representational difference analysis (Butler and Gallagher 1999), *Populus* and ESTs - expressed sequence tags (Kohler et al. 2003), *Pinus contorta* and microarrays (Brinker et al. 2004), and mutants of *Arabidopsis* associated with auxin measurements (Boerjan et al. 1995, Delarue et al. 1998, Sorin et al. 2005). Moreover, a proteomic study was carried out with *Arabidopsis thaliana* rooting *superroot* mutants 1 and 2, auxin overproducers (Delarue et al. 1998), *argonaute*, a mutant less sensitive to auxin and with altered miRNA regulation, and double mutants (Sorin et al. 2005). Eleven proteins altered in the mutants and involved in adventitious rooting were identified (Sorin et al. 2006). They included auxin-related proteins and light-related proteins positively or negatively associated with adventitious root development, as well as other proteins related to stress, cytoskeleton function, protein degradation and stress responses.

Similar putative functional profiles were observed in genes differentially expressed monitored in a microarray study during rooting of *Pinus contorta* (Brinker et al. 2004). In this last study, a time course pattern was observed. During the first 3 days after root removal and auxin treatment, an increase in protein synthesis and a decrease in protein degradation is observed, plants experienced water stress, weakening of existing cell walls and assembly of fewer new cells, the photosynthetic genes are down regulated, active auxin transport is reduced. The cell replication machinery is activated; transcript abundance of PINHEAD/ZWILLE-like, a gene involved in the regulation of cell fate is increased, indicating the beginning of root induction. In the next 3 days, root primordia are formed. From day 6 to day 9, root meristems differentiate, coincident with transcript increase of a B-box zinc finger-like protein. Activation of auxin transport and auxin responsive transcription are observed, cell wall synthesis increases, wall weakening decreases and a barrier for defense is built. Water stress reduces progressively in parallel to adventitious root function activation. From day 9 to 12, the development of root meristems is simultaneous with high rate of auxin transport and decreased cell wall reorganization. From day 12, roots start to elongate and there are increases in transcription factor gene transcripts. The cell replication machinery is less active and so is the expression of stress-related genes (Brinker et al. 2004).

Interestingly, PINHEAD/ZWILLE is the closest gene to AGO1 in *Arabidopsis*, suggesting at least a partial functional conservation of these genes in distant plant taxa, such as *Arabidopsis* and *Pinus* (Sorin et al. 2006). In fact, if the underlying mechanisms of adventitious rooting development have the evolutionary degree of conservation observed in some of the other root-related developmental processes, highly conserved molecular mechanisms of rooting would be expected. It has recently been shown that the control of

root hair and rhizoid formation in *Arabidopsis* sporophytes and *Physcomitrella* gametophytes, respectively, share the involvement of orthologs of basic helix-loop-helix transcription factors (Menand et al. 2007).

Conclusion

Although little is known on the molecular basis of adventitious root formation as compared to lateral root development, both processes seem to share at least some components, particularly auxin signaling and action as an essential step. However, adventitious rooting is strongly influenced by associated stress-related responses such as water stress and wounding response. On the basis of physiological, biochemical and molecular investigations using different plant species, it appears that among the most significant factors controling adventitious rooting response are auxins, mineral nutrients and light. Investigations combining proteomic and transcriptomic analyses with mutational studies and commercial-like minihedges approaches, carried out with both *Arabidopsis* and woody species, will shed further light on controling the challenging developmental task of making a root from scratch.

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OBJETIVOS

Considerando a relevância da rizogênese adventícia para a propagação de espécies de interesse econômico, bem como as vantagens potenciais de combinar abordagens básicas e aplicadas com plantas modelo e de interesse tecnológico, o objetivo geral deste estudo foi examinar diferentes fatores envolvidos no controle do desenvolvimento de raízes adventícias em *Eucalyptus globulus* Labill e *Arabidopsis thaliana* (L.) Heynh.

Os objetivos específicos foram:

Analisar o processo de enraizamento adventício em mini-estacas de *E. globulus* x *maidennii* em sistema de fertirrigação em canaletão de areia e de alagamento intermitente, avaliando possíveis marcadores bioquímicos para distinguir as diferentes fases da rizogênese.

Avaliar o uso de diferentes fontes de nitrogênio no enraizamento adventício de *E. globulus in vitro* e o desempenho das plantas enraizadas na aclimatação *ex vitro*.

Caracterizar mutantes supressores para a mutação *sur2* em *Arabidopsis*, visando melhor compreensão da homeostase de auxina e enraizamento adventício neste sistema genético.

Adventitious rooting of *Eucalyptus globulus* x *maidennii* minicuttings derived from ministumps grown in sand bed and intermittent flooding commercial production systems: a comparative study

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Manuscript to be submitted to New Forests (Springer Verlag)

Abstract

Eucalyptus globulus Labill and hybrids thereof have low lignin content, favoring cellulose extraction, but are often recalcitrant to clonal propagation. This work analyzed biochemical and morphological changes during adventitious rooting of minicuttings of *E. globulus* x *maidenni* obtained from ministumps cultured in drip fertigated sand bed system or intermittent flooding system. Morphological (% rooting, root number and length, mean rooting time) and biochemical parameters (peroxidase activity, total phenolic content and flavonoid content) were monitored to characterize the rooting phases. All of the rooting parameters had similar results in both systems, indicating equivalent efficiency of both methods in clonal propagation. Kinetic profiles of biochemical parameters were also similar, although the activity of peroxidases was an order of magnitude higher and the phenolic content an order of magnitude lower in cuttings derived from intermittent flooding-grown ministumps than in those derived from sand bed-grown ministumps. Taken together, results suggest that rooting phases were similar in both systems: induction (in the ministump or before day 5), formation (from day 5 to 15) and elongation from day 15 to 45. These data may contribute to the development of rooting phase-specific mineral nutrient solutions to maximize clonal propagation and plant survival.

Keywords: peroxidase activity, phenolic compounds, biochemical markers, adventitious rooting phases

Introduction

Eucalyptus has became the most widely planted hardwood genus in the world, with approximately 17.8 millions ha of planted area (FAO 2000; Turnbull 1999). In the Americas, eucalypt statistics are dominated by Brazil, where there are estimated 3 millions ha of plantations, the majority of which for pulp production (FAO 2000; Turnbull 1999). Brazil is the largest world producer of eucalypt pulp due to clonal forests formed by elite material with high productivity, that can yield values around 45 -60 m^3/ ha/ year (Mora and Garcia 2000). In southern Brazil, *Eucalyptus globulus* and its hybrids are interesting genotypes for this industry due to their relative high frost resistance and low lignin content, which facilitates cellulose extraction. On the other hand, *E. globulus* is generally considered recalcitrant for rooting (Serrano et al. 1996; Le Roux and Van Staden 1991).

Mass vegetative propagation has become an important tool for increasing the competitiveness of the forestry based industry. A super-intensive system has been successfully employed for commercial propagation of clonal *Eucalyptus*; it consists of minicuttings kept in indoor mini hedges and is based on the rooting of axillary shoots from rooted stem-cuttings – ministumps (Assis et al. 2004). There are two main hydroponic systems used by Brazilian companies to keep ministumps and to produce minicuttings: drip fertigated sand beds (that assures an adequate nutritional status for the ministumps and minicuttings derived thereof) and intermittent flooding, where the containers with the ministumps are temporarily immersed in a nutritive solution for fertigation (see Assis et al. 2004 for a review). Adventitious root formation is a key step in vegetative propagation (De Klerk et al.1999) and it is a developmental process consisting of a series of successive and interdependent phases (induction, initiation, formation), each with its own requirements and characteristics (Kevers et al. 1997). Knowledge of biochemical and morphological

events associated to root induction and formation may allow improvement in rooting procedures so as to limit losses, particularly towards the final stages of production. Therefore, it would be interesting to identify reliable biochemical marker(s) for rooting in commercial *ex vitro* systems.

Various studies on adventitious root formation have shown a fundamental role played by peroxidase on rooting in cuttings (Metaxas et al. 2004; Syros et al. 2004; Hatzilazarou et al. 2006; Husen and Pal 2007). Furthermore, these enzymes have been proposed to be biochemical markers of the successive rooting phases of several species, where rooting consistently occurred after the cuttings had reached and passed a peak of activity (Rout et al. 2000; Saxena et al. 2000; Caboni et al. 1997; Hand 1993; Fett-Neto et al. 1992; Gaspar et al. 1992). Peroxidase activity has been linked to the oxidation of auxin. Many basic peroxidases have indole-3-acetic acid (IAA) oxidase activity and other peroxidases have been shown to be effective in IAA oxidation, at least in vitro (Hand 1993 and references therein). Moreover, phenolic compounds are also known to be involved in rooting (De Klerk et al. 1999). Changes in phenolic compounds might be responsible for the control of peroxidase-IAA-oxidase activity, therefore, affecting the IAA content. However, it is not excluded that IAA itself might, by feedback, also control phenolic metabolism (Kevers et al. 1997). Flavonoids are a class of phenolic compounds that are potential biochemical markers of rooting (Hand 1993). High concentrations of flavonoids have been related to easy- to-root phenotype in *Eucalyptus gunnii* (Curir et al. 1990).

The aim of the present work was to characterize the phases of adventitious rooting in minicuttings of *Eucalyptus globulus* x *maidennii* obtained from ministumps commercially cultivated in drip fertigated sand bed or intermittent flooding system.

Material and methods

Eucalyptus globulus x *maidennii*

Minicuttings of *Eucalyptus globulus* x *maidenii* were collected during the winter/spring of 2005 every 5 days from the onset of the experiment (when the minicuttings are obtained) up to 45 days (when the minicutting is transferred from the greenhouse to the "hardening house") at Aracruz Celulose S.A (Guaíba, RS, Brazil) to evaluate the adventitious rooting process, both morphologically and biochemically. For these experiments, minicuttings were obtained from ministumps cultivated in indoor hydroponics clonal hedges using intermittent flooding or drip fertigated sand bed systems (Figure 1).

Ministumps cultivated in drip fertigated sand bed system received a nutritional solution containing (g/1000L): calcium nitrate – 1250, Krista K – 420, Krista MPK, magnesium sulphate – 505, iron chelate – 41.5, organic boron – 5, manganese sulphate – 4, copper sulphate – 0.4, zinc sulphate – 0.8 (Yara Adubos – Porto Alegre/RS – Brazil) . Mineral nutrient quantities (mg/L): N – 175.4; P- 36.4; K – 212.8; Ca – 242.5; Mg – 48; S – 66 ; B – 0.5; Fe – 5; Cu – 0.1; Mn – 1; Zn – 0.2. The frequency of fertigation was once a day and the volume fertigated was 1.5 to 2 L/m^2 of bed.

Ministumps cultivated in the intermittent flooding system received a nutritional solution containing 985 g Calcinit, 946 g Kristasol, 140 g Magnitra-L, 20 ml Boron Organic and 116 g HydroFerro (Hidro Fertilizantes – Barueri/SP – Brazil). Mineral nutrient quantities (mg/L): N – 195.3, NH_4 – 24, P_2O_5 – 113.5, K_2O – 340.5, Ca – 187.1, S – 18.9, B – 2.24, CuEDTA – 0.38, FeEDTA – 7.34, MnEDTA – 0.38, ZnEDTA – 0.24, Mo – 0.38. The frequency of flooding was once a day.

Minicuttings were cultivated in dark polyethylene tubes filled with carbonized rice hulls and vermiculite (1:1 v/v) as substrate with added fertilizers: 2.5 Kg/m^3 of PG mix (Yara Adubos – Porto Alegre/RS – Brazil), 1.5 Kg/m^3 of Osmocote Classic (Scotts – Ohio – USA) and 2 Kg/m^3 of Simple Super Phosphate (20% P_2O_5). Nutrient contents in Kg/m^3: phosphorus pentoxide – 0.4, ammonium sulphate - 1.04, potassium chloride – 0.208, zinc sulphate – 0.014, copper sulphate – 0.014, manganese sulphate – 0.014, boric acid – 0.028).

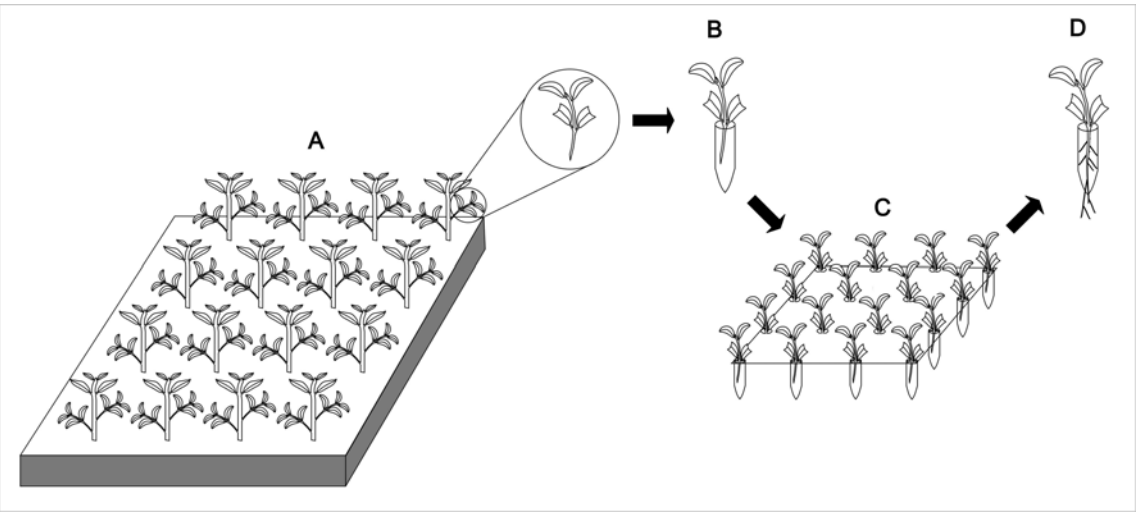

Figure 1: Vegetative propagation of *Eucalyptus globulus* x *maidennii*. A. Indoor hydroponics mini clonal hedge with ministumps maintained in sand bed or intermittent flooding trays. In detail, the production of minicuttings from ministumps. B. Minicutting in dark polyethylene tubes containing substrate (carbonized rice hulls and vermiculite 1:1). C. Rooting of minicuttings. D. Rooted minicutting ready to initiate adaptation to field conditions (hardening).

Morphological analyses

At each sampling time, 20 minicuttings from the intermittent flooding system and 80 minicuttings from the sand bed were evaluated at the same time. Parameters examined were percent of rooting, root number, root length and mean rooting time (Fett-Neto et al. 2001).

Biochemical analyses

Individual samples for biochemical analyses consisted of approximately 2 cm of the basal part of 4 minicuttings. Three replicate samples were used for each time point. Flavonoid content was evaluated only in cuttings obtained from the drip fertigated sand bed system.

Peroxidase activity

Approximately 100 mg of frozen plant tissues (basal part) were ground in liquid nitrogen. Peroxidase specific activity was quantified according to Fett-Neto et al. (1992), except that guaiacol was used as substrate. Crude protein extracts in phosphate buffer 0.2M, pH 7.0 were used to determine the enzyme activity at 420 nm. Protein was quantified by the method of Bradford (1976).

Total phenolic content

Approximately 50 mg of frozen plant tissues (basal part) were ground in liquid nitrogen, extracted in 1.5 ml HCl 0.1 N and submitted to sonication in a water bath for 30 min. The extracts were centrifuged at 12000 g for 30 min at 4°C. The supernatant was collected and the pellet was re-extracted. The supernatants were pooled and the volume

was completed to 5 ml with HCl 0.1 N. For quantification, 1 ml of $NaCO_3$ 20% (w/v) and 0.5 ml of Folin-Ciocalteu reagent were added, mixed and then incubated at 100°C for 1 min. After cooling, the extract was diluted to 50 ml with water and filtered through Whatman no.1 filter paper. Reading was at 750 nm. The standard curve was established with pirogalol 0.1% (w/v) in HCl 0.1 N (Fett-Neto et al. 1992).

Flavonoid content

The flavonoid content was determined by the aluminum chloride spectrophotometric method reported by Zhishen et al. (1999). Approximately 200 mg of frozen plant tissues (basal part) were ground in liquid nitrogen, extracted in 5 ml EtOH 95% and submitted to sonication in a water bath for 30 min in dark. The extracts were centrifuged at 7000 g for 5 min. The supernatant was collected, 1 ml of it was used and the volume was completed to 5 ml with H_2O. For quantification, 1 ml of $NaNO_2$ 5% (w/v) was added, mixed and then kept in R.T. for 5 min. Next, 0.3 ml of $AlCl_3$ 1% was added, mixed and then incubated at R.T. for 6 min. After, 2 ml of NaOH and 2.4ml of H_2O were added and mixed. Reading was at 510 nm. The standard curve was established with quercetin.

Results

The morphological parameters analyzed showed equivalent results for both systems at the end of the rooting process (Table 1). Considering all parameters together, intermittent flooding presented a slightly better performance. Minicuttings from both systems displayed callus formation (around 20% of the plants) after 10 days and the first roots appeared after 15 days in cuttings derived from both systems. At this time, 21% of sand bed-derived minicuttings were rooted versus 33% of intermittent flooding- derived

ones. Moreover, after 20 days the majority of the minicuttings were rooted: 70% of sand bed and 89% of intermittent flooding-derived cuttings. On the other hand, intermittent flooding led to a higher mortality rate (20.5%) than sand bed (14%).

Table 1: Effect of ministump culture system in adventitious root formation of minicuttings with 45 days of *Eucalyptus globulus* x *maidenni*. Parameters (means ± SEM) are not significantly different based on Student t-test or trust intervals overlap ($p \leq 0.05$).

	Sand Bed	Intermittent flooding
% of rooting	92,7	100
Number of adventitious root/ minicutting	6.9 ± 0.3	7.9 ± 1.3
Root length (cm)	5.11 ± 0.8	5.58 ± 2.1
Mean rooting time (days)	32.6 ± 3.2	29.7 ± 1.2

Minicuttings originated from both systems showed a peak in peroxidase activity after 15 days of culture, coincident with the emergence of the first roots. After that, peroxidase activity sharply decreased at day 20, remaining stable afterwards. This pattern was consistent in minicuttings derived from both culture systems (Fig. 2). In spite of the similar activity profile displayed by minicuttings derived from both ministumps operational systems, peroxidase activity of minicuttings obtained from ministumps under the intermittent flooding system was higher by about one order of magnitude (Fig. 2).

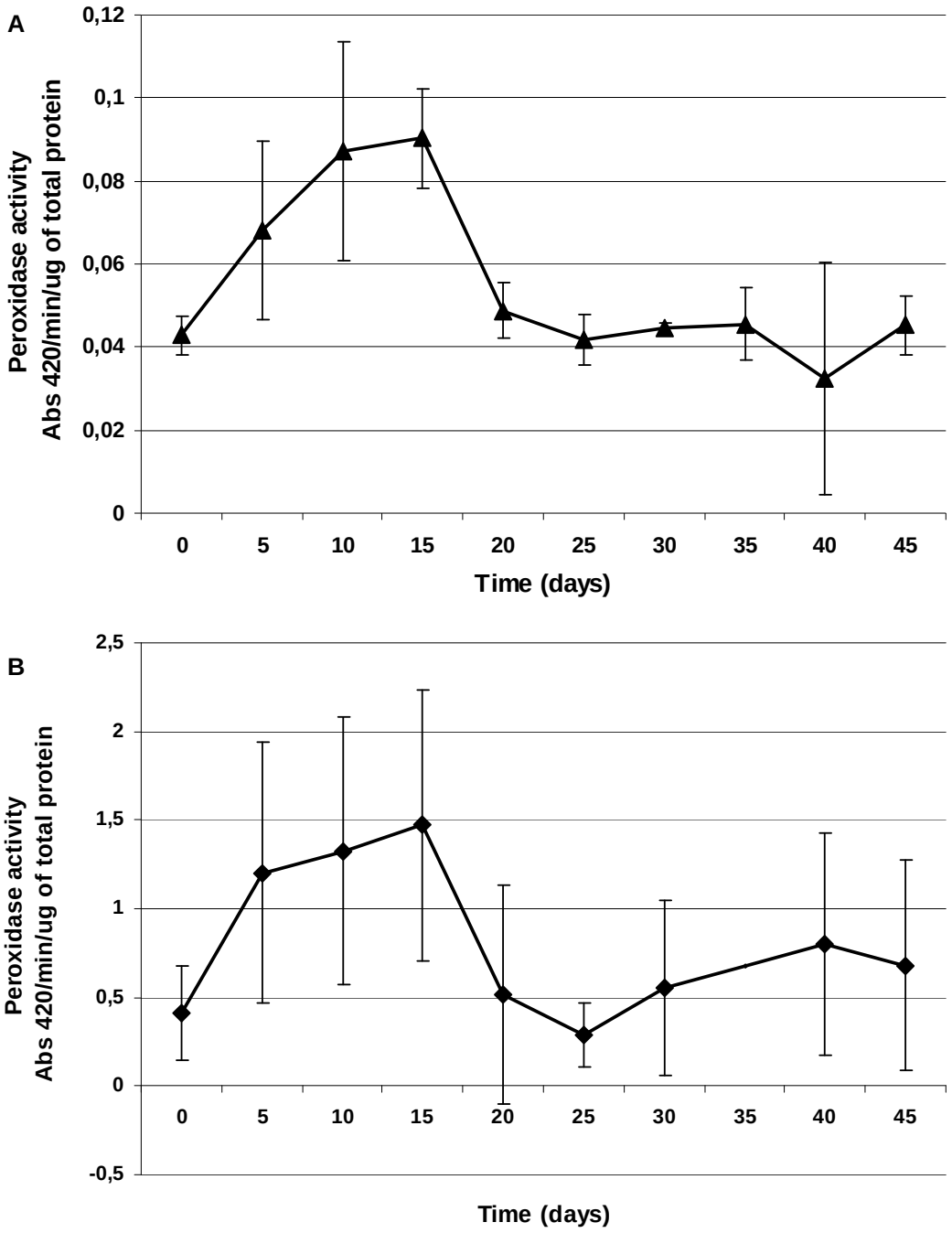

Figure 2: Peroxidase activity in minicuttings of *Eucalyptus globulus* x *maidenni* obtained from ministumps cultured in drip fertigated sand bed (A) and in intermittent flooding (B). Bars are standard deviations.

Changes in content of phenolic compounds were also similar between minicuttings derived from both culture methods. Phenolic content was low and stable from the time of cutting harvest to day 5, followed by a fast increase between day 5 and 20, reaching a variable plateau until the end of the experiment (Fig.3). The content of phenolic compounds was 2.5 to 3 times higher in minicuttings derived from ministumps grown in the drip fertigated sand bed system compared to those derived from intermittent flooding.

The analysis of flavonoid content in minicuttings derived from sand bed displayed a profile similar to that shown by total phenolics. The highest flavonoid content was observed between days 20 and 30 and a slow, but apparent decrease was observed until day 45 (Figure 4).

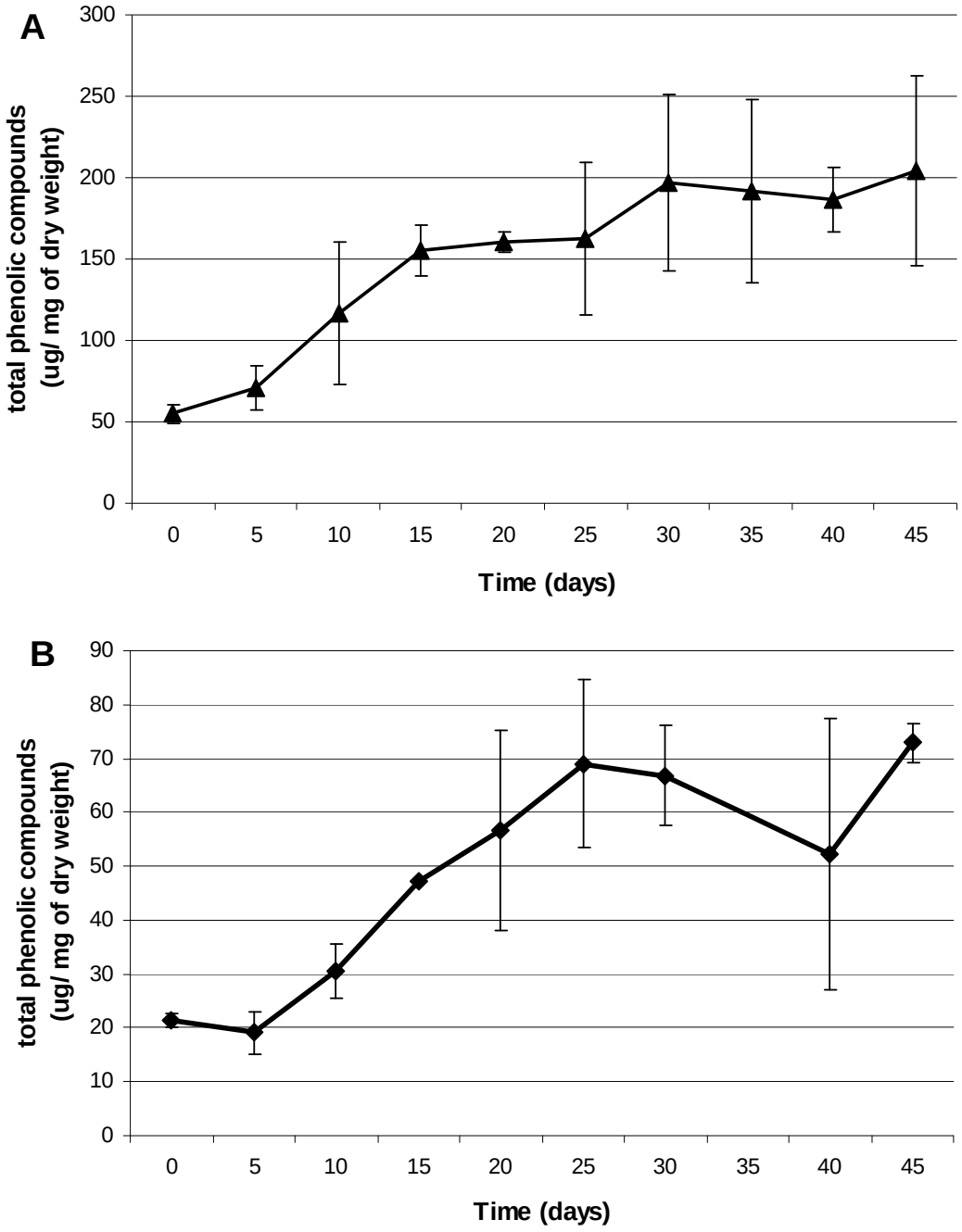

Figure 3: Total phenolic compounds in minicuttings of *Eucalyptus globulus* x *maidenni* obtained from ministumps cultured in drip fertigated sand bed (A) and in intermittent flooding (B). Bars are standard deviations.

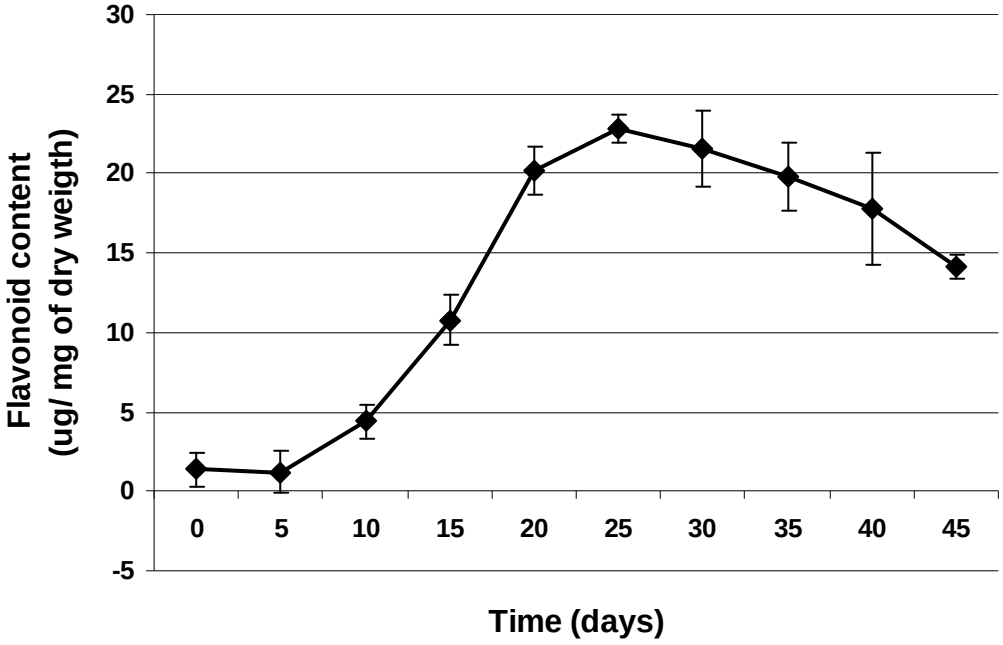

Figure 4: Flavonoid content in minicuttings of *Eucalyptus globulus* x *maidenni* obtained from ministumps cultured in drip fertigated sand bed. Bars are standard deviations.

Discussion

Gaspar et al. (1992) described a model for grapevine adventitious rooting that included three phases: 1) an induction phase, characterized by lower peroxidase activity (lack of morphological changes and high auxin content), 2) an initiation phase, occurring between the minimum and maximum of peroxidase activity (cell division and decrease in auxin content) and 3) an expression phase, characterized by a gradual decline in peroxidase activity followed by the first histologically visible signs of root primordia. Various studies have shown that peroxidase activity profiles during adventitious rooting of several species are consistent with this general model (Hatzilazarou et al. 2006; Metaxas et al. 2004; Qaddoury and Amssa 2004; Rout et al. 2000; Saxena et al. 2000; Rival et al. 1997). In the case of *Eucalyptus* minicuttings, it seems that the induction phase occurred in the first days after obtaining the cutting (before 5 days) or even when these were still attached to the

ministump. The initiation phase appears to have taken place from day 5 to day 15, during the increase and peak of peroxidase activity. Finally, the expression phase occurred between days 15 and 20, corresponding to the emergence of the first roots and rooting of the majority of minicuttings. At this phase, a decrease in peroxidase activity would be expected due to the stabilization of auxins at lower concentrations after the catabolic action of the enzymes on auxin at the initiation step. The same trends were observed when analyzing total peroxidase activity data (weight basis), indicating that changes were not the result of differences in protein content throughout the rooting process, but of enzyme activities and/or effectors (data not shown).

The much higher peroxidase activity in minicuttings derived from the intermittent flooding system can be related to a greater rooting ability (Gaspar et al. 1992 and references therein), not statistically significant when compared to sand bed system, and could have been induced by the temporary hypoxia and re-oxygenation phases of the intermittent flooding system. In plantlets of wheat, it was seen that periods of hypoxia, as well as hypoxia and aeration, where capable of increasing the activity of ascorbate peroxidase in roots (Biemelt et al. 1998). Studies in *Lupinus* showed that cycles of hypoxia and re-aeration represent sources of oxidative stress, as they increase the concentration of free radicals and induce antioxidant enzymes, such as superoxide dismutase (Garnczarska et al. 2004).

According to Berthon et al. (1993) phenolic compounds are involved in different steps of adventitious root formation. De Klerk et al. (1999) pointed out that phenolic compounds can act as antioxidants, thereby protecting IAA from oxidation and plant tissue from oxidative stress due to wounding. In the present study, it was not possible to identify a peak of total phenolic content during the early part of the experimental period as reported

in other studies with rooting of *Vigna radiata*, *Prunus dulcis* and *Sequoia sempervirens* (Nag et al. 2001; Caboni et al. 1997; Fett-Neto et al. 1992), this reinforces the possibility that the root induction phase happened during the first 5 days or in the ministump. During the initiation phase, the content of phenolics increased, which may reflect a need to reduce auxin transport from the shoot at this phase. Flavonoids have been shown to inhibit the basipetal transport of auxin in stems (Wasson et al. 2006; Peer et al. 2004). In fact, at least for the sand bed-derived microcuttings, the content of total phenolics and flavonoids increased in very similar fashions. In the expression phase, the content of phenolics remained high, but fluctuated, which may be due to differences in root growth between minicuttings.

The higher phenolic content in the sand bed system-derived microcuttings can also be related to differences in the nutrient solution application method in both culture systems. Studies with *Hypericum brasiliense* showed an increase in the content of total soluble phenolics in the shoots under hypoxia due to continuous flooding, whereas roots did not present a significant change (De Abreu and Mazzafera 2005). Ministumps under intermittent flooding are likely experiencing recurrent but transient oxidative stress and it is possible that phenolics could participate in the mitigation of this phenomenon by acting as antioxidants, leading to reductions in their steady-state content in shoots. The opposite magnitude of difference observed for peroxidase activity and content of phenolic compounds between minicuttings derived from ministumps grown in sand-bed and intermittent flooding supports the general view that these biochemical parameters display opposite behaviors in relation to the rooting process (Gaspar et al. 1992).

In conclusion, the rooting results obtained in both systems were similar. The choice for a system can be made based on operational convenience and production costs.

Furthermore, biochemical analyses showed a consistent profile for both culture systems, indicating that it is possible to use them as biochemical markers, especially peroxidase activity. Changes in time course profiles and relative contributions of peroxidase activity and content of phenolics appear more relevant for the final morphogenic rooting response than specific values of these *per se*. Although further studies with different clones and seasons will be necessary to confirm the use of the rooting markers herein evaluated, the identification of rooting phases may pave the way for a better modulation of the adventitious rooting process of *Eucalyptus* in the commercial scale set up employing the minihedge strategy. The development of rooting phase-specific nutrient solutions, for example, as has been succesfully done for in vitro rooting in *E. globulus* (Schwambach et al. 2005), may result in significant gains in efficiency of the propagation process.

Acknowledgments

The authors thank Aracruz Celulose S.A. for the opportunity of developing the work in the commercial miniclonal hedges, for the supply of plant material and for the grant-in-aid of research to carry out this investigation. The Brazilian government agencies CAPES (Coordenadoria de Aperfeiçoamento de Pessoal de ensino Superior) and CNPq (National Council for Scientific and Technological Development) provided graduate and research scholarships.

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Nitrogen sources and adventitious root development in *Eucalyptus globulus* microcuttings

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Manuscript to be submitted to Biologia Plantarum (Springer Verlag)

Abstract

Adventitious rooting is an essential step in the vegetative propagation of economically important woody species. Nitrogen sources have been shown to be important in the elongation of lateral roots and in primary root system branching in herbaceous species. Previous studies from our group pointed to a positive response of adventitious root elongation in *Eucalyptus globulus* microcuttings by the use of nitrate as the sole source of nitrogen, whereas ammonium had a negative impact on rooting. This work analyzed in further detail adventitious rooting and root branching in microcuttings of *E. globulus* in response to various sources of nitrogen considering both root induction and formation steps. The parameters analyzed were percent rooting, root density, root length, percent of adventitious roots showing branching and number of lateral roots per cm of branched root. Experiments confirmed the negative effect of ammonium and the positive effect of nitrate on root development, including root branching. Urea-treated microcuttings yielded a rooting response comparable to that of nitrate-treated ones. Induction of urease activity was observed on the second day of the root induction step and activity continued throughout the rooting process, with a peak in the root induction and another at the root formation step. Urea-treated microcuttings had a faster increase in protein content, indicating prompt assimilation of urea into amino acids. The use of glutamic acid as source of nitrogen promoted higher root number, but yielded shorter roots and did not affect root branching. Microcuttings rooted in urea, nitrate, or ammonium nitrate, acclimated to *ex vitro* conditions and transferred to soil, were exposed to drought stress and showed similar survival rates. Considering the overall rooting responses, the low cost of urea as a fertilizer and the potential properties of urease as a defense protein, urea represents a viable alternative source of nitrogen for the propagation of *E. globulus*.

Introduction

In forestry, vegetative propagation is widely used to multiply elite individuals obtained in breeding programs or selected from natural populations (Hartmann et al. 1990). Adventitious root formation is a key step in vegetative propagation (De Klerk et al.1999) and is a developmental process consisting of a series of successive and interdependent phases (induction, initiation, formation), each with its own requirements and characteristics (Kevers et al. 1997). Nutrition is a key factor affecting rooting predisposition, because of its involvement in determining the morphogenetic response of plants (Assis et al. 2004), such as lateral root formation and control of root length and density. Most plant species are able to uptake and assimilate nitrate, ammonium, urea and amino acids, but the response to a particular form of nitrogen varies from species to species (Márquez et al. 2005). Nitrogen plays a key role in the plasticity of the root system architecture mainly when provided as NO_3 and NH_4 (Zhang et al. 1999, Robinson 1994). Recently, it was demonstrated that exogenous *L*-glutamate is also able to elicit complex changes in root development of *Arabidopsis* and *Thlaspi caerulescens* (Walch-Liu et al. 2006a, 2006b), but other amino acids did not have the same effect.

Previous studies with nitrate and ammonium as nitrogen source for adventitious rooting of *Eucalyptus globulus* showed that nitrate produced a well-developed adventitious root system (Schwambach et al. 2005, Bennett et al. 2003). Besides, since urea is a popular form of N fertilizer, mainly due to its competitive price (Miller and Cramer 2004) it would be interesting to evaluate the impact of this form of N in the adventitious rooting and root branching of *E. globulus*.

Eucalypts have become the most widely planted hardwood species in the world, with approximately 17.8 millions ha of planted area (FAO 2000, Turnbull 1999). The American statistics is dominated by Brazil, where there are estimated 3 millions ha of plantations, most of them for pulp production (FAO 2000, Turnbull 1999). In southern Brazil, the cellulose pulp and paper industry has a specific interest in *Eucalyptus globulus* (Labill.) and its hybrids due to their relative resistance to frost and lower lignin content. Frost is a common feature of the winter in southern Brazil, and lignin interferes in the chemical extraction of cellulose. However, *Eucalyptus globulus* has poor rooting capacity and its cuttings are relatively difficult to propagate (Serrano et al. 1996, Le Roux and Van Staden 1991), underlining the need for studies that can result in improved root systems during propagation.

This work analyzed the adventitious rooting of *E. globulus*, applying a two-step sequential rooting medium protocol to microcuttings, in response to different sources of nitrogen. The objective was to identify a nitrogen source that can provide optimized root architecture for regenerated plants, and is amenable to use in large scale propagation.

Material and Methods

Plant material

Seeds of *Eucalyptus globulus* (Labill.) (batches from Chile and Australia supplied by Aracruz Celulose, Guaíba, RS, Brazil) were surface sterilized and aseptically cultivated. Microcuttings were obtained from three-and-a-half-month-old seedlings for use in the *in vitro* rooting experiments, as described by Schwambach et al. (2005).

Culture conditions

Rooting experiments were carried out with a two-step basal sequential medium protocol consisting of a 4-day induction step (0.3x MS salts – Murashige and Skoog [1962], 0.4 $mg.l^{-1}$ thiamine HCl, 100 $mg.l^{-1}$ inositol, 10 $mg.l^{-1}$ of indolyl-butyric acid [49.3 μM], 30 $g.l^{-1}$ sucrose and 6 $g.l^{-1}$ agar, adjusted to pH 5.8 ± 0.1 prior to autoclaving) and a 20-day formation step (induction medium without added auxin and supplemented with 1 $g.l^{-1}$ of activated charcoal) (Fett-Neto et al. 2001). In all experiments, a control treatment without auxin in the induction step was included to confirm that the micro-cuttings had significantly reduced or lost rooting capacity. All reagents were analytical grade and media were prepared with double-distilled water from a glass distiller. Media were sterilized by autoclaving at 121 °C and 1.5 atm for 20 min.

The experiments were carried out in 20 ml vials containing 6 ml of medium and double capped with aluminum foil. Two explants per vial were used and each treatment contained 30 explants. All experiments were performed in a growth room at temperature of 28 ± 2 ºC. The photoperiod was 16 h with a PAR of approximately 30 μmol m^{-2} s^{-1} at explant level, provided by cool white fluorescent lamps. The experimental design was completely randomized and the experiments were independently repeated at least twice with similar results. The presence of a root was scored if an approximately 2 mm long, polar whitish structure was visible. Only roots originating directly from the stem were considered in the analysis as adventitious root. Lateral roots were counted to analyze the ramification of the adventitious roots formed.

Nitrogen sources

Different nitrogen sources were evaluated (nitrate – $NaNO_3$, ammonium – NH_4Cl, urea – $CO(NH_2)_2$, glutamic acid, glutamine and asparagine), always in equimolar concentration (18 mM) to the nitrogen present in the control – 0.3x MS salts treatment (ammonium nitrate – NH_4NO_3), in the two-step rooting protocol. Changes in nitrogen supply between rooting phases were analyzed for combinations of nitrate and urea. Potassium concentrations were maintained the same among treatments by supplementing with appropriate amounts of KCl. All reagents were analytical grade.

Urease activity

Urease activity was quantified using a modified phenol-hypochlorite method (Weatherburn 1967). Approximately 100 mg of frozen tissue was ground in liquid nitrogen and 1 ml of phosphate buffer 0.2 M, pH 7.0, containing 0.062 g of soluble polyvinilpyrrolidone. The extracts were centrifuged at 14000 x g for 30 min at 4°C. The supernatants were harvested. For quantification, 50 µl of supernatant, 400 µl of distilled water, 25 µl of phosphate buffer 0.2M, pH 7.0, 25 µl of urea 100 mM and 400 µl of nitroprussiate-phenol were incubated at 37°C for 10 min. Next, 400 µl of alkaline sodium hypochlorite and 1 ml of distilled water were added, followed by incubation at 37°C for 60 min. Reading was at 570 nm. The standard curve was established with ammonium sulfate 0.01% (w/v).

Protein quantification

Protein was quantified according to Bradford (1976) using 5µl of the crude extract from urease activity samples .

Soil experiment

Acclimatization of rooted microcuttings was done as described in Schwambach et al. (2005). After two months of transfer to 2L plastic pots containing a mixture of soil and vermiculite (1:1 v/v), watering was stopped. Mortality rates were recorded every 5 days during soil acclimatization period and from the onset of the drought stress for a total period of 15 days.

Statistical analyses

Parameters examined included percent of rooting, root density (roots per rooted cutting), longest root length, percent of ramification or branching (plants with adventitious roots that presented lateral roots) and branching density (number of lateral roots per cm of adventitious root). Data were taken 20 days after transfer to root formation medium. Statistical analyses (Analysis of Variance, followed by Duncan tests when appropriate, $p \leq 0.05$) were done according to Sokal and Rohlf (1981). The experiments were repeated independently three times.

Results and Discussion

Ammonium and nitrate are the most important inorganic N sources in soils readily available to plants. Although NH_4^+ should be the preferred source of N because it requires less energy for assimilation in many higher plants, the supply of ammonium as the only N source often leads to physiological and morphological disorders (Zhang et al. 2007). Microcuttings that had ammonium as the only nitrogen source showed a poorer performance for most of the analyzed parameters. Rooting percentage was not significantly

different among treatments, but ammonium treatment showed 50% rooting whereas other treatments reached around 70%. The number of adventitious roots (Figure 1A) was negatively affected by the presence of ammonium (ammonium nitrate control and ammonium only treatment). Root length was severely reduced in ammonium only treatment (Figure 1B). The percentage of ramification in ammonium-treated microcuttings also showed a trend for reduction (Figure 1C). Root browning was also observed in the same treatment. These results confirm our previous findings with ammonium and extend a possible deleterious effect of this source of N to root branching. For many plants, NH_4^+, when supplied alone, is toxic and impairs plant growth (Britto and Kronzucker 2002), and this seems to be the case for *E. globulus* microcuttings (Schwambach et al., 2005).

Over the last decades there has been an increase in the utilization of urea-based fertilizers, so that urea is currently the predominant form of N fertilizer used (Miller and Cramer 2004). In this context, urea was added as one of the N sources to support adventitious root development in *E. globulus*. The nitrogen present in urea is unavailable to the plant unless hydrolyzed by urease and it has been proposed that its primary role is to allow the organism to use external or internally generated urea as nitrogen source (Sirko and Brodzik 2000). Urease activity has been detected in many plants and is reported to be inducible by urea in rice, jack bean, barley, potato and soybean [reviewed by Skokut and Filner (1980) and Witte at al. (2002)]. In the urea treatment, urease activity induction was observed between one and two days of the root induction phase, showing a higher level throughout the experimental period (1.4 to 1.9 times higher during rooting – evaluated until day 8) and a bimodal peak profile (Figure 2A). The data suggest that urease is inducible in *E. globulus* and that urea is being absorbed by the microcuttings or externally hydrolized. However, considering the apparent toxicity of external ammonium supply to

E. globulus cuttings, urea uptake seems to be the most likely scenario. Urea-treated microcuttings had higher contents of soluble protein than other treatments (Figure 2B), suggesting that N is quickly assimilated in amino acids. Urea treatment showed 70% of adventitious rooting, the same result obtained by the control and nitrate treatments. Plants treated with urea presented an intermediate result for root density, reaching numbers similar to those of nitrate but not differing from control and ammonium (Figure 1A). Root length displayed by urea-treated microcuttings was equivalent to that of nitrate or control-treated cuttings (Figure 1B). Furthermore, there was no difference in % of branching between treatments (Figure 1C) and the number of lateral roots in urea-grown cuttings was second only to that of nitrate-derived cuttings (Figure 1D).

These intermediate results for urea may be related to its hydrolysis to ammonium by plant ureases, which raises the pH (Witte et al. 2002). Moreover, urea can be hydrolyzed in the medium to ammonium (Miller and Cramer 2004), which makes ammonium available for uptake by the microcuttings, therefore affecting rooting. However, microcuttings rooted in the urea treatment presented white and thicker roots compared to the other treatments. Thicker roots are more costly to produce, but have greater transport capacity and are less vulnerable to desiccation, physical damage, pathogens and so are generally longer lived (Forde and Lorenzo 2001). White roots are often associated with intense physiological activity (Assis et al 2004). The induction of urease by urea supply as N source may also have important implications for plant resistance to insects and fungi in the greenhouse and under acclimation to field conditions. It has been shown that plant ureases also display defense-related functions both against insects and phytopathogenic fungi, and these defense properties are not related to ureolytic activity of the protein (Carlini and Grossi-de-Sá 2002, Becker-Ritt et al. 2007).

Nitrate, as one of the major sources of mineral N for many higher plants, has the advantages of being readily mobile in the xylem. It can also be stored in the vacuoles of roots and shoots, unlike ammonium, that has to be readily incorporated into organic compounds when absorbed (Marschner 1995). Plants also respond to nitrate as signals derived from internal and external content that modulate root growth and architecture (Stitt 1999). Besides, nitrate can act as an ion and modulate root initiation and branching independently of its effect as nutrient in nitrogen metabolism in a pathway related to auxin (Forde and Lorenzo 2001). Nitrate treated cuttings had significantly higher root density than those exposed to control or ammonium treatment, but were similar to urea treated cuttings (Figure 1A). Considering root length, nitrate allowed the development of roots with similar length to the control and urea treatment (Figure 1B). The microcuttings exposed to nitrate treatment had white roots. The percentage of plants showing root ramification was not significantly different between nitrogen sources, but nitrate-grown plants showed a tendency towards higher percentage of root ramification (Figure 1C). The number of lateral roots was greater in nitrate treatment when compared to the other N sources (Figure 1D). Similar results in adventitious rooting development were obtained by Bennet et al. (2003) and Schwambach et al. (2005). In this previous work it was suggested that the role of nitrate in the adventitious root formation would be an extension of its well established role in stimulating root branching and lateral root elongation in herbaceous species (Zhang et al. 1999). The data herein presented appear to confirm this relation, since nitrate improved the percentage of root branching and number of lateral roots.

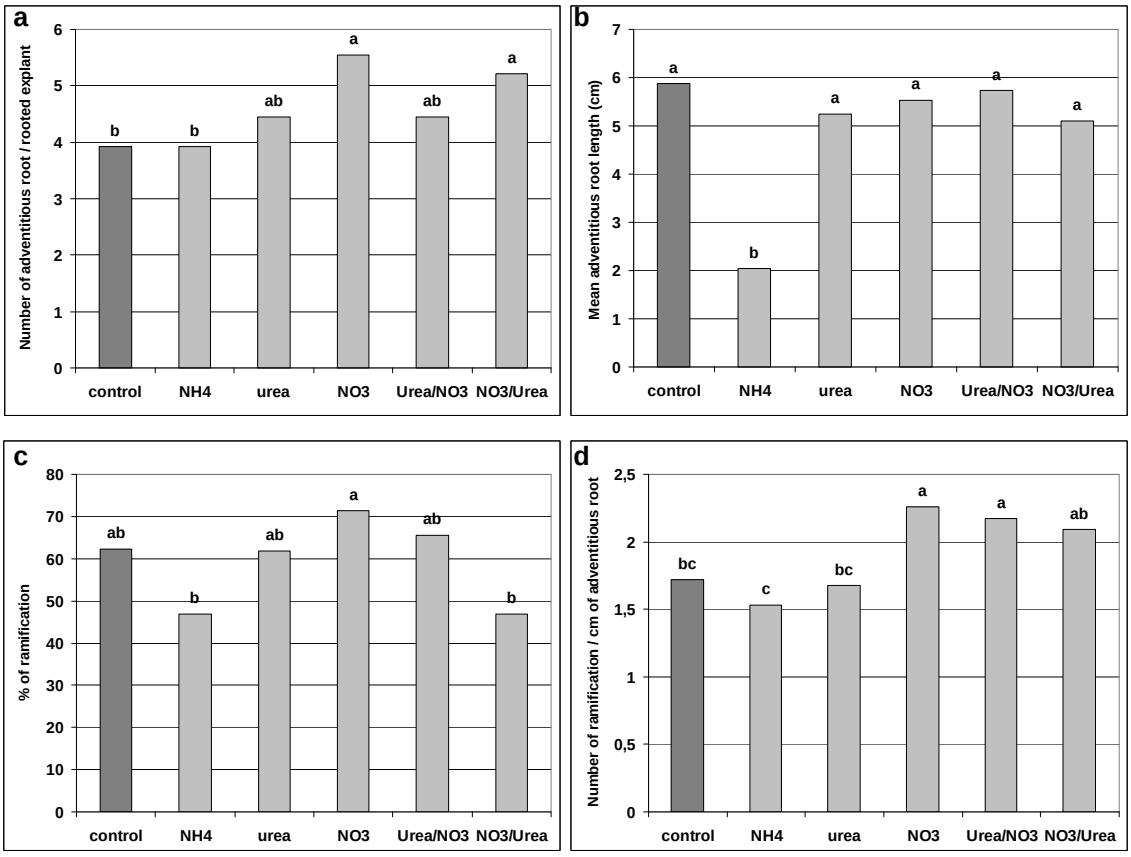

Figure 1: Adventitious rooting in micro-cuttings of *Eucalyptus globulus* after 20 days in formation medium growing in different sources of nitrogen: a. Root density; b. Root length; c. % of ramification; d. Number of ramifications per cm of adventitious root. Bars sharing the same letter are not different by a Duncan test ($p \leq 0.05$). Bars differently marked in each pool of bars indicate the control treatment (0.3x MS).

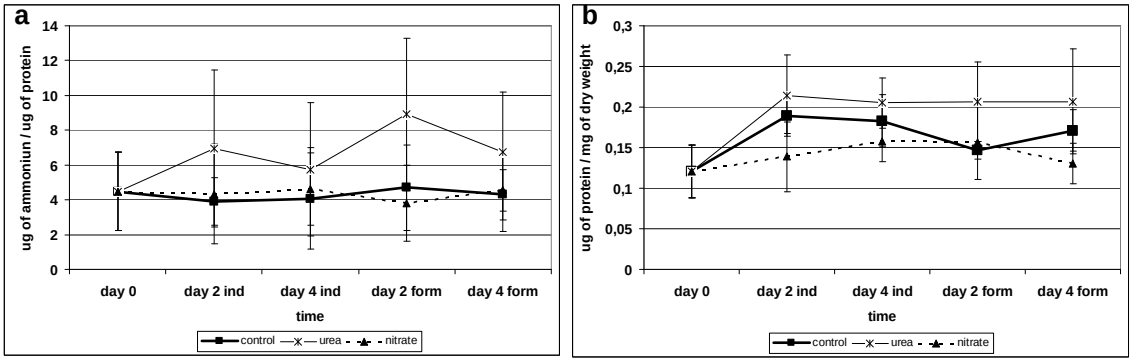

Figure 2: Urease activity and soluble protein content in micro-cuttings of *Eucalyptus globulus* from day 0 to day 8 (day 4 in formation medium) growing in different sources of nitrogen: a.Urease activity; b. Protein content. Each point represents the mean of three independent experiments and respective standard deviation.

Although nitrate is the major source of N for plants growing in aerobic soils, it is now recognized that organic forms of N can sometimes contribute to plant nutrition. Amino acids represent the largest fraction of low molecular weight organic N dissolved in soil and they can represent a significant source of N for plant growth in some ecosystems (Walch-Liu et al. 2006a and references therein). Given the importance of amino acids as a N source, different amino acids were tested to determine their role in adventitious rooting of *E. globulus*. The rooting percentage in the different treatments was similar to the control (75% of rooting) with glutamine yielding a lower proportion of rooted plants (55%). Adventitious root density was significantly increased when glutamate was provided as N source and root length was diminished when any of the amino acids were applied (Figure 3A and 3B). In addition, the percentage of ramification was not different among microcuttings rooted in amino acids and control, with a trend for asparagine exposed cuttings to have less lateral roots (Figure 3C). Glutamine and glutamate yielded microcuttings with similar number of ramifications compared to the control, whereas asparagine-treated cuttings had roots with less ramifications (Figure 3D).

There are few studies on the role of amino acids in root development. To investigate the possible interactions between amino acids and root development, Walch-Liu et al. (2006a) tested the effect of 21 amino acids and found that *L*-glutamate played an important role in embryonic root branching. Seedlings of *Arabidopsis* supplied with *L*-glutamate had the growth of the main root inhibited and an increase in lateral root formation. None of the other 21 amino acids tested showed an effect in root development. Similar responses were seen for embryonic root systems of *Thlaspi caerulescens* (Walch-Liu et al. 2006b). The results obtained in the present work suggest a related role of glutamate in adventitious root development of *E.globulus*, at least in terms of increasing

root numbers; however, no significant effect was observed on branching of adventitious roots.

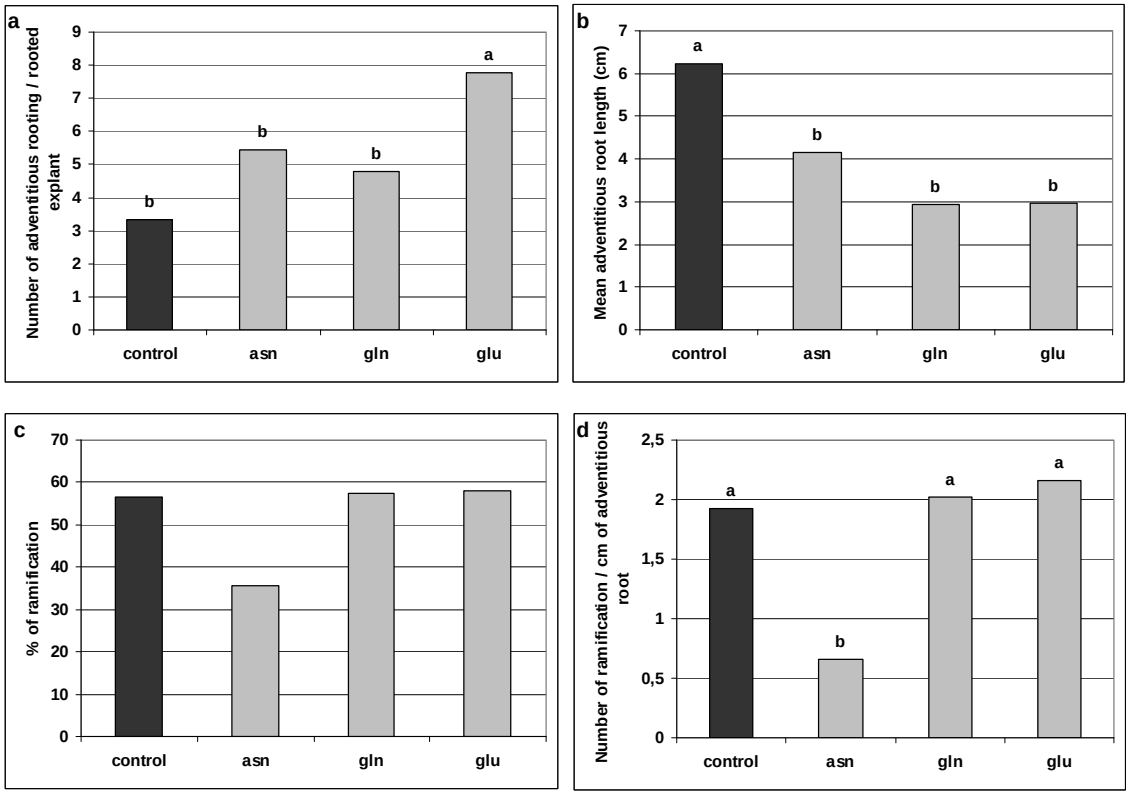

Figure 3: Root branching in micro-cuttings of *Eucalyptus globulus* after 20 days in formation medium growing in the presence of different amino acids: a. Root density; b. Root length; c. % of ramification; d. Number of ramifications per cm of adventitious root. Bars sharing the same letter or without letters are not different by a Duncan test ($p \leq 0.05$). Bars differently marked in each pool of bars indicate the control treatment (0.3x MS).

Plants rooted on nitrate, urea and control treatments, which were transferred to soil, were compared for survival after 2 months and during the 15-day water stress period. After 2 months of transfer to soil, survival of urea-derived plants was 92% compared to 73% of ammonium nitrate-derived plants; however, plant survival was not significantly different among the various treatments (data not shown).

In conclusion, nitrate is suggested as the source of choice to be used for *E.globulus* in the protocol of *in vitro* rooting. However, as the microcuttings rooted in the different sources of N, particularly urea, had a similar soil acclimatization performance and response under drought stress, it is possible to choose among the nitrogen sources (ammonium-nitrate, nitrate and urea) on the basis of cost reduction of operational processes.

Acknowledgments

The authors thank Aracruz Celulose S.A. for the gift of *E. globulus* seeds and for a grant-in-aid-of-research, and the Brazilian government agencies CAPES and CNPq for graduate and research bursaries.

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Auxin homeostasis and adventitious rooting: a search for suppressors of the *superroot2* mutation in *Arabidopsis thaliana*

Manuscript in preparation

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Introduction

One of the most successful approaches for investigating the underlying principles of plant development has been the analysis of morphological mutants of the small weed *Arabidopsis thaliana* (Bohmert et al. 1998). The availability of very large collections of *Arabidopsis* mutants has allowed many studies of root development during the last few years (Casson and Lindsey 2003). While development of the primary root from the embryonic stage has received a lot of attention and the processes involved are beginning to be unraveled, the formation of lateral and adventitious rooting is less well understood (Ludwig-Müller et al. 2005). Adventitious root formation is a key step in clonal propagation that is used in horticulture and forestry, and problems associated with cuttings considered difficult to root frequently result in significant economic losses. Identification

of genes in model species such as *Arabidopsis* can be expected to facilitate the improvement of root traits in due course, either by marker assisted breeding or genetic engineering (Casson and Lindsey 2003).

Among the many factors involved in the determination of rooting capacity, a central role appears to be played by auxin activity, *i.e.* the net result of the regulation of auxin metabolism and cell sensitivity to this phytohormone (Assis et al. 2004). Additionally, other differences such as uptake and transport can also account for the differences in rooting behaviour (Ludwig-Müller et al. 2005). There are several *Arabidopsis* mutants altered in auxin signal transduction, transport and conjugation. Among these, is the mutant *superroot2* (*sur2*) (Delarue et al. 1998) that overproduces auxin and therefore spontaneously develops adventitious roots on the hypocotyls (Delarue et al. 1998, Sorin et al. 2005).

Several lines of evidence indicated that the *SUR2* gene defines a key point in the regulation of endogenous auxin concentrations (Delarue et al. 1998). *SUR2* encodes the cytochrome P450, *CYP83B1*, which is responsible for oxidizing indole-3-acetaldoxime in the biosynthesis of indole glucosinolates (Barlier et al 2000). Endogenous free IAA concentrations in the *sur2* mutant were significantly higher than in the wild type and in most cases were correlated with a decrease in the level of bound IAA, leading to a reduction in the percentage of total IAA in the conjugated form compared to the wild type (Delarue et al. 1998). A certain control of the endogenous free IAA level, either by degradation or by a feedback regulation of IAA synthesis has been suggested (Delarue et al. 1998). Moreover, *sur2* explants are unable to sustain auxin-autonomous growth when grown on a medium lacking phytohormones, indicating that the *SUR2*$^-$ phenotype could be the consequence of auxin accumulation; this scenario is in agreement with a clear feedback

control of IAA synthesis in the *sur2* mutant (Delarue et al. 1998). Therefore, in spite of the recent evidence that *SUR2* is involved in the control of the pool of indole-3-acetaldoxime by acting downstream in glucosinolate biosynthesis (Barlier et al. 2000), significant differences in the *sur2* mutant detailed auxin phenotype indicate that important aspects of auxin homeostasis and rooting are yet to be unraveled by the study of this mutation and its suppressors.

Here we describe four *sur2-1* suppressors through a detailed phenotypical characterization together with genetic and biochemical analysis, suggesting that they are involved in the control of auxin homeostasis.

Materials and methods

Identification of sur2 suppressors

Homozygote seeds from the *superroot 2-1* (*sur2-1*) mutants (ecotype Wassilewskija – WS) were mutagenized with ethyl methanesulfonate (EMS) as described in Santoni et al. (1994). The M2 progeny of 1500 independent lines was screened as follows: seeds were sown in vitro as described in Santoni et al. (1994) and kept in the dark until the hypocotyls reached a size of 5-6 mm. Seedlings were then transferred to light for seven days as described in Sorin et al. (2005). The adventitious roots were counted on the hypocotyl. Seedlings which did not make or produced very few adventitious roots were transferred to soil and let set seeds. The suppressor phenotype was confirmed in the M3 progeny.

Complementation and Genetic Segregation Analysis

For the crosses, plants used as female parents were emasculated and cross pollinated. Progenies were harvested, stratified and germinated. The phenotype of the

seedlings was scored 10 days after germination. The suppressors were backcrossed three times to the original *sur2-1* mutant used as the female parent.

Mapping of sur2 suppressors

Mapping populations have been produced for 4 of the *sur2-1* suppressors (WS background) by crossing homozygous with *atr4-1* (an allele of *sur2-1* in the Columbia (Col-0) background). The mapping was done using the F2 recombinant populations using molecular markers such as CAPS, microsatellites and SFLP.

Growth conditions

For seed production and crosses, the plants were grown in a greenhouse as in Sorin et al. (2005).

For in vitro culture, seedlings were grown as previously described (Santoni et al. 1994). The conditions in the controlled-environment chambers were as follows: 140µmol.m^{-2}.s^{-1} irradiance provided by BIOLUX fluorescent tubes, 16 h of light, 60% relative humidity, 15°C night temperature and 22°C day temperature.

For dark growth conditions, Petri dishes were wrapped with three layers of aluminum foil and placed vertically.

For adventitious formation, the plants were grown in light for 7 days and then had their main root removed and were transferred to new medium for additional 7 days in light.

Hypocotyl and Root measurements

Hypocotyl and root measurements were performed as described by Gendreau et al. (1997). At least 30 seedlings were used for each data point. All measurements were done

on two independent biological replicates, using ImageJ software (http://rsb.info.nih.gov/ij/index.html). Errors bars in the figures indicate standard deviation.

Scoring of Adventitious Roots

Seedlings were etiolated in the dark for 2.5 days and then transferred to light. Emergent adventitious roots were scored at 7 d after transfer to light. At least 30 seedlings were used for each data point. This was repeated in two independent biological replicates. Error bars in the figures indicate standard error.

Determination of free and conjugated IAA concentrations

Seedlings were grown in light for 9 days. Entire seedlings were pooled to obtain an average of 15 mg of fresh weight. Samples were extracted, purified and analyzed by liquid chromatography-multiple reaction monitoring-mass spectrometry as described previously (Kowalczyk and Sandberg, 2001). The following metabolites were quantified: IAA, IAAsp, IAglu and oxIAA. A *t* test was performed according to http://graphpad.com/quickcalcs/ttest1.cfm.

Results

Mutant isolation and genetic characterization

To isolate the mutants, 1500 individually harvested M2 seed stocks derived from EMS mutagenized populations, were scored for a phenotype showing few or no adventitious root after dark etiolation as the wild type. Four suppressor mutations were identified and confirmed; and they were named 266, 420, 494 and 677. After outcrossing

the mutants with the *sur2-1* mutant, allelism tests and segregation analysis were performed and the phenotypes of *sur2-1* suppressors were scored in the F3 generation. The results showed that they are not allelic and that the mutations are recessive traits (data not shown).

The chromosomal map position of the *sur2-1* suppressors was determined. DNA was isolated from at least 48 homozygous plants of each suppressor and the segregation of markers was verified. Mutations 266 and 677 were located in the region of *nga151* marker at the top of chromosome 5. Mutation 420 was located in the region of *MSAT4.15* marker at the bottom of chromosome 4. For mutation 494, a map-based cloning had been initiated, using a recombinant population of 2.000 plants. Mutation 494 is located in a 170Kb region on the top of chromosome 4, containing 40 genes of unknown function. This region is comprised between markers CER461021 and CER457983. No polymorphic markers are available to reduce this region; the sequencing of the genes in that region is underway to identify the mutation.

Morphological characterization

Seeds of *sur2-1* suppressors, *sur2-1* mutant and wild type (WS) were surface sterilized and germinated in sterile medium in the light. Up to 4 days after germination (d.a.g) all the mutant seedlings showed a phenotype very closed to WT phenotype. From 5 to 7 d.a.g., all suppressors showed flat cotyledons as in wild type and light green hypocotyls as in *sur2-1* mutant. Development of 266 was similar to wild type showing comparable hypocotyl and root length, whereas 420 presented shorter root than the wild type and *sur2-1* mutant. Hypocotyl size of 494 was intermediate to *sur2-1* mutant and wild type, but root size showed a tendency to be shorter than in these. The mutant 677 presented half the length of wild type hypocotyl and shorter root than wild type (Figure 1A and1B).

None of the suppressors developed adventitious roots in light as the wild type seedlings. All the seedlings developed lateral roots; *sur2-1* mutant presented twice more lateral roots than wild type and mutant 266 had 1.4 times more than the wild type, whereas the other suppressors showed fewer lateral roots than wild type (Figure 1C). Similar results were obtained when wild type, *sur2-1* mutant and suppressors where grown on horizontal plates i.e. the root was growing in the agar and not only on top of it (data not shown). In addition, only *sur2-1* mutant was capable of developing adventitious roots in light with 25% of the seedlings showing one to three roots.

Seedlings were grown in the dark to analyze hypocotyl growth kinetics, and as expected, *sur2-1* mutant showed shorter hypocotyl than wild-type. The 266 suppressor mutant grew as wild-type, whereas the 420 and 494 were comparable to *sur2-1* mutant (Figure 2). Mutant 677 is female sterile and only the progeny of heterozygous plants could be analyzed. Since it was not possible to identify the phenotype of the homozygote mutant in a segregating population, in the dark, we concluded that mutant 677 has a *sur2-1* mutant phenotype in the dark. Seedlings of all suppressors developed normal apical hook in the dark except for 494 that exhibited opened cotyledons from the fourth day (Figure 3).

Adventitious root development was examined in seedlings etiolated in the dark until their hypocotyl reached 5 to 6 mm and then transferred to the light for 7 days. The *sur2-1* mutant had in average 14 adventitious roots and the wild type an average of 1.5. Mutant 266 developed 50% less adventitious roots than the *sur2-1* mutant; mutants 420 and 494 were almost wild-type phenotype and mutant 677 had no adventitious roots (Figure 4A). The lateral root formation at these conditions presented a similar profile to the one observed when the plants were grown directly in light (Figure 4B). Since mutant 677 did not develop adventitious roots on intact seedlings we checked whether it could

regenerate adventitious roots from the hypocotyl after removal of the main root. Seven-day old seedlings from wild type, *sur2-1* and *677* grown in the light had the main root removed, were transferred *in vitro* on fresh medium and let to root in light for 7 additional days. In these conditions 677 suppressor could regenerate adventitious roots but 3 times less then wild type and *sur2-1* seedlings (Figure 5).

IAA quantification

To determine whether the suppressor mutations would affect the endogenous auxin levels in the *sur2-1* background, liquid chromatography-multiple reaction monitoring-mass spectrometry analyzes were performed. For measurements, homozygous seed stocks of wild type (WS ecotype), *sur2-1* mutant and suppressors (for 677 it was heterozygous, but just the seedlings with 677 phenotype were harvested) were and grown in light for 9 days.

Comparing the entire seedlings, the suppressors appear to be involved in the auxin homeostasis and/or synthesis. The 420, 494 and 677 suppressors showed free IAA level similar to the one in *sur2-1* mutant, but the content of conjugated auxin was significantly lower in 420 and 494 (Figure 6). In the mutant 266 the free auxin and conjugated IAA contents were back to the wild-type levels (Figure 6).

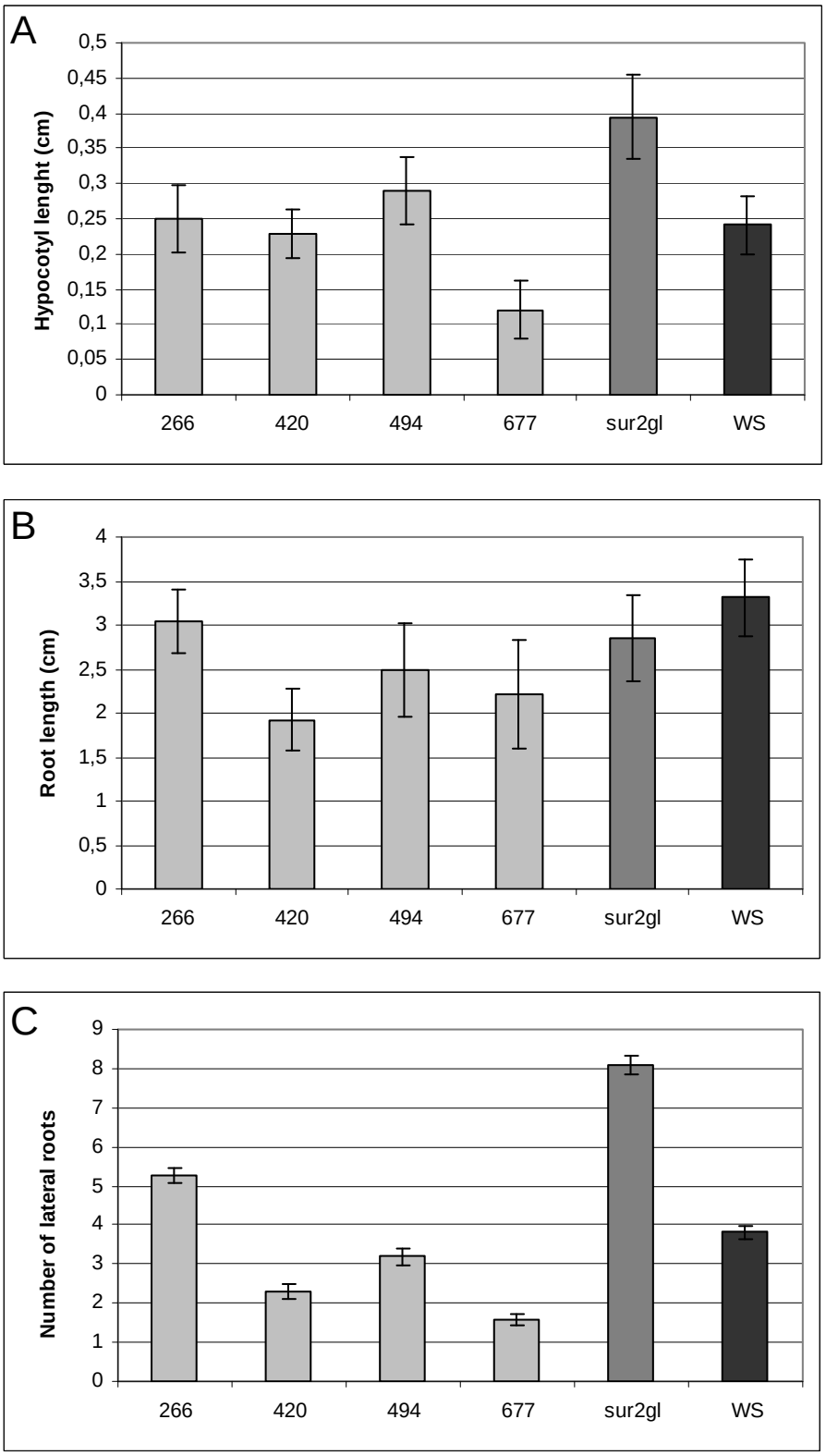

Figure 1: Phenotypic characteristics of *sur2-1* suppressors 7 days after germination on light compared to wild-type (WS) and *sur2* mutant. (A) Hypocotyl length. (B) Root length. Error bars indicate SD. (C) Number of lateral roots. Error bars indicate SE.

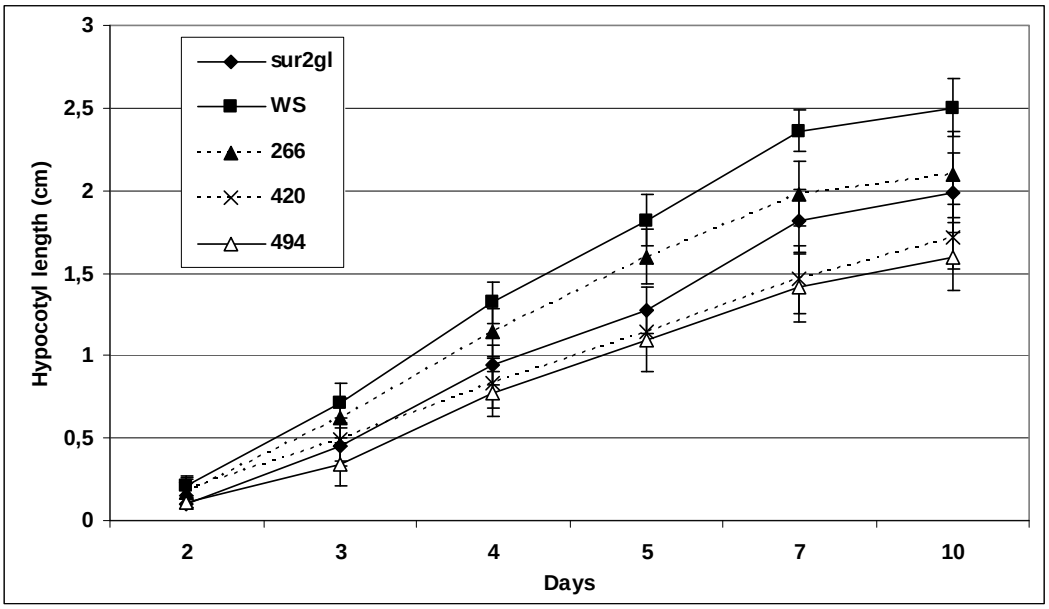

Figure 2: Hypocotyl length of wild-type (WS), *sur2-1* mutant and *sur2-1* suppressors grown in vitro in the dark. The hypocotyls were measured at different time points. Error bars indicate SD.

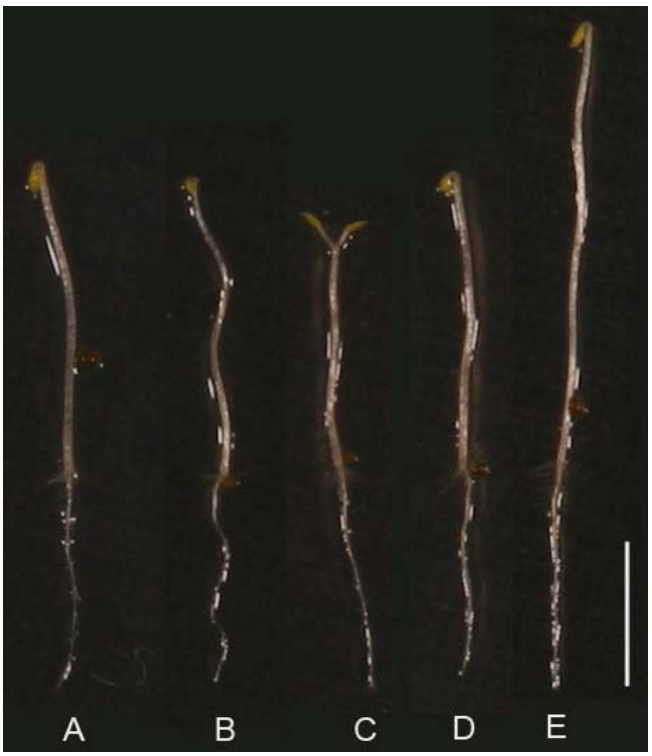

Figure 3: Phenotype of in vitro dark grown seedlings with 4 days. (A) 266. (B) 420. (C) 494. (D) *sur2-1*. (E) WS.

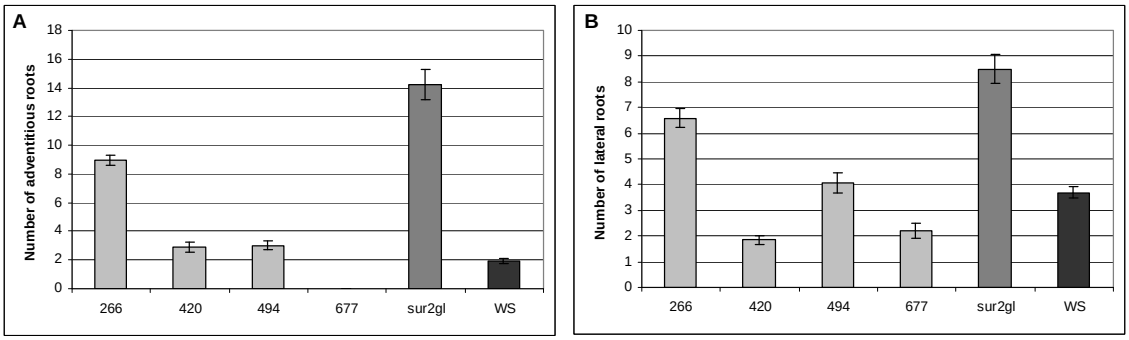

Figure 4: Phenotypic characteristics of *sur2-1* suppressors compared to wild-type (WS) and *sur2-1* mutant Seedlings were germinated and grown in dark until the hypocotyls reached 5 to 6 mm then transferred to light for 7 days. (A) Number of adventitious roots. (B) Number of lateral roots. Error bars indicate SE.

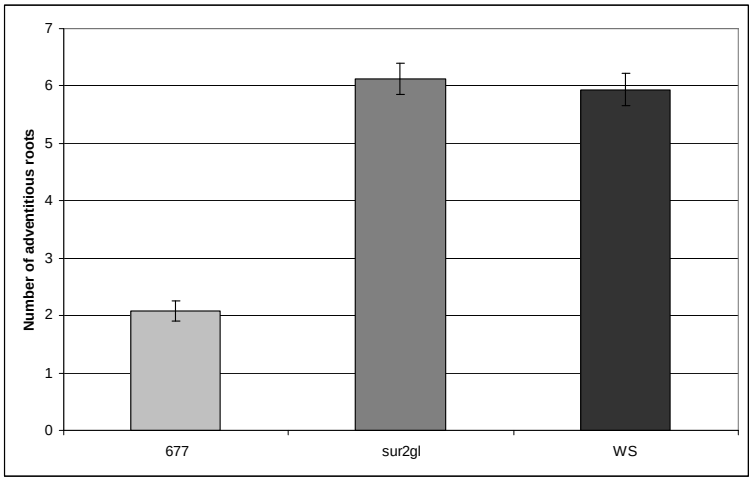

Figure 5: Number of adventitious roots formed after 7 days on light when the main root of wild-type (WS), *sur2-1* mutant and 677 suppressors were removed at 7 days germination in light. Error bars indicate SE.

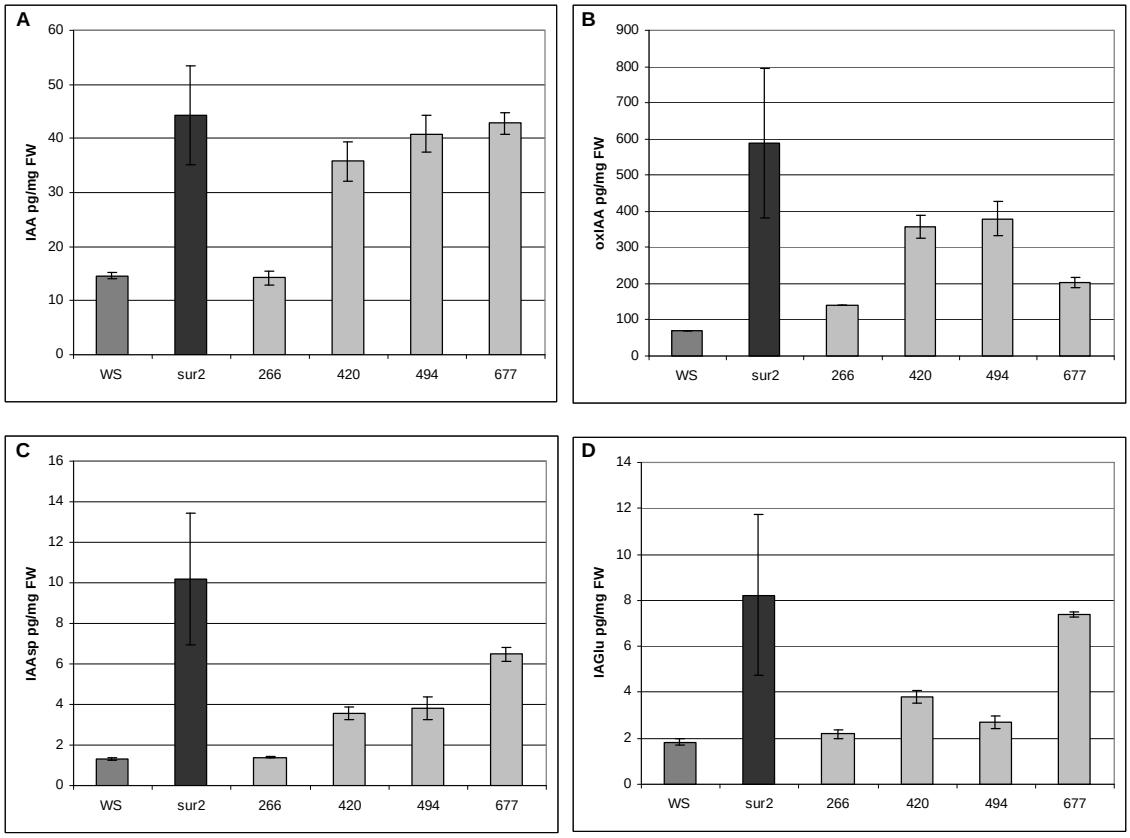

Figure 6: Free and conjugated IAA content in entire seedlings of wild-type (WS), *sur2-1* mutant and *sur2-1* suppressors after 7 days germinated in light. (A) IAA content. (B) oxIAA content. (C) IAAsp content. (D) IAGlu content. Error bars indicate SD.

Discussion

Auxin plays a central role in controlling adventitious rooting (Blakesley 1994). Indeed, *sur2-1* mutant over-produces auxin and spontaneously produces adventitious roots (Delarue et al. 1998), what makes this mutant a very useful tool to study the role of auxin in adventitious rooting. For that, *sur2-1* mutant was mutagenized and suppressors mutants were identified based on the number of adventitious roots produced being lower than in *sur2-1* mutant and possibly similar to wild type. This approach was supposed to lead to the identification of new genes either involved in the regulation of auxin homeostasis or in the regulation of auxin signaling. So far 12 suppressor mutants have been isolated. The

characterization of 4 of them is described in this article. All these suppressors develop fewer adventitious roots than *sur2-1*, but each of them with singular characteristics.

When grown directly in the light the suppressor mutant 266 is close to wild type: same hypocotyl and root length but slightly bigger cotyledons (data not shown). Nevertheless after being etiolated and transferred to light it produces more adventitious roots than the wild type but significantly less than *sur2-1* single mutant. This would suggest that similarly to *sur2-1* it would still produces more auxin than the wild type. Interestingly when free and conjugated IAA contents were measured in entire seedlings of mutant 266 they turned back to the wild-type levels. The mutation, therefore affects a gene related with auxin biosynthesis. Since 266 is a recessive mutation, presumably acting as a loss-of-function mutation, it could be suggested that 266 wild-type protein acts in the up-regulation of auxin biosynthesis pathway. Although mutant 266 has wild-type levels of auxin, it still develops 4 times more adventitious roots than the wild type. This can be explained either by an increase in the sensitivity to auxin or to a different compartmentation of the hormone. The auxin content was measured using entire seedlings; therefore the quantification of free and conjugated auxin in the apical part and root could bring some more information. Moreover, the formation of adventitious roots in mutant 266 can also be related to an alteration in the interaction between light and auxin transport, as this difference just appears after the period of etiolation.

The 420 suppressor has an intermediate phenotype when compared do *sur2-1* mutant and wild type. Morphological parameters show seedlings smaller than wild type and *sur2-1* mutant when submitted to etiolation, but when they are grown directly in light the hypocotyl looks like wild type and the cotyledons are fully expanded as in WT. But the root is shorter and the number of lateral roots is lower than that of *sur2-1* and WT.

Considering the number of adventitious roots, the mutant develops similar number to wild-type; consequently it could be expected a decrease in the auxin content. However, free IAA content is equivalent to the background genotype *sur2-1* and the conjugated forms are decreased compared to *sur2-1* mutant, suggesting that the mutation could affects the auxin conjugation pathway. Because the number of adventitious roots in the mutant 420 is similar to wild type it can be suggested that the mutation 420 affects auxin sensitivity in addition to its putative role in the regulation of auxin conjugation. Likewise, 494 suppressor presents similar phenotype and auxin contents to 420 suppressor, implying related role for 494 protein.

Morphological parameters of suppressor 677 show that its hypocotyl is shorter than in wild type and *sur2-1* mutant when grown directly in light as well as after an etiolation period. Mutant 677 does not develop any adventitious root in entire seedlings although it is able to regenerate adventitious roots when the main root is removed; they are fewer than in WT or *sur2-1* mutant treated in the same conditions. These results suggest that the mutation could affect auxin transport. Interestingly the silencing of *PIN1* gene in rice, involved in polar auxin transport, resulted in inhibition of adventitious root formation (Xu et al. 2005). Since, 677 suppressor has similar free and conjugated auxin contents than *sur2-1* mutant, the 677 mutation might affect auxin sensitivity or response.

Considering the lateral root formation in the conditions presented here for 266, 420 and 494 suppressors, *sur2-1* mutant and wild-type it would be possible to consider that they have a similar behaviour with respect to adventitious root formation, showing that these mutations affect both developmental processes and that auxin is playing a wider role in this suppressors. On the other hand, 677 suppressor presents less lateral roots than wild type, but still produces it, whereas there is no adventitious roots. In this case, the mutation

affects differently these developmental processes, playing an important role in the adventitious root formation and how it is affected by auxin.

All the characterization was made in the *sur2-1* mutant background that presents an overproduction of auxin due a partial silencing of the indol-glucosinolate pathway (Barlier et al. 2000). It is still necessary to characterize the single suppressor mutation, after backcrossing out the *sur2-1* mutation in order to better understand the function of the mutated genes.

Supplementary experimental data – Preliminary results

Light quality experiments

The growth condition is the same as described above. For experiments in different light qualities, the plates were placed under continuous irradiance at a constant temperature of 20°C. Blue light (468 - 470nm, 38 $\mu mol.m^{-2}.s^{-1}$) was provided by Kingbright L-7113QBC-D. Red light (660 nm, 24 $\mu mol.m^{-2}.s^{-1}$) was provided by Kingbright L-1513SRC-F. Far-red light (735 nm, 9 $\mu mol.m^{-2}.s^{-1}$) was provided by Epitex Inc. L735-03AU (Techmark).

Response to light quality

A characterization of the behavior of these suppressors in different light qualities indicated that they respond differently to them, confirming the link between light and adventitious root formation and suggests that the new genes are also likely to act at the cross talk of auxin and light signaling. The suppressor 677 had the behavior altered for all the qualities of light presenting a short hypocotyl for all. Whereas 420 and 494 presented the shorter hypocotyl just in blue light, similar to *sur2* mutant, 266 suppressor showed the

same behavior as the wild-type (Figure 6). It is necessary to highlight that the seedlings from the 494 suppressors were the only ones that displayed the hypocotyl with a high number of adventitious roots in all different qualities of light. Moreover, the results with red light for *sur2* mutant were different from the expected (Hoecker et al. 2004), probably due a problem in the red light lamps. In the same way, it is suggested that 266 have a behavior similar to wild-type while 420 and 494 are closer to *sur2* mutant performance.

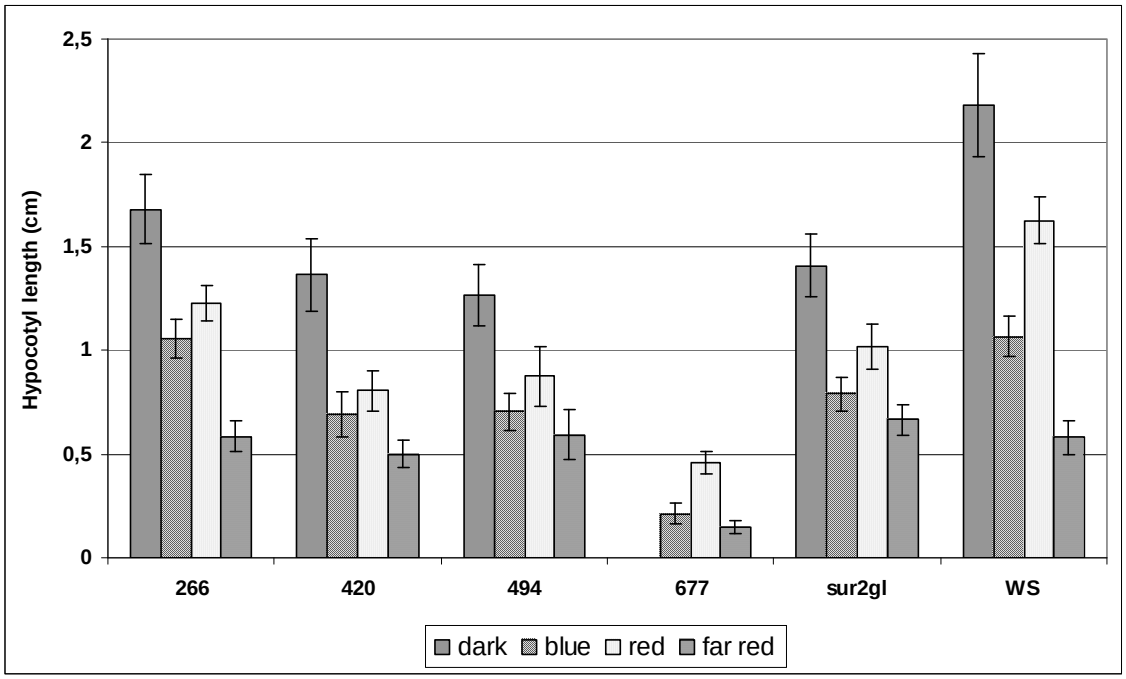

Figure 6: Hypocotyl length of wild-type (WS), *sur2* mutant and *sur2* suppressors grown in different light conditions for 7 days. Error bars indicate SD. For growth conditions see Supplementary experimental data.

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CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Os experimentos conduzidos com mini-estacas obtidas a partir dos sistemas de cultivo hidropônico indicaram que é possível utilizar os marcadores bioquímicos propostos na identificação das fases do processo de enraizamento adventício. As análises bioquímicas mostraram um perfil consistente e coincidente para ambos os sistemas de cultivo, principalmente para atividade de peroxidase. Os perfis de atividade de peroxidase e conteúdo de compostos fenólicos se mostraram mais importantes do que os valores obtidos *per se* para a resposta morfológica final de enraizamento. Os resultados de enraizamento foram similares nas mini-estacas oriundas de ambos os sistemas. Desta forma, a escolha pelo sistema de cultivo pode ser feita de acordo coma conveniência operacional e os custos de produção.

Estudos futuros com diferentes clones e estações serão necessários para confirmar o uso dos marcadores bioquímicos de enraizamento avaliados neste trabalho. A identificação das fases de enraizamento permitirá uma melhor modulação do processo de enraizamento adventício de *Eucalyptus* em escala comercial empregando a estratégia de mini-jardins, especialmente no que tange à nutrição mineral de minicepas e miniestacas.

Quanto à eficácia das diferentes fontes de nitrogênio no enraizamento adventício de *E. globulus*, é possível dizer que nitrato é a fonte preferencial a ser utilizada. No entanto, como as plantas enraizadas nas diferentes fontes de N (uréia, nitrato de amônia e nitrato de sódio) apresentaram o mesmo desempenho na aclimatação *ex vitro* é possível escolher a fonte de acordo com os custos de operação. Uréia pode ser uma alternativa interessante

devido ao baixo custo, menor restrição de comercialização e possivelmente por estimular a produção de urease, a qual, além de atividade ureolítica, ao menos em espécies de Fabaceae, possui propriedades de defesa contra insetos e fungos patogênicos. O uso de glutamato resultou em um sistema radicular mais denso, com raízes mais curtas, porém não mais ramificadas, indicando que a rizogênese adventícia em *Eucalyptus globulus* responde a esta fonte de N orgânico em parte de forma similar ao sistema radical embrionário.

Experimentos com as diferentes fontes de nitrogênio em mini-estacas de *Eucalyptus* no sistema de enraizamento comercial podem trazer vantagens na produção de mudas modulando a arquitetura da raiz e assim produzindo um sistema radicular mais robusto.

O estudo com supressores de *sur2* permitiu a identificação e caracterização de quatro supressores envolvidos no mecanismo de regulação da biossíntese e homeostase de auxina. Como a caracterização foi feita no background de *sur2* ainda é necessário caracterizar cada mutação no tipo selvagem para a melhor compreensão da função dos genes mutados. A caracterização destes mutantes propiciará um quadro mais detalhado da relação entre metabolismo e sensibilidade auxínicos e resposta rizogênica em *Arabidopsis thaliana*.