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Effect of Indole-3-Butyric Acid on Root Formation in *Alnus glutinosa* Microcuttings

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A study of the in vitro rooting process in mature alder (*Alnus glutinosa* (L.) Gaertn.) shoots is described. Microcuttings from shoots cultured in vitro were transferred to a half-strength Woody Plant Medium containing 0 or 0.1 mg l^{-1} indole-3-butyric acid (IBA) for 0 to 7 days. The presence of IBA in the medium increased the rooting percentage, number of roots, percentage of lateral roots, and length of the shoots. Histological studies were carried out with shoots treated with 0 or 0.1 mg l^{-1} IBA for 7 days. According to these criteria, treatment with IBA for 2–3 days proved to be the most successful. In both treatments, substancial reactivation of cell division was observed at the base of the shoots after 1 day. Some cambial zone and adjacent phloem cells became dense cytoplasm, having nuclei with prominent nucleoli. The first cell divisions were also observed at this time. In the treatment with IBA (0.1 mg l^{-1} for 7 days), meristemoids became individualized, consisting of densely staining cells, by day 3. Identifiable conical shaped root primordia with several cell layers were visible after 4–5 days. Roots with an organized tissue system emerged from the stem after 6 days in the IBA-treated shoots. Meristemoid formation was delayed until the fourth day and root emergence until the eight day in the control treatment (no IBA).

Keywords alder, histology, mature trees, rooting
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1 Introduction

The productivity of forest plantations can be enhanced by traditional breeding techniques involving the identification of superior trees with desired traits and the production of offspring with these traits. However, an alternative method of tree improvement, through vegetative or clonal propagation, has the advantage of capturing all the genetic superiority without involving any gene segregation. Clonal material can be propagated via rooted cuttings or through biotechnological methods, as tissue culture techniques provide appropriate tools for rapid production of clones to regenerate trees with desired traits (Ballester et al. 2009).

Adventitious root formation is a key step in plant propagation. From an economic point of view, rooting is an important phenomenon in horticulture, agriculture and forestry. From a scientific point of view, rooting is a highly interesting developmental pathway. It is one of the three main routes of organ regeneration (along with shoot formation and somatic embryogenesis). In all three routes, cells undergo an apparent reversal of differentiation and again become meristematic. The formation of adventitious roots from cuttings has been known since ancient times and is used for vegetative propagation of elite plants that have either been selected from natural populations or obtained in breeding programs (De Klerk 2002). The rooting capacity varies with the genotype, and is generally lower in woody species than in herbaceous species (Hackett 1988). Efficient rooting treatment yields a high percentage of rooted shoots and a high-quality root system. The latter involves number of roots per shoot, length of roots and absence of callus formation, and determines the performance after planting in soil (De Klerk et al. 1997).

One of the main achievements in the study of adventitious rooting has been the recognition of successive phases, generally called induction, initiation and expression (Gaspar et al. 1997). Auxins have been known to be involved in the process of adventitious root formation for a long time (Haissig and Davis 1994), and the interdependent physiological phases comprising the rooting process are associated with changes in endogenous auxin concentrations (Heloir et al. 1996).

Some plants regenerate roots on cuttings spontaneously. However, in these plants, endogenous auxin produced in the apex and transported basipetally to the cut surface acts as trigger; removal of the apex reduces both the level of endogenous auxin in the basal portion of a cutting and the number of regenerated roots. Moreover, in these plants, application of auxin strongly increases the number of roots (Nordström et al. 1991, Liu and Reid 1992). In many species, application of exogenous auxin is required to achieve rooting (Díaz-Sala et al. 1996, Henrique et al. 2006, Ballester et al. 2009, Hunt et al. 2011) and auxin may be more beneficial for increasing rooting in difficult-to-root rather than in easy-to-root cuttings (Hunt et al. 2011). It is well known that exogenously applied natural or synthetic auxins favour rooting, and there is evidence that this hormone is the most effective inducer of the process (Lyndon 1990). Application of auxin, particularly indole-3-butyric acid (IBA), is one of the most common and effective means to enhance rooting of cultures (Hartmann et al. 1990, Leaky 2004, Han et al. 2009). Cuttings often respond optimally to auxin application during the dedifferentiation phase of adventitious root formation, and may be less responsive to earlier or later application (Smith and Thorpe 1975, De Klerk et al. 1995, De Klerk et al. 1999, Luckman and Menary 2002). It was recognized early, just after the discovery of indole-3-acetic acid (IAA) and its rhizogenic action, that at concentrations that are favorable for rooting, auxin blocks the growth of roots (Thimann 1936). These data show that various phases in rooting may have different or even opposite hormone requirements.

Many basic studies on rooting are now carried out in vitro. This has several advantages as tissue culture conditions facilitate the administration of auxins and other compounds, prevent microbial degradation of applied compounds, allow addition of inorganic nutrients and carbohydrates (so that secondary causes of poor root formation, e.g. low photosynthetic capacity of cuttings, are avoided), and enable experiments with small, simple explants, such as stem slices (De Klerk et al. 1999).

Histological studies of adventitious rooting have been carried out i) to identify the cells/tissues from which the roots originate (and hence

are the target for auxin and other root-inducing factors); ii) to establish whether preformed root primordia are present or a site for root primordium formation must be created; iii) to determine if low rooting capacity is related to anatomical features (e.g. the presence of a continuous ring of sclerenchyma); iv) and to relate physiological and biochemical data with the anatomical stages of root formation (Altamura 1996).

The ecological value of alder has been widely recognized (Cech 1998). *Alnus glutinosa* (L.) Gaertn. has considerable lanscape value along waterways, plays a vital role in riparian ecosystems, and the root system stabilize riverbanks (Gibbs et al. 1999). Unfortunately, this species has declined drastically in number as a result of disease caused by the fungus *Phytophthora alni.* In this context, micropropagation methods would be beneficial for the large-scale multiplication, improvement and conservation of the species.

In this study, we analyzed the effect of IBA application for different lengths of time on adventitious root formation and the ontogeny of in vitro rooting using microshoots from a mature *A. glutinosa* tree. Analysis of sequential series of histological preparations may provide information leading to a better understanding of this rooting process from the very first stage (induction) to complete development of the root. Anatomical descriptions of this in vitro process have not previously been reported for *Alnus* spp.

2 Material and Methods

2.1 Plant Material and Culture Conditions

Shoot cultures were established in vitro from the crown of a 30 to 40-year-old *A. glutinosa* tree. Branch segments (20–25 cm long) were set upright in moistened perlite and forced to flush axillary shoots, in a growth cabinet at 25 °C and 80–90% relative humidity under a 16-h photoperiod (90–100 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps). After 3–4 weeks, newly sprouted shoots (3–10 cm in length) were used as a source of explants. Flushed shoots were surface sterilized by immersion for 20 s in 70% ethanol and for 5 min in a 0.6% solution of free chlorine (Millipore Tablets®) containing 2–3 drops of Tween 80®, and rinsed three times in sterile distilled water. Apical and nodal explants (8–10 mm) were cut from the shoots and used as initial explants for establishing shoot cultures. Shoots developed in the establishment stage were used for clonal shoot multiplication. Apical and nodal segments (8–10 mm) were subcultured in 500-ml glass jars containing 70 ml of Woody Plant Medium (WPM; Lloyd and McCown 1980) supplemented with 20 g l^{-1} glucose, 0.7 g l^{-1} Bacto Difco agar, 0.1 mg l^{-1} N^6-benzyladenine (BA) and 0.5 mg l^{-1} indole-3-acetic acid (IAA). The multiplication cycle consisted of a 9-week period with transfer every three weeks to fresh medium. Cultures were maintained in a growth chamber with a 16-h photoperiod (50–60 µmol m^{-2} s^{-1} provided by cool-white fluorescent lights), and 25 °C day/20 °C night temperature (photoperiodic conditions). These standard culture conditions were also applied to the cultures described below. The pH of all media was adjusted to 5.7 prior to autoclaving at 115 °C for 20 min.

2.2 Rooting Treatments

Shoots (15–20 mm in length) were harvested at the end of the multiplication period (9 weeks). The shoots were placed on WPM medium with half-strength macronutrients and supplemented with 20 g l^{-1} glucose and 0.7g l^{-1} Bacto Difco agar (rooting medium). In a first set of experiments, shoots were placed on rooting medium with either 0 (Control) or 0.1 mg l^{-1} indole-3-butyric acid (IBA). After 7 days, shoots were transferred to auxin-free medium of the same composition for the remainder of the 4-week rooting period.

In view of the results of the histological study, we decided to determine the optimal duration of treatment with IBA. Shoots were placed on rooting medium supplemented with 0.1 mg l^{-1} IBA for 1, 2, 3, 5 or 7 days, and were then transferred to auxin-free medium for the remainder of the 4-week rooting period. A control treatment without IBA was included. The experiments were carried out in 300-ml glass jars, each containing 60 ml of rooting medium.

2.3 Histological Analysis

For histological examination, basal segments (0.5 cm) were collected from the shoots in the control treatment and the treatment with 0.1 mg l^{-1} IBA for 7 days. Samples were collected daily from Day 0 to Day 10 after the start of the experiment. Six samples were collected every day, and the entire experiment (except for day 0) was repeated twice. Explants were fixed in a mixture of formalin, glacial acetic acid and 50% ethanol [1:1:18 (v/v/v)], dehydrated through a graded *n*-butanol series and embedded in paraffin wax (Jensen 1962). Sections (10 µm) were cut and stained with either safranin-fast green for general examination or with periodic acid-Schiff (PAS)-naphtol blue-black, which is commonly used to reveal total insoluble polysaccharides and total protein content of the cells (O'Brien and McCully 1981). Photographs were taken in a Nikon-FXA microscope equipped with an Olympus DP71 camera.

2.4 Data Collection and Statistical Analysis

Ten shoots were used for each treatment, and the experiments were repeated three times (30 shoots per treatment). In the 1st experiment the rooting percentage was determined at the end of the 4-week rooting period, while in the 2nd experiment root emergence was monitored daily during the 4-week rooting period. In both experiments, the following rooting characteristics were recorded at the end of the rooting period (4 weeks): mean root number, mean root length, percentage of rooted shoots with lateral roots, and the mean shoot length. The results were statistically analyzed by application of a Student's test (MedCalc version 10.3, Marekarke, Belgium) and differences were considered significant at $p \leq 0.05$.

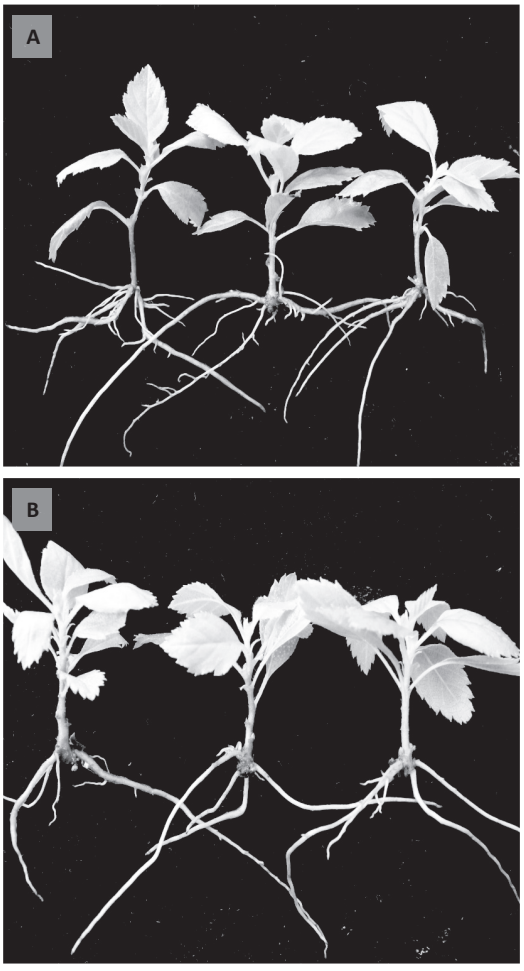

Fig. 1. Adventitious root formation in *Alnus glutinosa* microcuttings rooted in medium containing IBA 0.1 mg l^{-1} for 7 days (A) or without IBA (Control, B). Development of lateral roots was lowest in the control treatment.

3 Results

3.1 Rooting Treatments

Although the material used was obtained from the crown of an adult alder tree, the rooting percentage was high in both treatments, 76.7% in the control and 90% in the IBA treatment, respectively. The mean number of roots that developed in each shoot increased from 1.9 to 3.0 in the presence of IBA, which also favoured root elongation. The mean length of the roots was 26.4 mm in the control specimens, and 35.7 mm in the shoots treated with auxin. In both treatments, the roots were reddish in colour and no callus was observed at the base of the shoots. In the shoot treated with auxin 63% of the roots developed lateral roots, whereas in the control only 43% of

Table 1. Effect of the duration of treatment (1–7 days) with 0.1 mg l^{-1} IBA on rhizogenesis in *Alnus glutinosa* microcuttings (15–20 mm long). Data were recorded after 4 weeks.

Days	Rooting (%)	No. roots	Length of the longest root (mm)	Lateral roots (%)	Shoot length (mm)
Control[+]	70.0 ± 0.00a[*]	2.2 ± 0.06a	29.2 ± 1.81a	47.6 ± 4.73a	23.1 ± 0.50a
1d	86.7 ± 3.33b	2.5 ± 0.24a	36.4 ± 3.62bc	80.6 ± 4.24cd	27.8 ± 0.67bc
2d	100.0 ± 0.00d	3.0 ± 0.13b	37.2 ± 0.58c	85.0 ± 3.33d	27.4 ± 1.04bc
3d	96.7 ± 3.33cd	3.1 ± 0.15b	37.6 ± 2.88c	85.9 ± 7.06d	28.3 ± 0.64bc
5d	93.3 ± 3.33bc	2.9 ± 0.06b	32.3 ± 1.05ab	74.8 ± 4.12c	28.7 ± 0.43c
7d	96.7 ± 3.33cd	2.9 ± 0.09b	32.9 ± 0.96b	58.5 ± 2.13b	27.2 ± 0.49b

[+] In the control treatment no auxin was added to the medium.
[*] In each column, values (means ± SE) followed by different letters are significantly different ($p \leq 0.05$).

the roots developed lateral roots. The presence of a greater number of lateral roots increased the length of the shoots to 27.3 mm, in comparison with the control in which mean shoot length was 21.2 mm. In both treatments, the shoots had a healthy appearance, with well-developed green leaves and actively growing green apices (Fig. 1).

On the basis of this preliminary experiment and the histological study of the formation of adventitious roots on the microcuttings, we carried out further studies to investigate how the length of time that the microshoots remain in medium containing IBA (1–7 days) affects the process. The data show that the presence of auxin in the medium favoured rooting of microcuttings (Table 1). The best results were obtained with the microcuttings placed in medium containing auxin for 2–3 days, which coincided with the appearance of the meristemoids observed in the histological study. Independently of the duration of the treatment, the presence of IBA in the medium increased the number and length of the roots, the best results were obtained when the auxin was added to the medium in the first three days. The presence of auxin in the medium also favoured the development of lateral roots. In the shoots treated with IBA for 3 days, 85% of the roots developed lateral roots. Development of lateral roots and the length of the roots were negatively affected by the treatment with IBA for more than 3 days. As in the preliminary experiment, shoot development was enhanced by treatment with IBA, possibly due to a greater adsoption capacity conferred by the greater development of lateral roots. Root emergence was also favoured by the presence of auxin in the rooting medium (Fig. 2); in the control treatment the first roots were visible in 6.7% of the shoots from day 8, whereas in the treatments including auxin, root emergence occurred on day 6, and the rooting percentage ranged between 13.3 and 20%. In control treatment, the process was completed by around day 20, with a maximal rooting percentage of 70%, whereas in the different treatments with IBA, the highest rooting percentage (86.7–100%) was reached between day 16 and 18. After 14 days, the rooting percentage ranged between 50% for the control and 83.3–93.3% for the different treatments including IBA, which illustrates the greater speed of root development in the presence of IBA.

3.2 Histological Analysis

At the time of excision for root initiation, the anatomy of the microshoots was typical of dicotyledons stems (Fig. 3A). Transverse sections revealed the epidermis surrounding the cortex, which mainly consisted of large highly vacuolated parenchyma cells; the largest of these cells were situated close to the vascular system or the epidermis. The inner layer of cortex formed an ill-defined starch sheath. The vascular system consisted of collateral bundles of xylem and phloem that formed a continuous ring around the pith. The xylem was formed by isodiametric cells with thick walls, which gradually increased in size from the pith towards the cambium. The xylem was regularly crossed by strands of parenchyma cells, which formed the medullary rays. A cambial zone with three to four layers of flattened vacuolated cells was observed between the xylem

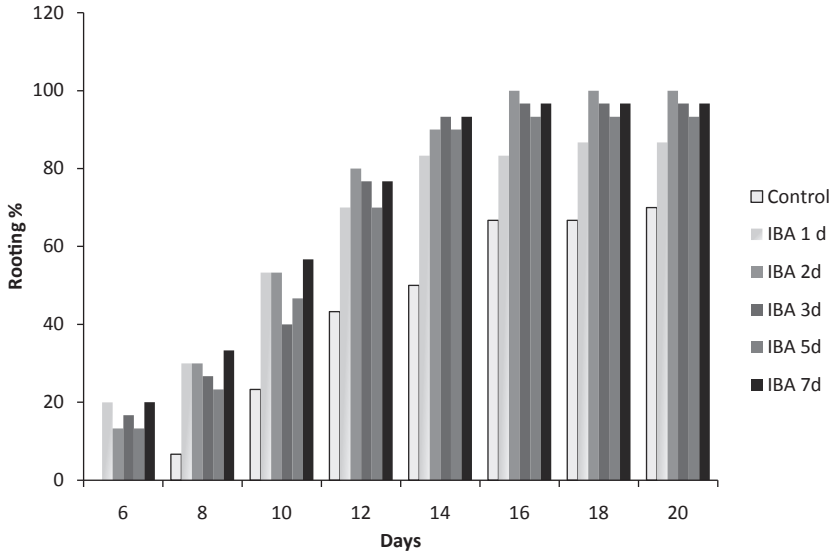

Fig. 2. Rooting percentage in alder microcuttings in relation to time in rooting medium containing IBA (0.1 mg l^{-1}) (1–7 days). The control treatment did not contain IBA.

and phloem. The phloem consisted of sieve elements, companion cells and parenchyma cells, and was surrounded by groups of sclerenchyma fibres separated by zones of parenchyma cells. The pith was consisted of highly vacuolated parenchymatic cells containing small starch grains.

3.2.1 IBA Treatment

One day after excision, some cells in the cambial zone and adjacent phloem showed signs of meristematic activity. These cells were less vacuolated, contained nuclei and prominent nucleoli, and stained more intensely. The first meristematic divisions were observed in these zones, mainly in the periclinal plane. The amount of starch also increased, mainly in the cortex, pith and in the cells that formed the vascular rays.

By 48 hours, the number of dedifferentiating cells and mitotic figures had increased (Fig. 3B), and short radial rows of three-four successive dedifferentiated cells were observed in the phloematic region. Dedifferentiation was evident in some cells of a variety of tissues including cambium, inner cortex and vascular parenchyma. Some cells in the xylem also displayed signs of meristematic activity, but no divisions were observed. The amount of starch in the cells decreased sharply.

Three days after the onset of treatment, regions of densely stained cells appeared, as a result of active cell division. Numerous periclinal divisions were observed together with some oblique and anticlinal division of cells located in the cambial zone, and in the outermost layers of the phloematic region. These divisions produced small groups of isodiametric, densely-stained cells with a prominent central nucleus containing a large nucleolus (Fig. 3C). These isodiametric cells resembled cells in meristems, and were assumed to make up the organization of meristemoids. The meristemoids showed no overall polarity at this stage and generally developed in the phloem region close to the sclerenchyma fibre ring. They were initially formed by periclinal cell division and the involvement of dedifferentiated cells of the surrounding parenchyma. Activity was restricted to the basal 1000–1200 µm of the microcuttings, and transverse sections above the meristematic activity were similar to those of the control.

After 4–6 days, transverse sections showed that root meristemoids had differentiated, through polarization of cell division, into a typically dome-

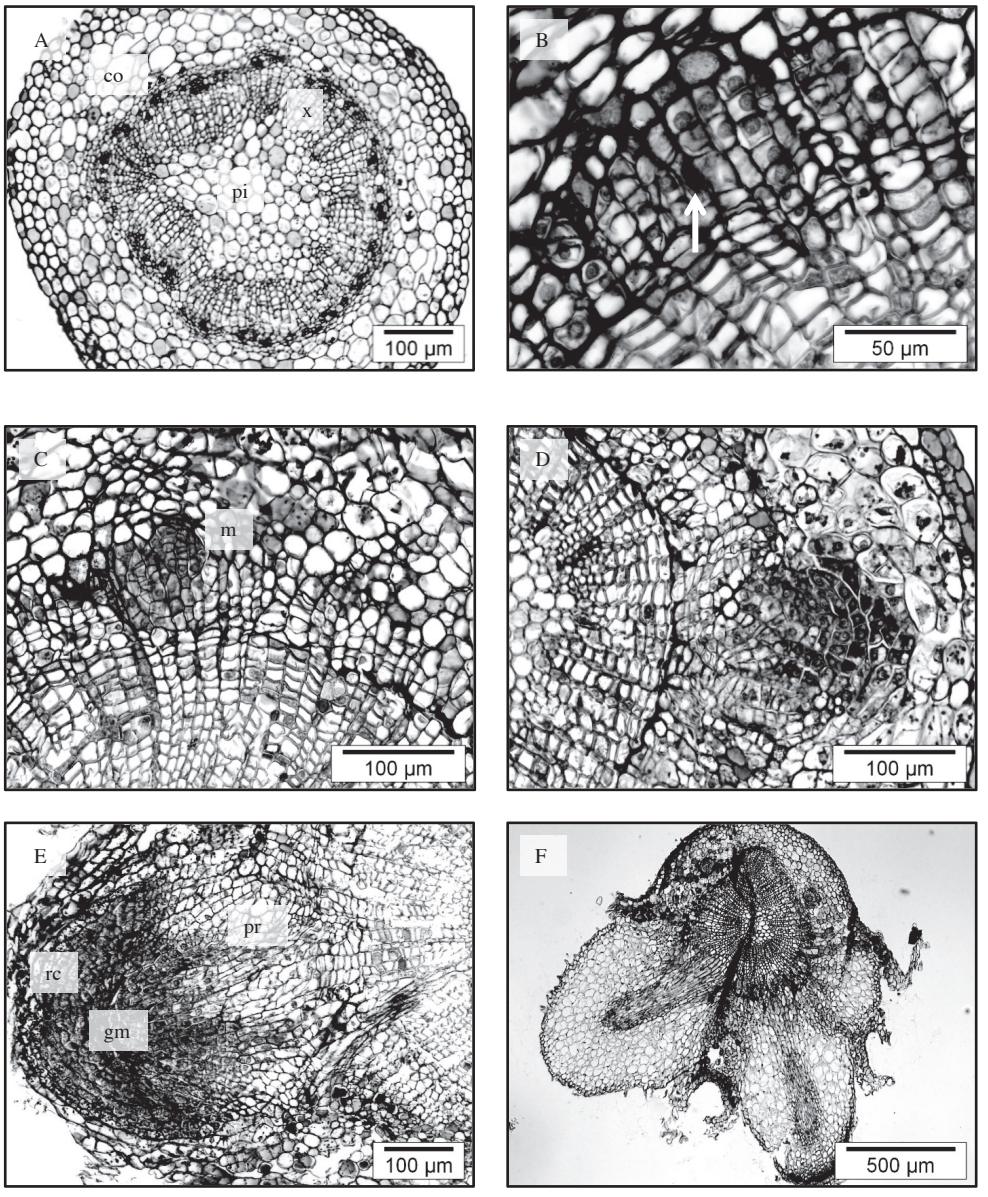

Fig. 3. Transverse sections of the stem base of alder microcuttings after root inducing treatment with 0.1 mg l^{-1} IBA for 7 days. A. Day 0. Anatomical structure at the time of excision (pi: pith, co: cortex, x: xylem). B. Day 2. Cell activation with enlarged nuclei and nucleoli in the cambial zone and adjacent phloem. Mitotic figures were observed (arrow). C. Day 3. More cells were involved in cell reactivation and a localized cluster of densely stained meristematic cells (meristemoid) (m) was observed. D. Day 4. Early root primordium showing a tendency to form dome-like structure. The distal end of the primordium passed through the ring of sclerenchyma fibres. Several starch grains were observed in the cortex close to the primordium. E. Day 6. Protuding root primordium showing the formation of procambium (pr), ground meristem (gm), and root cap (rc). F. Day 8. Emerging adventitious roots showing complete differentiation of the vascular system, which was continuous with that of the stem.

shaped root primordium that pushed through the sclerenchymatic fibre ring to reach the cortex (Fig. 3D). An increase in the starch content was observed in the cortex cells, especially those closest to the primordium. In the following hours, the root primordium started to differentiate internally, procambium and ground meristem becoming distinguishable from the apical meristem. Further divisions at the distal end of the apical meristem gave rise to a root cap consisting of two-three layers of cells that were more vacuolated than centrally located cells. Divisions continued in the cells behind the developing primordium, with differentiation of tracheary elements orientated perpendicular to the vascular systems of the main stem and the emergent root primordium. Root primordia were asynchronously formed, and the first adventitious roots emerged at the surface as early as 6 days after the IBA treatment (Fig. 3E). These had a developed vascular system which was continuous with that of the stem. In the following days, numerous roots had emerged from the base of microcuttings (Fig. 3F). All roots appeared to have developed normally.

3.2.2 Control Treatment

Formation of adventitious roots in the control treatment was similar to that already describe, although the time intervals differed. Thus, cell activation and the first divisions were also observed after the first 24 h, but formation of the meristemoids did not take place until day 4. Organization of the primordia was delayed until day 6 and the first roots emerged to the exterior on day 8. As observed in the microcuttings in the IBA treatment, the starch content increased during the first 24 hours, then decreased after 48 hours and increased again after day 6 in the cells in the cortex, close to the root primordia.

4 Discussion

Adventitious root formation is an important step in plant vegetative propagation and is mainly controlled by genetic, physiological, physical and chemical factors (De Klerk et al. 1999). The effects of plant regulators on rooting have been extensively examined in various plant species (Kevers et al. 1997, De Klerk et al. 1999, Steffens et al. 2006). Phytohormones, especially auxins, play an essential role in regulating root development. Auxins have long been known to be involved in the process of adventitious root formation (Haissig and Davis 1994), and the interdependent physiological phases comprising the rooting process are associated with changes in endogenous auxin levels (Heloir et al. 1996). Auxins have been shown to be effective inducers of adventitious roots in many woody species (Díaz-Sala et al. 1996, Pruski et al. 2005, Ballester et al. 2009, Vieitez et al. 2009).

Although the addition of auxins is not always essential for induction of rooting in young material of *Alnus* spp. (Périnet and Lalonde 1983, Tremblay and Lalonde 1984, Tremblay et al. 1984, Tremblay et al. 1986, Périnet and Tremblay 1987, Barghchi 1988), the presence of IBA in the rooting medium enhances the number of roots per shoot and reduces the time required to obtain the maximum percentage of rooting (Périnet and Lalonde 1983, Tremblay et al. 1986). In a study with adult material of *A. glutinosa*, Lall et al. (2005) observed successful rooting of shoots in medium without auxin, although the authors did not provide the corresponding data. They consider that the high content of auxin in this species is an adaptation to its natural habitat, which suffers from frequent flooding. Like other species that are subject to flooding in their natural habitats, alder is able to produce adventitious roots at the base of mature plants in response to inundation of the roots and lower stems (Gill 1975). In the present study, carried out with material obtained from the crown of an adult tree, the rooting percentage was high, even in the medium that did not contain auxin. However, inclusion of IBA in the medium increased the rooting percentage, number of roots per rooted shoot, and length of the roots; the roots also developed more lateral roots, which increased the overall length of the shoots. Lateral root formation is important for the establishment of root architecture in higher plants. Many classic and recent studies have shown that auxin plays a central role during lateral root development (Casimiro et al. 2003). The roles of auxin include the selection of specific pericycle cells,

and conversion of these into founder cells and determination of the spatial pattern during lateral root formation (Dubrowsky et al. 2008). In most studies carried out with *Alnus* spp., cuttings were usually maintained in medium with auxin until the end of the rooting period (3–4 weeks), with good results. In this study, the best results were obtained when the microcuttings were kept in the medium with auxin for 2–3 days. Barghchi (1988) reported that in *A. cordata* (Loisel.) Loisel., transfer of cuttings to medium without auxin after 10 days improved the rooting success. De Klerk et al. (1999) suggested that various phases in rooting may have different or even opposite hormone requirements. Thus, the auxin that is required to induce roots, also inhibits the outgrowth of root primordia, growth of roots and growth of cuttings. Most woody plants require a sequence of two rooting media. Thus, Naija et al. (2008) found that a period of 5 days in rooting medium with IBA was sufficient to induce 97% rooting in microcuttings of *Malus* × *domestica* Borkh. rootstock MM 106, and the exposure to IBA for periods longer than five days produced undesirable side effects on shoots, such as callus formation and leaf necrosis. Likewise, Metivier et al. (2007) indicated that the percentage of rooted shoots of *Cotinus coggygria* Mill. increased with increasing time of exposure to the IBA-containing medium, from 40% after one day of exposure, to 100% after 5 days in the presence of IBA.

Micropropagation rooting protocols have been developed for most species by testing different culture media and environmental conditions. However, most of these trials did not include histological studies, which provide a better understanding of tissue differentiation processes underlying rhizogenesis in microshoots from woody species. The duration and location of these events may vary between taxa and even between conventional and micropropagation material (Lowell and White 1986).

The present results showed that factors that induced root formation in microcuttings of alder did so by triggering the initiation of cell division, leading to formation of root primordia (root initiation) in regions where they did not exist previously. The first histological signs of the formation of adventitious roots in alder were the appearance of large nuclei and denser cytoplasm in the cambial zone and adjacent phloem cells. The early activation of cells by altered nuclear/nucleolar characteristics, altered staining properties of the cytoplasm and early cell division activity have been reported as the first cytological events leading to root primordia formation in a number of rooted in vitro cultures (Vidal et al. 2003, Naija et al. 2008, Ballester et al. 2009). In other studies of woody species, the site of origin has been located close to or in the vascular cambium (Harbage et al. 1993, Ballester et al. 1999, Vidal et al. 2003, Naija et al. 2008). The region of the tissue in which cells become activated is thought to depend partly on physiological gradients of substances entering the shoot from the medium, and on the presence of competent cells to respond (Ross et al. 1973).

In alder microcuttings, first discernible response to root induction occurred within 1 day, in both treatments (Control and IBA). Meristemoid formation occurred after 3 days in the IBA treatment, and was delayed until 4 days in the control treatment. Jasik and De Klerk (1997) observed that in apple slices not supplied with auxin, signs of activation were delayed until 96 h after the start of culture. De Klerk et al. (1999) reported that microcuttings are not very sensitive to auxin and cytokinin during the initial 24 h after the microcuttings have been taken. Dedifferentiation is thought to occur during this lag period, and the cells become competent to respond to the rhizogenic stimulus (auxin). Some previously activated cells then become committed to the formation of root primordia by the rhizogenic action of auxin in the induction phase. The time required for the initiation of meristematic activity in response to inductive treatment varies between species, with examples ranging from 1 day for apple rootstock 'Gala' (Harbage et al. 1993) to 8 days for *Camellia japonica* L. (Samartín et al. 1986). Since activation occurs only a few millimetres from the cut surface, wound factors have been suggested to play an important role, at least at the beginning of the rhizogenic process (De Klerk et al. 1999).

Different authors have observed variations in the starch content of cells during rhizogenesis (De Klerk et al. 1999, Li and Leung 2000, Megre et al. 2011). In alder, the starch content of the cells increased after the first 24 hours, and was most evident in the microcuttings treated with IBA. In

both treatments, the starch content decreased after the first 48 hours and then increased again after 4 or 6 days (IBA and Control, respectively), especially in the cortex cells close to the developing primordia. Histochemical studies in IBA-treated *Pinus radiata* D. Don hypocotyl cuttings showed that starch began to build up preferentially in cells involved in or situated close to potential sites of new root formation, before organized root primordia were visible, and began to disappear during root primordia formation (Li and Leung 2000). Jasik and De Klerk (1997) suggested that the starch grains that had been accumulated during the first day of culture functioned as a transient storage for carbohydrates before cells entered division. Adventitious root formation is an energy-demanding process, and starch stored in the rooting zone of cuttings is utilized to provide the energy required (Husen and Pal 2007).

No callus growth was detected during root formation. The absence of callus represented a clear advantage, since this undifferentiated tissue may interfere with the normal vascular connection in the formed roots. The absence of callus indicated that root development is an example of direct organogenesis

Anatomical observations, such as those presented in this study reveal events related to rooting and timing, thus enabling the refinement of techniques for manipulating final root performance.

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