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Starch Accumulation Is Associated with Adventitious Root Formation in Hypocotyl Cuttings of *Pinus radiata*

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Abstract

This study of the internal physiology of adventitious root formation in *Pinus radiata* was performed without the potential complications from microbial contamination and nutritional stress. Hypocotyl cuttings of radiata pine were cultured in half strength MS nutrient medium supplemented with IBA (indole-3-butyric acid), IBA + kinetin, kinetin, or without phytohormones (control). Averages of 8.35 and 0.08 roots per cutting were formed in IBA and in growth regulator–free treatments, respectively. No roots were formed in IBA + kinetin, kinetin, or sucrose-free treatments at day 30 after excision of hypocotyls. Changes in fresh weight, sugar, and starch content were measured at established developmental stages associated with adventitious root formation. Sucrose-supplemented medium was required for higher levels of sugar, starch, and root formation in rooting region of IBA-treated hypocotyls. Starch accumulation, in particular, seems to have potential as a biochemical marker before root primordium emergence in light of the following observations in the IBA treatment. Starch began to build up preferentially in cells involved in or in close proximity to potential sites of new root primordium formation (that is, the cells on the inside of the cortex and the pith) before any visible organized root primordia and then began to disappear during root primordium formation. The substantial starch accumulation associated with IBA treatment was not observed in the IBA + kinetin, kinetin alone, growth regulator–free, sucrose-free treatments (nonrooting treatments) or in the nonrooting region of hypocotyls treated with IBA.

Key words: Adventitious root formation; IBA (indole-3-butyric acid); Starch; Sucrose; Sugar; *Pinus radiata*

Introduction

Adventitious root formation in radiata pine (*Pinus radiata* D. Don), a species of considerable economic importance in several southern-hemisphere countries (Smith and Thorpe 1975a), can be induced with varying plant tissues, including hypocotyls (Smith and Thorpe 1975b), mature shoots (Thulin and Faulds 1968), micropropagated shoots from embryos (Aitken-Christie and others 1985; Horgan and Aitken 1981; Reilly and Washer 1977), seedling shoot tips (Horgan and Aitken 1981), and mature

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shoots (Horgan and Holland 1989). In New Zealand, commercial clonal production of radiata pine plantlets relies on the capacity of seedling shoot-derived clones to form adventitious roots under tissue culture conditions (Gleed and others 1995). However, many clones are difficult to root, thus limiting the potential development of otherwise desirable clones. A better understanding of the internal physiology of the rooting process and knowledge of potential biochemical markers of this process in radiata pine would be advantageous for further development of clonal forestry based on radiata pine shoot cuttings. Research on other species suggests that biochemical changes, such as protein, enzyme, and carbohydrate changes, are involved in the rooting process (Bhattacharya 1988; De Klerk 1996; González and others 1991; Haissig 1986; Hand 1994; Jarvis 1986). To date reports on radiata pine in this area, however, are lacking.

Development of the present experimental system for biochemical investigations into adventitious root initiation in radiata pine was based on two factors. First, many past studies were performed under nonaseptic conditions, making it impossible to rule out interference from microbial metabolism. Second, to avoid studying root formation in starving or senescencing tissue, a basal nutrient medium was considered appropriate. The choice of plant material was based on a previous study showing that hypocotyls of derooted radiata pine seedlings formed adventitious roots in response to IBA (Smith and Thorpe 1975b). This is a simple and convenient method for providing large amounts of experimental materials compared with rooting from *in vitro* clonal shoot culture. This study examined changes in sugar and starch levels of derooted hypocotyls of radiata pine after treatment with IBA (indole-3-butyric acid, an inducer of adventitious root formation), or kinetin (counteracts IBA action).

Materials and Methods

Rooting Treatments under *in vitro* Condition

Seeds used in all experiments were collected in 1995 from the same population of open-pollinated *Pinus radiata* D. Don trees grown in Canterbury, New Zealand. Seeds were surface-sterilized in 70% (v/v) ethanol for 30 s, rinsed briefly in sterile water, then soaked in 50% (v/v) of a commercial bleach (containing 31.5 g/L active sodium hypochlorite) for 30 min before being rinsed thoroughly with sterile water. The sterilized seeds were sown in autoclaved vermiculite in tissue culture jars and stratified in a cold room (4°C) for 1 week. The jars were then maintained in a warm dark room (26°C) until seedlings emerged before transfer to a plant growth room at 22°C with continuous lighting at 80 μE · m^{-2} · s^{-1}.

Uniform seedlings with hypocotyls about 1 mm in diameter and 2.5–3 cm long were selected to prepare cuttings. The original roots and part of the hypocotyls of the seedlings were aseptically removed at 2.5 cm below the cotyledonary nodes and were discarded. Then seven derooted seedling cuttings were transferred to one tissue culture jar (250 mL, clear polycarbonate plastic container from Labserv, Biolab, New Zealand) and were cultured upright with cut-ends of the hypocotyls inserted into 0.5–1 cm of a basal medium comprised of half strength mineral salts and vitamins as described by Murashige and Skoog (1962) and 2 % (w/v) sucrose. This was supplemented with 44.3 μM IBA, 46.5 μM kinetin, a combination of these two growth regulators, or no phytohormone. All media were set at pH 5.8, solidified with 0.8% (w/v) agar, and then autoclaved.

The lowermost 0.5 cm of hypocotyl tissue cuttings were harvested for all analyses because adventitious roots formed in this region. Fresh weight determination involved at least 100 segments from each treatment.

Determination of Carbohydrates

The methods of McCready and others (1950) and Jermyn (1975) were used for sugar and starch extraction and determination.

Starch Localization

Free hand-sections were stained for starch with an I-KI solution (Gates and Simpson 1968).

Results and Discussion

Rooting Response

First, we examined different basal media, including MS, 1/2 MS, 1/5 MS, GD (Gresshoff and Doy 1972) modified by Reilly and Washer (1977), RIM (Rancillac and others 1982), different IBA concentrations and treatment times, and different sucrose concentrations to establish a reliable *in vitro* adventitious rooting system. The best combination for root formation was 1/2 MS nutrient medium with 0.8% agar, 2% sucrose, supplemented with 44.3 μM IBA for 10 days. Average number of roots per cutting (mean root number of the total rooted and nonrooted cuttings) and percentage of rooted cuttings from this treatment compared with the same medium without IBA were 8.35 vs. 0.08 and 95% vs.

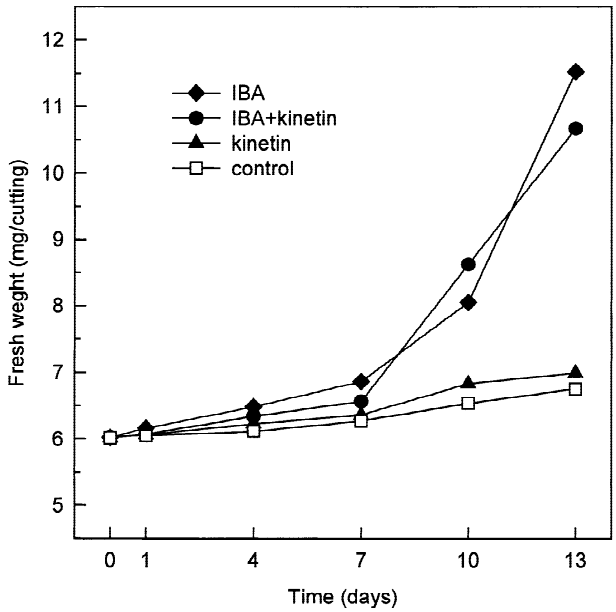

Figure 1. Fresh weight changes in the rooting region of *Pinus radiata* hypocotyls during the rooting process. Each data point is the mean weight of at least 100 segments (the lowermost 0.5 cm of the cuttings) from each treatment. All treatments had media supplemented with 2% (w/v) sucrose.

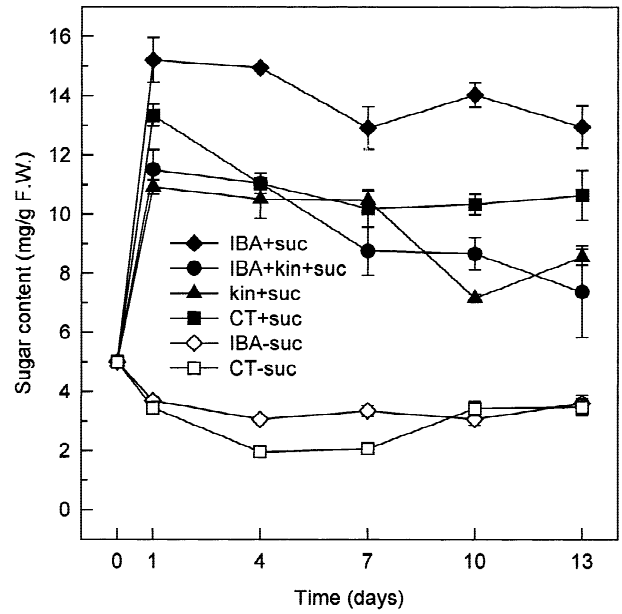

Figure 2. Changes in content in the rooting region of *Pinus radiata* hypocotyls during the rooting process. Treatments are IBA and/or kinetin (kin) and growth regulator–free control (CT) with (+) or without (−) sucrose (suc). Vertical bars represent mean ± SE value for three determinations from three individual extracts. Where no bars are shown, SE values are smaller than the legend symbols.

8% at day 30 after excision of the hypocotyls, respectively. Root formation was not observed in the medium supplemented with IBA + kinetin (callus was formed instead), kinetin alone, or IBA minus sucrose.

Light microscopic observation of developmental changes (data not shown) indicated that the first cell division, appearance of root primordium initials (a cluster of meristematic cells), well-organized root primordia (dome-shaped with organized root caps), and root emergence occurred at days 4, 7, 10, and 13, respectively during *in vitro* culture of cuttings (Li and Leung, unpublished observation). Results were similar to those from experiments performed under nonaseptic conditions (Smith and Thorpe 1975a). Samples were harvested with reference to the histologic observations.

Fresh Weight and Soluble Sugar Changes

The fresh weight of the basal hypocotyl region increased very little in kinetin and control treatments throughout the duration of the experiment. In IBA and IBA + kinetin treatments, fresh weight increased slightly during the first 7 days of culture and then increased sharply (Figure 1). This increase was probably IBA-induced but not related to adventitious root formation because a similar increase was also observed in the nonroot-inducing treatment (IBA + kinetin).

Free sugar levels dramatically increased during the first day in all treatments, except in those with sucrose-free medium, reaching different levels but remaining relatively high throughout the experiment (Figure 2). This initial increase and maintenance of higher levels of free sugar in different treatments after culturing is likely dependent on sucrose rather than the type of plant growth regulator in the media (Figure 2). However, it appears that sugar content was higher in IBA treatment than in other treatments throughout the process, suggesting that free sugar content might be associated with root formation. This is consistent with the findings of many researchers (see review by Haissig 1974).

Changes in Starch

Interestingly, starch content was very low at day 0 but increased sharply during the first day. After that starch content remained almost constant in all treatments except in IBA + sucrose (Figure 3). Under this root-inducing condition, the increase in starch content continued until day 4, then starch remained

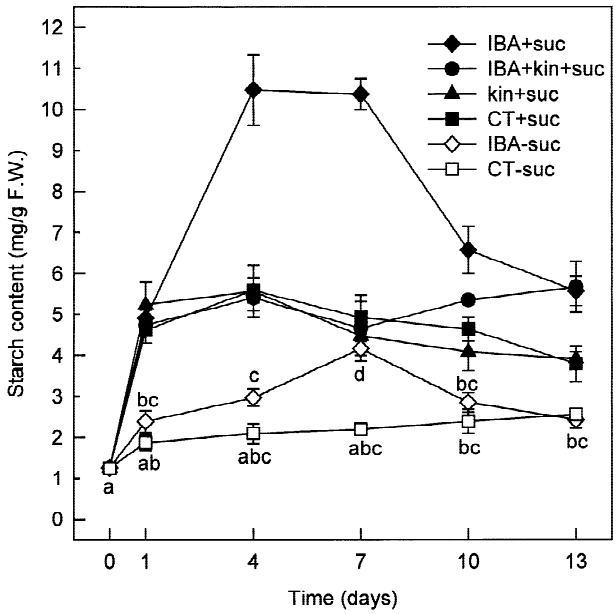

Figure 3. Changes in starch content in the rooting region of *Pinus radiata* hypocotyls during the rooting process. Treatments are IBA and/or kinetin (kin) with (+) or without (−) sucrose (suc), and controls (CT). Vertical bars represent mean ± SE value for three determinations from three individual extracts. Where no bars are shown, SE values are smaller than the legend symbols. The bars with different letters are significantly different at $p < 0.05$. Multiple comparison test was performed using Tukey's method following ANOVA. Statistical analysis was performed using the SPSS for Windows statistical software package (SPSS Inc., Version 8.0, 1998).

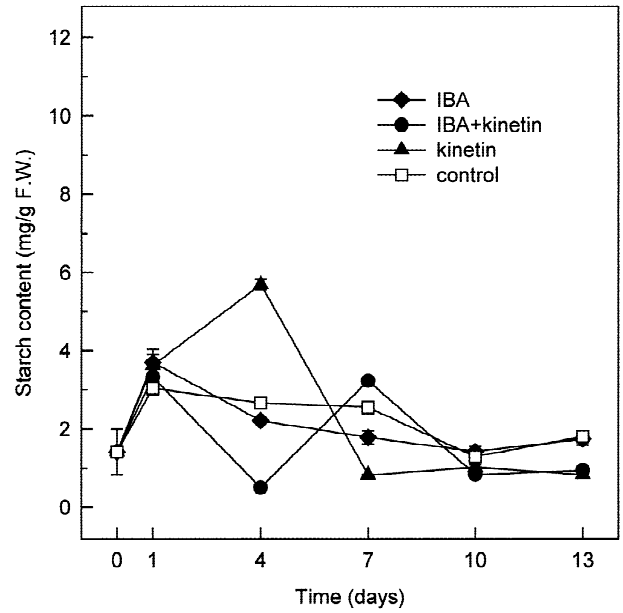

Figure 4. Changes in starch content in the nonrooting region of *Pinus radiata* hypocotyls during the rooting process. Vertical bars represent mean ± SE value for three determinations from three individual extracts. Where no bars are shown, SE values are smaller than the legend symbols.

constant until day 7 before subsequently declining. This preferential accumulation, at critical times in the rooting process, of substantial levels of starch in the rooting region of IBA-treated (no kinetin) hypocotyls and not in the nonroot-inducing treatments suggests that starch accumulation before root primordium organization could have potential as a biochemical marker for this differentiation event in radiata pine. This postulate is consistent with the conspicuous absence of a predominance of starch accumulation in the nonroot-forming region of hypocotyls in the IBA treatment (Figure 4). Overall, starch levels in all treatments were lower in the nonroot-forming than in the root-forming region of radiata pine hypocotyls. It might be that the rooting region was closer to the externally supplied sucrose. This and the wider question of the potential sugar uptake gradient along the hypocotyl require further investigation.

Higher levels of starch accumulated in the hypocotyls when cultured in sucrose-containing rather than sucrose-free media (Figure 3). Another point to note is that more starch did seem to accumulate in IBA-treated rooting regions of hypocotyls than in growth regulator–free treatments, particularly from day 4 to day 7, even when sucrose was omitted from the media (Figure 3). This much-reduced starch accumulation in the IBA minus sucrose treatment was apparently associated with root primordium formation (Li and Leung, unpublished histologic observations) but was not sufficient for root emergence. These data support the contention that nutrient deficiency, in general, and carbohydrate status, in particular, should be important considerations in studies of adventitious root formation.

When cuttings were made, most cells were devoid of starch, except for some endodermis cells (Figure 5A). Starch grains were completely absent from cortical cells. Initially, slight starch accumulation occurred in endodermis and pith cells in hypocotyl cuttings treated with IBA at day 1 (Figure 5B, *arrows*). At day 4, starch deposition was more pronounced in hypocotyl cuttings treated with IBA (Figure 5C) than in nonrooting controls (growth regulator-free) (Figure 5D), particularly in the pith and the area around differentiating resin ducts or so-called inner cortex (Smith and Thorpe 1975a). In contrast, fewer starch grains were detected in the cortex (Figures 5C,D).

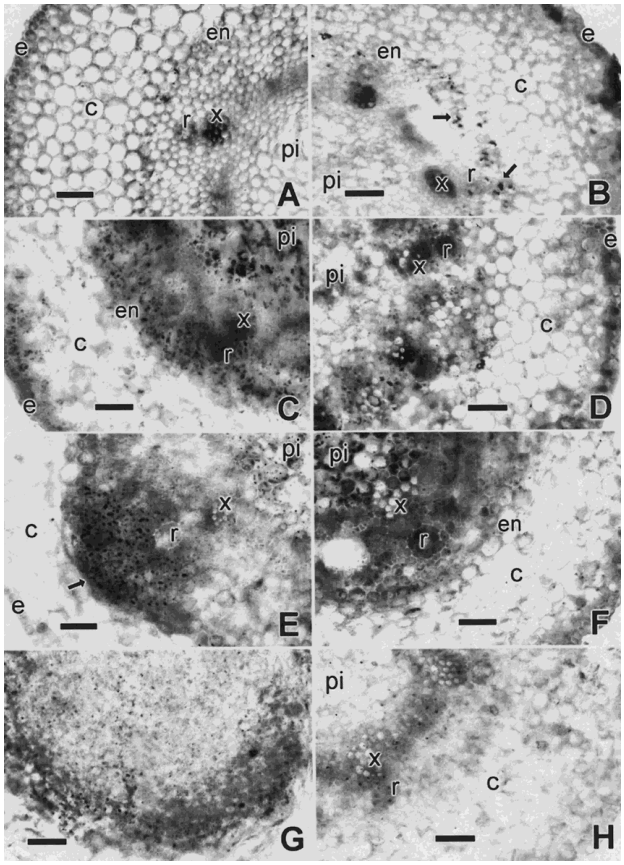

Figure 5. Histochemical localization of starch grains in hypocotyls of *Pinus radiata.* c, cortex, e, epidermis, en, endodermis, pi, pith, r, differentiating resin duct, x, protoxylem. All bars = 100 µm. Transverse sections at day 0 (A), at day 1 (B), at day 4 (C), at day 7 (E), and at day 10 (G) after IBA treatment, and nonrooting control (growth regulator-free) at day 4 (D), at day 7 (F), and at day 10 (H).

At day 7, starch grains were still preferentially associated with the newly formed putative root primordium meristematic tissues within the hypocotyl cuttings treated with IBA but reduced levels were observed in the pith and at the periphery of the meristematic tissues (Figure 5E, an arrow indicates the root primordium meristematic tissue). In the hypocotyl cuttings of the nonrooting control, the starch content was apparently similar to that at day 4, but with a slight decrease within the endodermis cells (Figure 5F). At day 10, when root primordia were well formed, starch levels in the pith and the periphery of root primordia appeared to decrease further, although some starch grains were still associated with the primordia (Figure 5G). These starch grains are expected to be consumed as energy and carbon skeletons for the further development of adventitious roots (De Klerk 1996; Haissig 1974; Haissig 1986; Jásik and De Klerk 1997). Very few starch grains were observed in the section from the hypocotyl cuttings of nonrooting controls (Figure 5H).

Further Discussion and Conclusion

The close agreement of the temporal and spatial evidence in this study suggests that starch accumulation may be a biochemical marker for early critical stages of root formation (that is, root initiation and primordium formation) in hypocotyl cuttings of radiata pine. Further research is required to evaluate more fully this possibility.

Interestingly, starch accumulation has frequently been correlated with *de novo* induction of shoot primordia (Mangat and others 1990; Thorpe and Murashige 1968). Furthermore, similar to our study, the phytohormone-containing shoot induction media required the addition of sucrose (Burritt and Leung 1996; Ramage and Leung 1996). It is therefore possible that organ initiation and development might be fundamentally the same with respect to energy requirements.

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