THE IMPORTANCE OF MORPHOLOGICAL AND PHYSIOLOGICAL PROPERTIES FOR THE ACCLIMATIZATION OF TISSUE-CULTURED DOUGLAS-FIR [*PSEUDOTSUGA MENZIESII* (MIRB.) FRANCO]

by

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THE IMPORTANCE OF MORPHOLOGICAL AND PHYSIOLOGICAL PROPERTIES FOR THE ACCLIMATIZATION OF TISSUE-CULTURED DOUGLAS-FIR [PSEUDOTSUGA MENZIESII (MIRB.) FRANCO]

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ABSTRACT

These studies showed that the morphology of Douglas-fir plantlets was related to performance during acclimatization, and that morphology could be controlled by cultural manipulation at the rooting stage.

A harsh acclimatization environment (i.e. low humidity, high temperature) adversely affected survival and growth of plantlets. However, such effects were mitigated by certain morphological features, including a high root number, tall shoots, upright needles, considerably elongated roots, and active root tips. Physiological performance was correlated to plantlet morphology. Root system morphology was related to CO_2 uptake, respiration rates, relative water content, water uptake, P- and M-level fluorescence, and the rate of quenching of fluorescence. Shoot height and needle surface area were positively related to CO_2 uptake. However, a well-developed root system relative to the shoot was a requirement. Average plantlet performance was inferior to seedlings, but beneficial morphologies narrowed this gap.

Initially, plantlets and seedlings were morphologically distinct, with plantlets having reduced root systems and a high ratio of needle surface area/root surface area. But by 7 weeks after transfer, plantlet morphology had improved through the production of new tissue. There was evidence to

indicate that original needles may have served as nutrient sources for new root growth during acclimatization.

The frequency of plantlets bearing beneficial traits was improved through cultural manipulation at the rooting stage. Shoot height and rooting medium constituents, such as boric acid, sucrose, and iron, had profound effects. The most feasible approach was to select 3-cm-tall shoots, rather than shorter ones, for rooting. Shoots of this height yielded both a high frequency of plantlets with advantageous features, and a high (>90%) rooting percentage.

DEDICATION

To my husband, Dan

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A. GENERAL INTRODUCTION

All of the stages in the micropropagation of plants present their own unique and challenging problems. Typical stages include explant establishment, bud production, shoot elongation, root induction, root elongation, acclimatization, hardening off, and field planting. Much of the work in tissue culture of gymnosperms has concentrated on enhancing survival of explants in culture and obtaining shoots. While these two objectives are still important, there is currently more of an effort to determine requirements for the subsequent phases of rooting, acclimatization and field planting. These stages are generally considered to be difficult ones for gymnosperms, but our knowledge is growing steadily [Mohammed and Vidaver 1988].

Acclimatization is the focus of this study. During this stage, plantlets are gradually exposed to harsher environmental conditions than were present *in vitro*. These new conditions are lower relative humidity, higher light intensity and more extreme temperatures. In addition, plantlets must undergo a transition from heterotrophism or mixotrophism to autotrophism. Keeping plantlets alive until they become fully and independently functional is not always easy and certainly is not cheap. The main approach has been to seek rigorous control of atmospheric conditions in the greenhouse so that plantlets are subjected to only gradual

environmental changes. Another approach is to try to produce plantlets which can better withstand stress. A combination of both approaches is probably best.

Research on the production of hardy plantlets is still in its infancy. All approaches aim to produce plantlets which are more like their conventionally-grown counterparts. It is well-known that tissue culture-derived plantlets usually display reduced morphology, anatomy, and physiology as a result of the culture environment. These differences have been reported for a number of species, of both angiosperms [Grout and Aston 1978; Smith et al. 1986; Dhawan and Bhojwani 1987; Marin et al. 1988; Sutter 1988] and gymnosperms [Von Arnold and Eriksson 1984; McKeand 1985; John 1986; Krogstrup et al. 1988]. There are gradual changes in morphology, anatomy, and physiology following transfer of plantlets to soil, in response to the new environment. The phenomenon has been well-documented for red raspberry [*Rubus idaeus* L.] [Donnelly 1984; Donnelly et al. 1984; Donnelly and Vidaver 1984a,b; Donnelly et al. 1985]. The challenge in producing hardy plantlets is to get them to appear and function more like controls as early as possible.

One approach is to facilitate autotrophism in culture, by reducing or removing sucrose and providing higher concentrations of carbon dioxide. This approach is being

used with tobacco [*Nicotiana tabacum* L.] and potato [*Solanum tuberosum* L.] [Pospisilova et al. 1988], strawberry [*Fragaria* sp.] [Fujiwara et al. 1988], and other species [Kozai 1989]. This kind of system has promise in automation utilizing batch culture. However, in some ways this approach simply transfers the requirement for rigorous environmental control from the greenhouse to the laboratory. For example, CO_2 must be delivered to plants within culture vessels, while maintaining asepsis. Sucrose is likely to be absent, but microorganisms can use other nutrients for food; thus, contamination of large batches of plants is likely. Therefore, while this approach is desirable idealistically, it still has a long way to go before it can be implemented safely and economically.

From a practical perspective, a second approach may be better in the short term, namely the production of hardier plantlets through simple manipulation of nutrient media and control of shoot growth and quality. Beneficial features could be targeted which might confer a higher resistance to stress. This approach and the one described above are similar since both are intended to produce plantlets whose form and function are better suited to *ex vitro* conditions. But, at present, it may be the more accessible option, from a technical and economic standpoint.

There is already some evidence that certain features

confer an advantage during the transfer stage. For example, plantlets of Norway spruce [*Picea abies* L. Karst.] were easier to acclimatize when roots were present [Von Arnold and Eriksson 1984]. Vitrification of foliage was shown to severely reduce survival following transfer of Norway spruce [Von Arnold 1982], radiata pine [*Pinus radiata* D. Don] [Aitken-Christie and Jones 1985] and *Rosa* sp. [Langford and Wainwright 1987]. Healthy, vigorous shoots were apparently a requirement for good post-transfer performance of loblolly pine [*Pinus taeda* L.] [Wisniewski et al. 1986]. Several authors have suggested that development of the root system prior to acclimatization is likely to be an important factor, but more research is necessary [David et al. 1979; Amerson et al. 1984; McKeand and Allen 1984; McKeand 1985; Gronroos 1987; Timmis and Ritchie 1988]. Thus, methodical investigation into the relevance and control of morphology for various species appears warranted.

One such species is Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco], which is a valuable timber resource in western Canada and the northwestern United States. In British Columbia alone more than 16 million seedlings are planted each year [B. Jaquish, B.C. Ministry of Forests and Lands, pers. comm.]. Research efforts on the micropropagation of Douglas-fir have been underway at AgriForest Technologies Ltd. in Kelowna, B.C., since the

early 1980's [Dunstan et al. 1986; Mohammed and Patel 1989]. In addition, Douglas-fir has been the subject of considerable research in other laboratories [review by Goldfarb and Zaerr 1989]. One reason for micropropagating this species is to increase quantities of planting stock from limited supplies of superior seed. [For reviews of the roles of tissue culture in forestry, refer to Bonga 1977; Biondi and Thorpe 1981; Powledge 1984; Dunstan 1988; and Haissig 1989.]

The objectives of this research are: (i) to establish whether morphology can be linked to survival and growth during acclimatization; (ii) to characterize the physiology of plantlets according to morphological features; and (iii) to investigate the importance of cultural treatments, at the rooting stage, on root and shoot morphology.

B. GENERAL MATERIALS AND METHODS

SEED SOURCE

These studies utilized one seedlot (#952) of open-pollinated Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco], which was obtained from the B.C. Ministry of Forests and Lands Surrey Seed Centre. Geographical details of this seedlot are:

Latitude/Longitude 50°00'N/125°20'W
Forbes Landing, B.C.
Elevation 152 m
Seed orchard planning zone: dry maritime
Nat. topog. grid ref.: 92K3

PLANTLET PRODUCTION

This protocol is based on the methods reported in Mohammed and Patel [1989]. Seeds were surface-sterilized in 70% ethanol for 30-60 s followed by 20% (v:v) Sunbrite^{R} (1.05% sodium hypochlorite final concentration) for 20 min, then rinsed four times, 5 min each, in sterile, distilled water. Embryos were dissected out and laid lengthwise on sterilized, solid medium (20 ml per 100 X 15 mm petri dish) which was autoclaved at 138 kPa for 15 min. The medium for conifer morphogenesis (MCM) [Bornman 1983; see Appendix] was used with 2.25 $mg \cdot l^{-1}$ benzyladenine (BA) and 3% sucrose, for shoot induction. The pH of the medium was adjusted to 5.6, then 0.6% agar (Sigma Chemical Co., St. Louis, MO) was added for solidification. Approximately fifteen intact, white

embryos were placed in each dish. Dishes were sealed with Stretch and Seal^{R} (Dow Chemical) plastic wrap.

After 17 d, embryos with multiple adventitious buds were transferred to BA-free medium for 21 d, for shoot elongation. Finally, they were transferred to BA-free medium containing 0.1% activated charcoal (Sigma, C-4386) for a further 21-42 d. Subsequently, cultures were transferred every 21 d to charcoal-free medium which was contained in 500-ml glass jelly jars with metal screw-type lids. Each jar contained 100 ml of medium solidified with 0.3% Gelrite^{R} (Kelco, San Diego, CA).

When adventitious shoots had elongated to 1 cm in height, they were remultiplied. Single shoots were cultured in jars (7-8 shoots/jar) containing MCM (pH 5.6) with 3% sucrose, 1.12 $mg \cdot l^{-1}$ BA and solidified with 0.3% Gelrite. BA concentration was reduced to 0.11 $mg \cdot l^{-1}$ after 21 d, and omitted after a further 21 d. Axillary shoots were excised 21-42 d later (total time for one remultiplication cycle was 63-84 d). Axillary shoots from the fifth to the twentieth remultiplication cycle were harvested for rooting. Only shoots from the same cycle were used in a given experiment.

Shoots measuring 1.5-2 cm in height were rooted, *in vitro*, in 150 X 25 mm test tubes, each containing 15 ml of Sunshine^{R} Mix No.4 (60:40 peat:perlite, Fisons Western Corporation, Vancouver, B.C.) and 9-10 ml of liquid medium.

For root induction, the medium contained 1/5-strength MCM, 1% sucrose and 0.5 $mg \cdot l^{-1}$ naphthaleneacetic acid (NAA). Medium pH was adjusted to 5.0 prior to adding to the substrate and autoclaving. Tubes were capped with Kaputs (Bellco Glass Inc., Vineland, N.J.). After 7 weeks, rooted and unrooted shoots were transferred, *in vitro*, to NAA-free substrate with 1/5-strength major and minor MCM salts and organics, full ferrous sulphate (and Na_2EDTA), 3% sucrose and 0.1% charcoal, for 21-28 d, to undergo root elongation.

Any variations from this basic treatment are detailed in the relevant chapters. Shoots or plantlets represented a mixture of clones, but individual clonal responses were not followed.

Culture conditions *in vitro*

The culture regime during shoot production and elongation was 25 $\pm$ 2°C, 16 h daylength and 67.6 $\mu mol \cdot s^{-1} \cdot m^{-2}$ photosynthetic photon flux density (PPFD). During rooting, lighting was reduced to 47.3 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Lighting was provided by high-output, cool white fluorescent bulbs (Sylvania F96 T12).

ACCLIMATIZATION (TRANSPLANTATION) OF PLANTLETS TO BE USED IN COMPARISONS WITH SEEDLINGS

Plantlets were removed from test tubes and transplanted to tapered plastic pots of 100 ml volume and dimensions of 5 X 5.5 X 6 cm high. Pots had been filled to 1 cm from the top with a 3:1 mixture, by volume, of peat:vermiculite. Fine gravel was added to fill the pot. Pots were arranged in a tray and covered with a clear plastic lid to maintain 70% relative humidity. Transplants were watered, misted with a hand sprayer, and incubated in a growth chamber with a PPFD of 13.5 $\mu mol \cdot s^{-1} \cdot m^{-2}$ from cool white fluorescent lights (Sylvania F20 T12), 16 h daylength, and 22:20°C day:night temperature. After 3 d, lighting was increased to 27 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Transplants were hand-misted twice daily for the first 3 d, then once daily for the next 9 d, and finally on alternate days for the next 3 d. Thirteen days after transplantation, the relative humidity was reduced to 60-65% by adjusting the lid to permit air intake, and plantlets were fed weekly with Peters conifer fertilizer (20:19:18 N:P:K; Edmonton, Alta.) mixed to 120 $mg \cdot l^{-1}$ nitrogen. Eighteen days after transplantation, the plastic lid was removed and R.H. averaged 55-60%. Then, 5 d later, the tray was placed in a greenhouse where conditions were 24 $\pm$ 6°C, a natural summer daylength of about 13 h, R.H. 50-70%, and a maximum PPFD of 555.6 $\mu mol \cdot s^{-1} \cdot m^{-2}$.

SEEDLING PRODUCTION

Seeds were soaked for 24 h in tap water containing a few drops of detergent. Floating seeds were then discarded, and the rest were patted dry with paper towel prior to placing in vials or Petri dishes and sealing with Parafilm^{R} (American Can Co., Greenwich, CT). Seeds were refrigerated at 4-5°C for 21-28 d for stratification. Stratified seeds were rinsed for 1 min in 1.5% hydrogen peroxide and a few drops of detergent, then rinsed with tap water. They were germinated on filter paper in sterilized, covered plastic trays containing wet perlite, and incubated in indirect light at 21 $\pm$ 3°C. Seeds were misted daily. When they had germinated and approximately 5 mm of radicle was visible, they were planted into pots as described above for plantlets, then incubated at 23 $\pm$ 3°C and natural indirect lighting of approximately 37 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Germinants were hand-misted twice daily until the seed coats fell off, at which time the plastic lid was removed and seedlings were transferred to the greenhouse, under the same environmental conditions as described above for plantlets. Seedlings were fed weekly, for 6 weeks, with Miracle-Gro^{R} fertilizer (15:30:15; Stern's Nurseries Inc., Geneva, N.Y.), mixed to 70 $mg \cdot l^{-1}$ N, and thereafter, weekly with Peters mixed to 120 $mg \cdot l^{-1}$ N. Seedlings which were 7-9 weeks old post germination were used in all comparisons with plantlets.

CALCULATIONS OF SURFACE AREA

The following formulae were developed after calibration with a sample of ten shoots of each plant source, using individual needle measurements. To calculate total needle surface area (NSA, in dm^2):

i) seedlings:

$$NSA = 27.37 \times \text{Shoot height (mm)} \times 10^{-4}$$

ii) plantlets prior to transplantation:

$$NSA = \{[37.23 \times \text{Shoot height (mm)}] + [50 \times \text{No. of axillary shoots}]\} \times 10^{-4}$$

iii) plantlets at 5-7 weeks after transplantation:

$$NSA = \{[(37.23 \times \text{mm length of main shoot with persistent needles}) + (19.96 \times \text{mm length axillaries with persistent needles}) + (37.89 \times \text{mm lengths of main shoot and axillaries with new needles}] \text{ minus estimated \% browning}\} \times 10^{-4}$$

Root surface area (RSA, in dm^2) was derived by considering each root as a cylinder. Hence, surface area was defined by the formula $[2\pi r \times \text{length (mm)}] \times 10^{-4}$. The average[1] diameters (mm) that were used in the formula were:

i) seedlings: 0.8 for main roots
0.4 for laterals

ii) plantlets prior to transplantation: 0.6

iii) plantlets at 5-7 weeks after transplantation:
0.8 for main roots
0.4 for laterals

[1]Fifty to 100 roots measured for calibration.

DATA ANALYSIS

Significance was determined using the chi-square test of independence for results expressed as a percentage, t-tests or Duncan's multiple range test for means, or linear regression analysis for relationships [Ott 1977]. Statistics were generated using Number Cruncher Statistical System Version 5.01. Unless indicated otherwise, $p \leq 0.05$.

C. THE IMPORTANCE OF MORPHOLOGICAL PROPERTIES FOR THE ACCLIMATIZATION OF TISSUE-CULTURED DOUGLAS-FIR

CHAPTER I

THE INFLUENCE OF ACCLIMATIZATION TREATMENT AND PLANTLET MORPHOLOGY ON EARLY GREENHOUSE-PERFORMANCE OF DOUGLAS-FIR

INTRODUCTION

In tissue culture, the acclimatization treatment is almost always stressful and is regularly associated with plantlet mortality [Mohammed and Vidaver 1988; Sutter 1988]. Attention has been directed to the possible relationship between morphology and acclimatization success of gymnosperm cultures, and useful preliminary information has emerged. Important parameters in survival and growth have included shoot height [Leach 1979] and shoot quality [Wisniewski et al. 1986] for loblolly pine, and the presence of roots for Norway spruce [Von Arnold and Eriksson 1984]. We do not know the potential effects of plantlet morphology on the acclimatization of Douglas-fir.

In this study, plantlets of Douglas-fir have been exposed to one of two environmental regimes based on water stress during acclimatization: (i) high stress: low humidity and high temperatures, or (ii) low stress: high humidity and moderate temperatures. The objectives were to determine both the effect of stress on survival and growth, and

whether certain morphological characteristics are associated with greater capacity to withstand stress.

MATERIALS AND METHODS

Plantlets were produced according to the general protocol described in Section B. Acclimatization was carried out in the greenhouse. Rooted plantlets were removed from test tubes and thoroughly rinsed free of the substrate and medium. Morphological features of the plantlets were recorded prior to planting in Styroblock^{R} No.6 trays (Beaver Plastics Ltd., Edmonton, Alta) with 112 cavities per block, and 100 ml volume per cavity. Trays contained a 3:1 volume ratio of peat:vermiculite. Fine gravel was spread over the substrate. Each tray was covered with a hard plastic lid containing two adjustable air vents which were kept closed at the beginning. The lid did not provide a perfect seal around the plantlets but it did minimize air disturbance. Plantlets were misted twice daily and watered every 2-3 d. The daylength was 18 h, achieved with a combination of natural and artificial lighting (high pressure sodium lamps, Philips SON T400). Initially, plantlets were shaded to produce a PPFD of only 18.3 $\mu mol \cdot s^{-1} \cdot m^{-2}$.

The low stress treatment consisted of 80-90% relative

humidity at plant level and an average day:night temperature of 20:15°C. The high stress treatment, carried out 35 d later in the same greenhouse during hotter weather, consisted of 40-70% R.H. and 28:22°C. Eighty-four and one hundred and five plantlets, split into two replicate Styroblocks per treatment, were included in the low and high stress treatments, respectively.

In both treatments, air vents were opened half-way after 10 days, and plantlets then received Peters conifer fertilizer mixed to 120 $mg \cdot l^{-1}$ N, twice weekly. The lid and shading were removed after a further 10 d. Plantlets were then exposed to natural light which registered approximately 560 $\mu mol \cdot s^{-1} \cdot m^{-2}$ at midday.

Seven weeks after transfer to the greenhouse, plantlet survival, shoot growth, frequency of plagiotropism, needle form, activity of the shoot apex, survival of the shoot apex, and waxiness of new needles were assessed. These were then tested statistically for any relationships to initial morphology. Initial morphological features were: shoot height, shoot colour, activity of shoot apex, needle form, caliper, root number, length of the longest root, root thickness, root tip activity, and the diameter of the shoot:root junction.

RESULTS AND DISCUSSION

Survival

The extent of survival following transfer to the greenhouse was related to the degree of environmental stress. Under high humidity and low temperatures (low stress treatment), 89% of the plantlets survived after 7 weeks. Under the reduced humidity and high temperatures of the high stress treatment, survival was only 33%. However, maintaining such a low stress environment during the critical first weeks can be a problem, one that is sometimes solved only with expensive apparatus [Griffis et al. 1983; Pocock 1983]. It may be more practical to try to produce plantlets which are better able to withstand stress initially.

Three morphological features were associated with better survival under high stress: upright needles (Table 1, Figure 1), more than 10 roots/plantlet (Figure 2) and shoot height exceeding 40 mm (Figure 3). Under low stress, upright needles and stem caliper were important (Table 1). Plantlets with at least two of the three beneficial features had significantly better survival under high stress compared with those lacking any features (Figure 4). Fifty-two percent of the plantlets with more than 10 roots survived the high stress treatment, compared to the average survival

Table 1. Influence of initial plantlet morphology on survival under two stress environments.

Morphological feature	% Survival	
	Low stress	High stress
Majority of roots		
(i) thin (<1 mm)	90.6	39.1
(ii) thick (>1 mm)	87.1	22.2
	NS	NS
Majority of root tips		
(i) white	93.0	36.8
(ii) brown	81.5	16.7
	NS	NS
Length of longest root		
(i) <1 cm	86.7	25.8
(ii) >1 cm and <3 cm	87.5	33.3
(iii) >3 cm	95.5	38.7
	NS	NS
Diameter shoot:root junction		
(i) <5 mm	91.5	33.9
(ii) >5 mm	84.0	32.6
	NS	NS
Shoot (i) deep green	90.0	40.5
(ii) pale tip	88.9	29.4
	NS	NS
Shoot apex (i) active	88.6	37.9
(ii) dormant	92.9	25.6
	NS	NS
Needles (i) upright	97.2	41.7
(ii) drooping	58.3	22.2
	S	S
Shoot caliper (i) >1 mm	93.0	32.6
(ii) <1 mm	69.2	40.0
	S	NS

Note: n=10 to 95. Chi-square test conducted for each set of observations in a column.

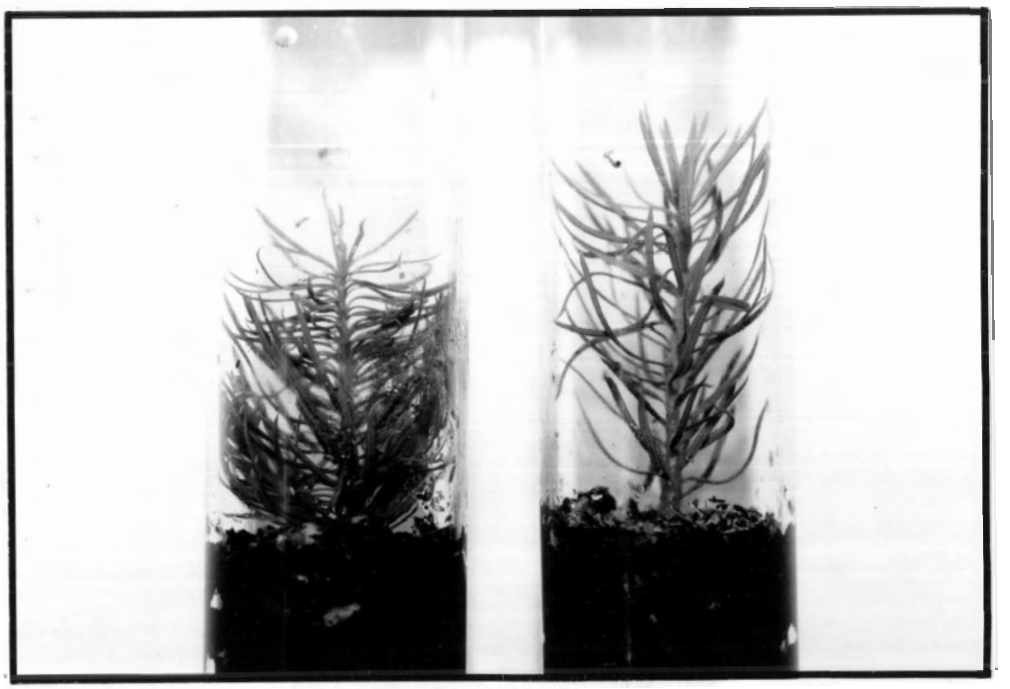
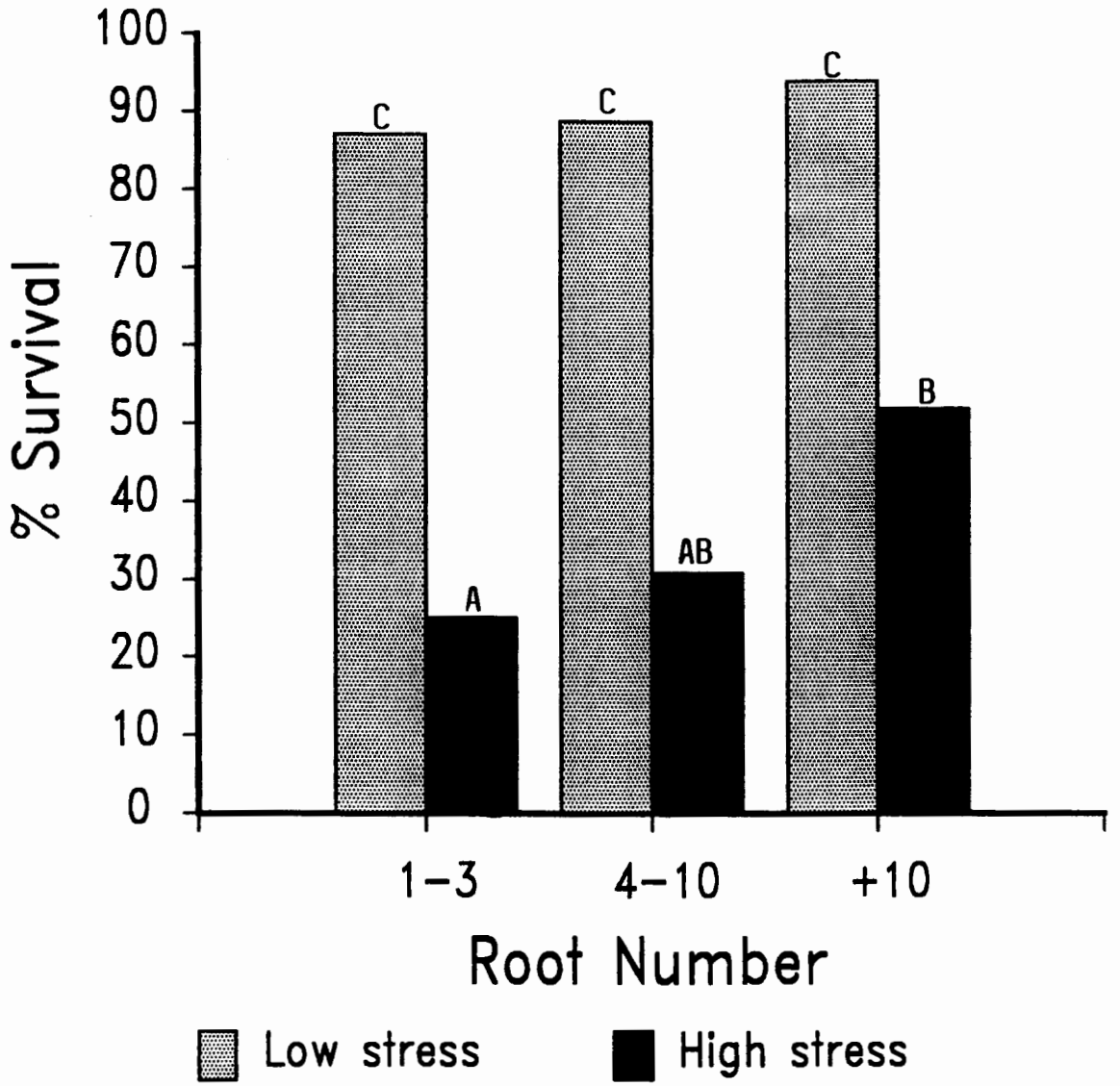


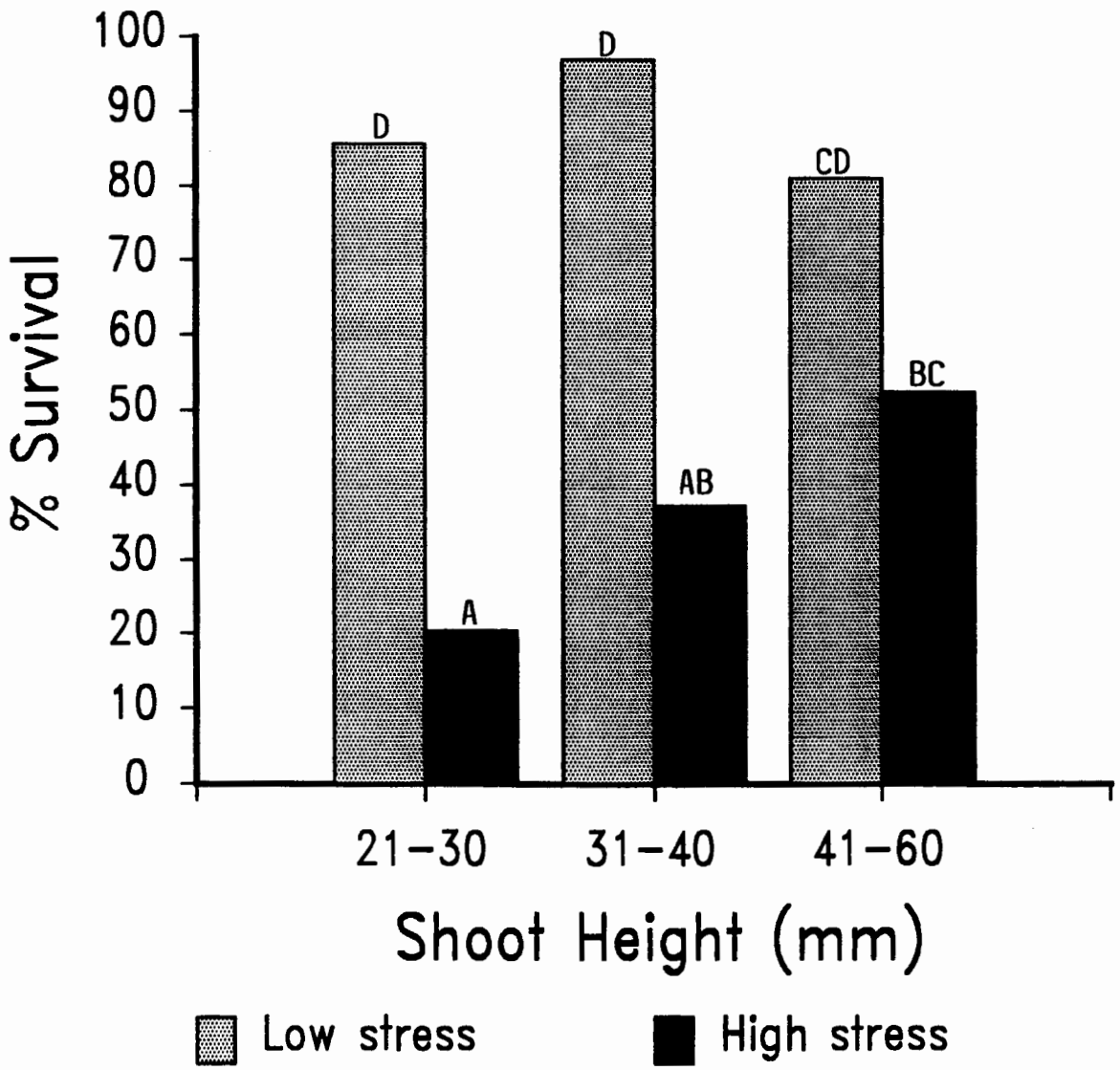
FIGURE 1. Needle form on plantlets, prior to transplantation. Permanently wilted [*left*] or upright [*right*] needles. Upright needles were associated with higher survival during acclimatization.

Figure 2. Relationship between survival of plantlets and initial root number.



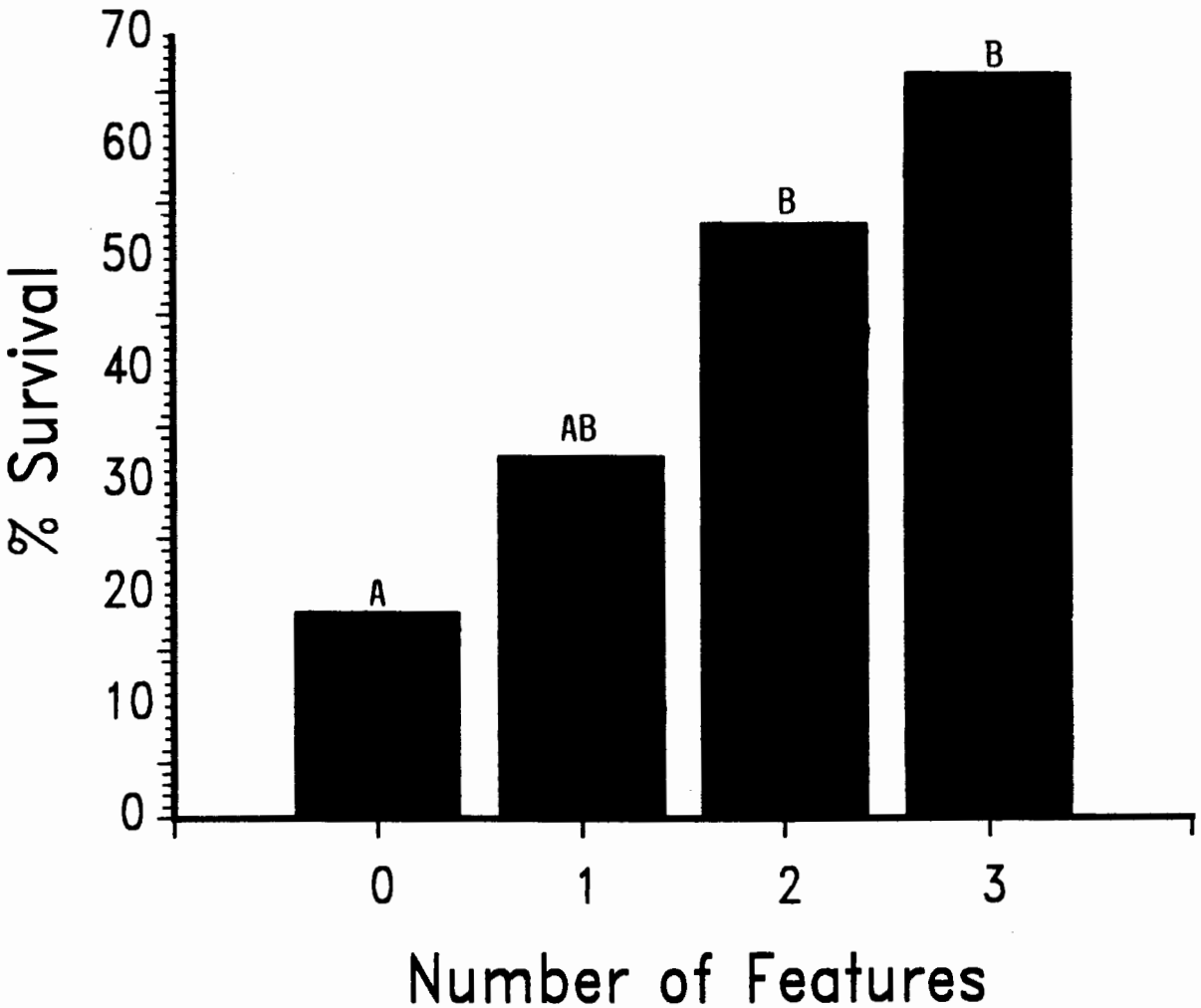
Note: n=17 to 52. Bars with the same letter(s) were not significantly different according to the chi-square test.

Figure 3. Relationship between survival of plantlets and initial shoot height.



Note: n=16 to 49. Bars with the same letter(s) were not significantly different according to the chi-square test.

Figure 4. Plantlet survival under high stress, based on initial number of beneficial features.



Note: n=30 to 58, except '3 feature' category, n=9. Features are 10+ roots/plantlet, upright needles, and 41-60 mm shoot height. Bars with the same letter(s) were not significantly different according to the chi-square test.

rate of 25% or 31% for those with 1-3 or 4-10 roots, respectively. Tall plantlets (41-60 mm) had a survival rate of 53%, compared to 20% or 37% for heights of 21-30 or 31-40 mm. In another study, the survival of loblolly pine plantlets was also highest when shoots were tall [Leach 1979]. Topics for further investigation might include assessments of the relative amounts of nutrient reserves, the degree of vascular development, and the efficiency of water transport in tall versus short shoots.

Table 2 lists the frequency of occurrence for traits found to be beneficial for survival. Sixty-four percent of the plantlets had at least one of the traits, but only 9% possessed all three features. Certain traits indicated the probable occurrence of others. For example, 72% (18/25) of the plantlets with 10+ roots also had upright needles. Similarly, 90% (18/20) of the tall plantlets had upright needles. The corollaries were not true, i.e. plantlets with upright needles did not consistently have many roots or tall shoots.

Features which were not related to survival were root thickness, white root tips, root length, the diameter of the shoot:root junction, chlorosis and shoot tip activity (Table 1). The shoot:root junction was not observed to exceed 10 mm, and consisted of non-friable tissue.

With loblolly pine it was reported that shoot quality

Table 2. Frequency of plantlets possessing morphological features beneficial for survival.

Morphological feature	Percentage of plantlets
Upright needles (U)	57.1
10+ roots (R)	23.8
41-60 mm stem height (T)	19.0
U+R	17.1
U+R+T	8.6
R+T	11.4
U+T	17.1
U, R or T	63.8

Note: n=105.

was much better correlated to survival than root number [Wisniewski et al. 1986]. In that study, plantlets with varying degrees of chlorosis and necrosis were sampled. The present study utilized an *in vitro* treatment that produces primarily green shoots [Mohammed and Patel 1989]. Any chlorosis was slight and tended to be restricted to the shoot tip. Whereas shoot morphology was the essential factor under low stress, root characteristics became important under water stress.

Shoot growth and morphology

New shoot growth was visible within 2 weeks following transplantation. Morphological features did not differ substantially between the two stress treatments 7 weeks after transplantation (Table 3). All new needles were of the upright form, and appeared longer than those from tissue culture, but actual measurements of length were not kept at this time.

At 7 weeks, elongation of the main shoot was greater under low stress. Average shoot growth was 18.2 $\pm$ 1.9 mm, compared to 9.3 $\pm$ 1.7 under high stress. Also, an average of 3.2 $\pm$ 0.3 axillary shoots per plantlet were produced under low stress, compared to 2.0 $\pm$ 0.3 shoots under high stress. These differences were significant ($p=0.05$) according to t-test analyses.

Table 3. Morphology of plantlets, 7 weeks after transplantation.

Morphological feature	Percentage of surviving plantlets	
	Low stress	High stress
Shoot apex active	80.0 a	97.1 b
Axillary shoots[1] present	88.0 a	88.6 a
Main shoot tip alive	88.0 a	97.1 a
Plagiotropic	16.0 a	20.0 a
New growth waxy	100	100
New growth with upright needles	97.3 a	97.1 a

Note: n=75 and 35 plantlets for the low- and high-stress treatments, respectively. Results within a row followed by the same letter were not significantly different according the chi-square test.

[1]Avg. 3.19 $\pm$ 0.27 axillaries per plantlet (low stress) and 2.03 $\pm$ 0.26 (high stress), significant at p=0.05, t-test.

The following section describes findings for the low stress treatment, but similar patterns were observed under high stress.

Shoot growth(y) was positively correlated with initial height(x), according to the equation: $y=0.5x$ ($p<0.01$, $R^2=0.56$, range of x=20-58 mm, y=5-60 mm). Root number was again important, and shoot elongation was greatest on plantlets with 10+ roots (Table 4). Other features which were associated with better growth were white root tips, active shoot tips, and root length greater than 1 cm (Table 4).

The frequency of plantlets having morphological features found to be beneficial for growth is shown in Table 5. Plantlets with 10+ roots tended to possess at least one other beneficial feature. The presence of any one of these other features indicated 10+ roots only 20-50% of the time. White root tips were usually associated with +1-cm-long roots. Also, white root tips or +1-cm-long roots were usually accompanied by active shoot apices.

Plagiotropism

At 7 weeks, there were occurrences of apparent plagiotropism (Table 3) with no significant difference between the two stress treatments. Shoots were classified as plagiotropic if stem curvature was at least 30° from the

Table 4. Morphological features of plantlets which significantly influenced shoot growth under low stress treatment.

Morphological feature	Average shoot growth[1] (mm)
Majority root tips	
(i) white	22.8 b
(ii) brown	6.7 a
Length of longest root	
(i) <1 cm	11.3 a
(ii) >1 cm and <3 cm	22.1 b
(iii) >3 cm	21.3 b
Number roots (i) 1-3	13.4 a
(ii) 4-10	16.1 a
(iii) +10	31.5 b
Shoot apex (i) active	19.3 b
(ii) dormant	13.2 a

Note: n=12 to 54. Results for each feature followed by the same letter were not significantly different according to the t-test or Duncan's multiple range test.

[1] Seven-week height minus initial height.

Table 5. Frequency of plantlets possessing morphological features beneficial for shoot growth.

Morphological feature	Percentage of plantlets
Majority of root tips white (W)	67.9
Length of longest root>1 cm (E)	64.3
10+ roots (R)	20.2
41-60 mm stem height (T)	19.0
Active shoot tip (A)	83.3
W+E+R+T+A	6.0
W+E	58.3
W+R	16.7
W+T	14.0
W+A	56.0
E+R	16.7
E+T	12.0
E+A	52.4
R+T	10.0
R+A	16.7
T+A	16.7
W, E, R, T, or A	98.0

Note: n=84.

vertical axis. The frequency of curvature was 44% among shoots with less than 1 mm caliper (measured prior to transfer), significantly higher than the 12% observed for thicker stems. Curvature was also more prevalent on initially taller shoots, and was recorded at 3%, 16% or 38.5% for heights of 20-30 mm, 31-40 mm or 41-60 mm, respectively. Tall shoots and slender stems rarely occurred simultaneously.

Early plagiotropism has been observed with loblolly pine [McKeand 1985] and radiata pine [cited in McKeand 1985], and although the cause is uncertain, stress during transfer or root system morphology may be responsible. Plagiotropism is also a problem in rooted cuttings from older donor sources and it varies with species [Brix and Van den Driessche 1977], with Douglas-fir being highly susceptible. At 7 weeks, plagiotropism could not be linked to initial root characteristics or acclimatization stress.

SUMMARY AND CONCLUSIONS

The degree of stress encountered during the acclimatization treatment was found to affect survival and shoot growth, with poor survival and less growth occurring under low humidity and high temperatures. Features found to be beneficial for survival under high stress were tall

shoots (41-60 mm), 10^{+} roots, and upright needles. Features which were beneficial for growth were 10^{+} roots, tall shoots, white root tips, active shoot tips, and root systems greater than 1 cm in length. The majority of plantlets with many roots tend to display other beneficial traits. Thus, this single feature is worth targeting in the production of plantlets.

At 7 weeks, some shoots appeared plagiotropic, and these were more prevalent from initially tall plantlets. However, assessment of the same plantlets after 7-8 months [Mohammed, unpublished results] revealed that the early incidence of plagiotropism was not indicative of later results, and that tall plantlets eventually showed a lower tendency for lateral growth. In contrast, the frequency of plagiotropism increased markedly with the initially short plantlets. These later observations also indicated that stress was the most significant cause of plagiotropism. Since initially tall shoots grew more than short ones, the former may have appeared plagiotropic at 7 weeks because newer stems were not yet sufficiently lignified to physically support the new growth.

CHAPTER II

THE IMPORTANCE OF PLANTLET MORPHOLOGY IN REGULATING NET WATER LOSS IN DOUGLAS-FIR

INTRODUCTION

A major cause of mortality during the acclimatization of tissue cultures is poor control of water loss [Brainerd and Fuchigami 1982; Dhawan and Bhojwani 1987; Pospisilova et al. 1987; Sutter 1988]. In angiosperms, this problem has generally [Brainerd and Fuchigami 1982; Sutter 1988] but not always [Marin et al. 1988] been related to poor stomatal functioning. However, in gymnosperms, e.g. white spruce [*Picea glauca* (Moench) Voss], stomatal functioning appeared normal [Toivonen 1985]. Other reasons for substantial water loss in angiosperms have included reduced amount and abnormal structure of epicuticular wax [Grout and Aston 1977; Dhawan and Bhojwani 1987; Sutter 1988], and altered internal anatomy [Donnelly and Vidaver 1984a; Donnelly et al. 1985]. Normal gymnosperm tissue culture-derived plantlets are more waxy [Aitken-Christie and Jones 1985], yet they are still subject to water stress. One reason for this problem may be relatively unspecialized internal anatomy, e.g. in tissue cultures of Norway spruce [Von

Arnold and Eriksson 1984].

Certain morphological features have been linked to improved survival under conditions of water stress [Chapter I; Mohammed and Vidaver 1990]. These included: a high (10+) root number and tall (41-60 mm) shoots. The present study was conducted to investigate the possible relationship between morphology and the control of net water loss, as measured by relative water content and the rate of water uptake under stressful conditions. Plantlet performance was compared to that of conventionally-grown seedlings.

MATERIALS AND METHODS

Plantlets and seedlings were obtained using the general protocol described in Section B. The combination of a plant, its container, and water are referred to as a "unit".

Comparative net water loss in plantlets and seedlings

Plantlets at the end of the rooting treatment were selected for this study. Two plantlets (with 5 or 14 roots) and one seedling were included. Plants were removed from their containers and their roots were rinsed and patted dry with paper towels. The plants were then placed in pre-weighed plastic Kaputs containing 15 ml of tap water, with roots submerged completely and shoots exposed to the

air. Units were weighed on an analytical balance to 10^{-5} g. In addition, three Kaputs with water were weighed periodically to determine evaporative water loss.

Units were set out on a laboratory bench with atmospheric conditions of 54-62% relative humidity and 23 $\pm$ 2°C. Lighting was a combination of natural (through window) and artificial (fluorescent ceiling lights) to give a total PPFD of approximately 32 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Units were weighed at hourly intervals. The weight of the plant at each time was calculated as follows:

Plant weight (mg) = [Unit weight] - [Initial weight of container and water] - [weight of water lost by evaporation]

At the end of 5 h, the final weight of the plant was obtained by removing it from the container, towel-drying its roots, and weighing the plant directly.

For dry weights, plants were oven-dried at 80°C for 24 h and cooled in a desiccator. Relative water content (RWC) was calculated for each plant at each weighing time as follows:

$$RWC\ (\%) = [(W_t - W_d)/(W_i - W_d)] \times 100$$

where W_t is the fresh weight at time t, W_i is the initial fresh weight, and W_d is the dry weight.

The experiment was repeated once.

Relative water content and water uptake in plantlets

Fifteen plantlets were selected at the end of the rooting treatment, representing a random distribution of root numbers and shoot heights. Unit preparation and atmospheric conditions were the same as those described above. Five containers with water were included and weighed regularly to determine evaporative water loss. Weights were recorded hourly for 3 h. The final weight of the plant was obtained by direct weighing. The final weight of the container and water was derived by subtracting the final weight of the plant from the final weight of the unit.

Morphological features were recorded prior to oven-drying. These were: root number, root surface area, length of the longest root, needle surface area/root surface area, and shoot height. Relationships were then sought between morphology and either relative water content at 3 h or the hourly rate of water uptake. The latter was determined as follows:

$$\text{Rate uptake (mg or } \mu l \cdot h) = (C_i - C_f - C_e)/3$$

where C_i is the initial weight of the container and water, C_f is its final weight, and C_e is the weight of water that evaporated.

Index of water potential

This study involved seedlings and 0-, 3-, 5-, and 7-week-old transplants. Needles were selected at random from several plants to give a total of ten per sample. Needles were removed at 1 cm from the shoot tip. On transplants with new growth, samples were taken from both persistent and new needles, at 1 cm below the start of new growth and at 1 cm below the tip of new growth, respectively. All samples were taken from dominant shoots with actively growing apices.

A correlate of needle water potential was measured using a J-14 Leaf Press (Decagon Devices Inc., Pullman, WA). Upon application of hydraulic pressure, the endpoint was identified at the first appearance of moisture from unwounded areas. Pressure readings (p.s.i. on gauge) were converted to -bars by dividing by 14.97.

Needle epicuticular wax (SEM)

To examine epicuticular wax on abaxial and adaxial needle surfaces, needles were sampled from plantlets and seedlings. Plantlets were selected at the end of the rooting treatment, and at 7 weeks following transplantation. Needles were removed from positions as described in the experiment on water potential. Five seedlings and five plantlets were used to provide ten needles per sample. Needles were mounted fresh on stubs with silver adhesive,

and allowed to air-dry overnight. Following a conventional gold-coating procedure in a vacuum evaporator, epicuticular wax of abaxial and adaxial surfaces was examined with an ETEC Autoscan SEM, at an operating voltage of 20 kV. Qualitative comparisons of wax structure, density, and distribution patterns were made between samples.

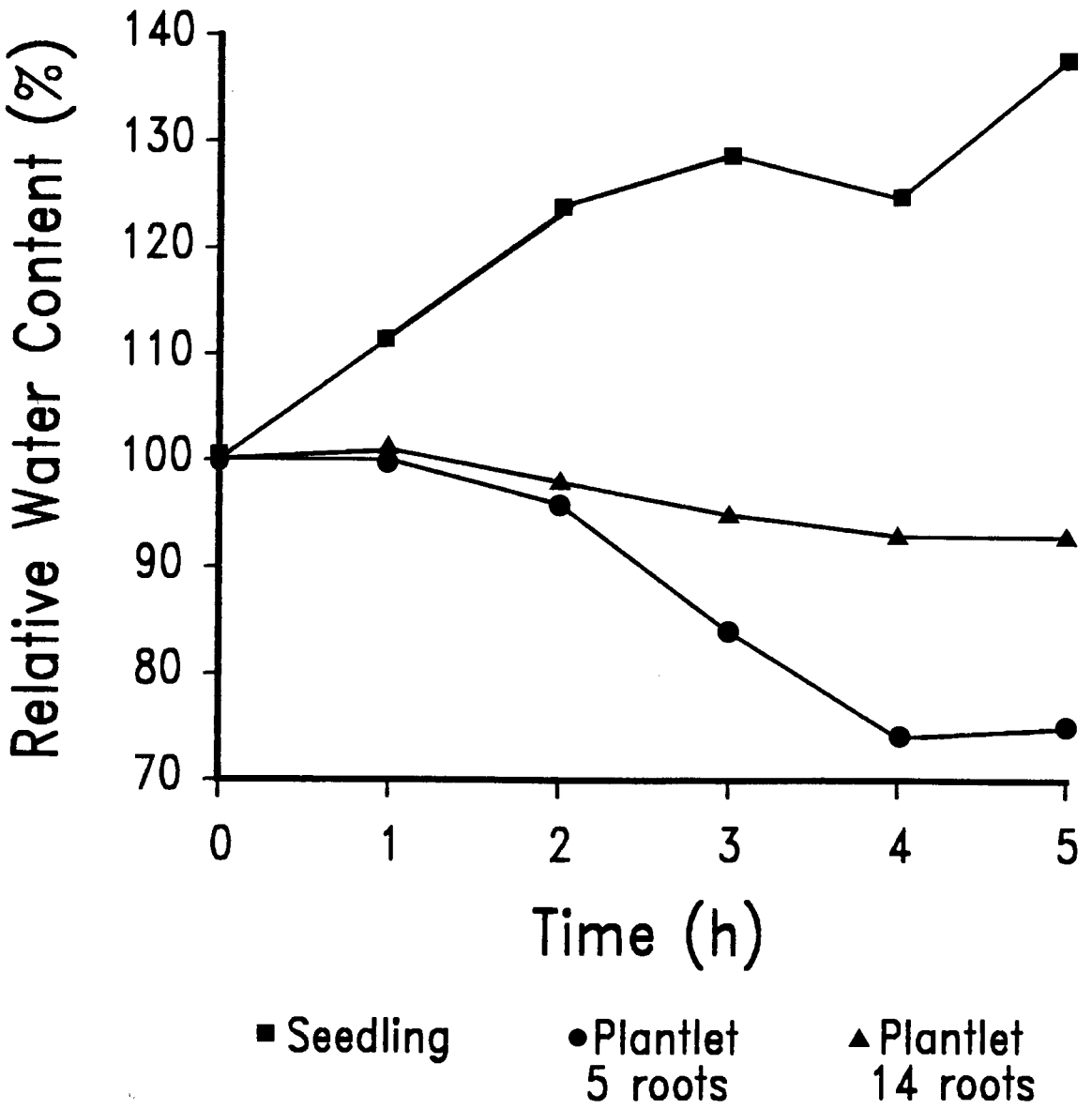
RESULTS AND DISCUSSION

Comparative net water loss in plantlets and seedlings

The observed patterns of response were similar between both trials of this experiment. Results for one trial have been presented (Fig.5). During the experiment, seedling RWC increased. This observation may have been a result of: (i) normal diurnal or circadian fluctuations; (ii) modifications in stomatal behaviour caused by the disruption of transfer; or (iii) higher rates of water uptake because of the higher availability of water in the root zone. No visible wilting was observed, suggesting little or no stress.

Plantlets showed a progressive decrease in RWC, which was mitigated by the presence of many roots. RWC of the plantlet with 14 roots decreased by only 7% while that of the 5-root plantlet dropped by 25%. The latter was also observed to wilt considerably.

Figure 5. Changes in relative water content of seedling and plantlets under stress.



Note: n=1.

Comparisons of water loss among plantlets and seedlings have been made mainly with angiosperms [Grout and Aston 1978; Sutter and Langhans 1982; Dhawan and Bhojwani 1987; Pospisilova et al. 1987; Marin et al. 1988; Sutter 1988]. In all cases, plantlets had considerably higher rates of water loss. But these rates have not been correlated previously to root system characteristics.

Relative water content and water uptake in plantlets

This experiment provided more quantitative information on relationships between morphology and the control of net water loss. Table 6 summarizes these relationships. Both RWC after 3 h and the hourly rate of water uptake were positively correlated with root number, root surface area, and length of the longest root. The equations for these relationships were not straight lines, but tended to be logarithmic.

Root number was a good indicator of root surface area. These variables were related as follows:

$$RSA\ (mm^2) = 13.8\ (Root\ \#),\ R^2=0.95,\ p<0.01$$

The positive relationship between shoot height or needle surface area and water uptake may have been due to a higher overall rate of transpiration and, consequently, a greater demand for water to supply the transpiration stream. It has been reported [Chapter I; Mohammed and Vidaver 1990]

Table 6. Plantlet morphology and the control of net water loss under moderate stress.

Morphological feature [x]	Relative water content after 3 hours [Range 73-91%] [RWC]	Rate of water uptake [24-97 $\mu l \cdot h^{-1}$] [W]
Shoot height [Range 20-35 mm]	not related	$W=10.8\sqrt{x}$ (0.93)
Needle surface area [850-1670 mm^2]	not related	$W=1.6\sqrt{x}$ (0.92)
Root number [1-20]	RWC=84 log(x) (0.92)	$W=18.5\sqrt{x}$ (0.95)
Root surface area [1-500 mm^2]	RWC=38.8 log(x) (0.95)	$W=4.6\sqrt{x}$ (0.96)
Needle surface area/root surface area [4-150]	RWC=466.7/x (0.68)	W=32.6+191.9/x (0.86)
Length of longest root [5-39 mm]	RWC=64.7 log(x) (0.95)	W=20 ln(x) (0.97)

Note: n=15, $p \leq 0.01$.

that as many as 60% of plantlets with tall (41-60 mm) shoots also have 10+ roots, so tall shoots or large total needle surface areas may be suggestive of extensive root systems. The requirement for a well-developed root system is indicated by the inverse relationships between RWC or rate of water uptake and the ratio of needle surface area/root surface area. Poor control of net water loss resulting from the absence of roots would help account for the higher rates of desiccation and mortality which have been observed during *in vivo* rooting of tissue culture-derived plantlets [Mohammed and Vidaver 1988].

Water potential

The correlate of seedling water potential averaged -18 ± 0.3 bars, which was significantly lower than the range observed for plantlets. In addition, plantlets with 2 roots (-6.5 ± 0.3 bars) had a significantly higher reading than did those with 9 roots (-10.9 ± 1.8) or 14 roots (-11.2 ± 2.6).

The water potential correlate of persistent needles had decreased slightly, but not significantly, by the 7th week following transplantation. However, at each sampling date during this period, the readings for new needles were progressively more negative, and, by 7 weeks, the average value was only 3.3 bars higher than that of seedlings. This

decline was not accompanied by any visible indications (e.g. wilting) of stress. Also, new needles resembled those of seedlings. These observations may reflect morphological and anatomical developments during acclimatization. Differences between plantlets (prior to transfer) and seedlings might also result from morphological and/or anatomical variation between these two groups. This aspect warrants further study.

Epicuticular wax

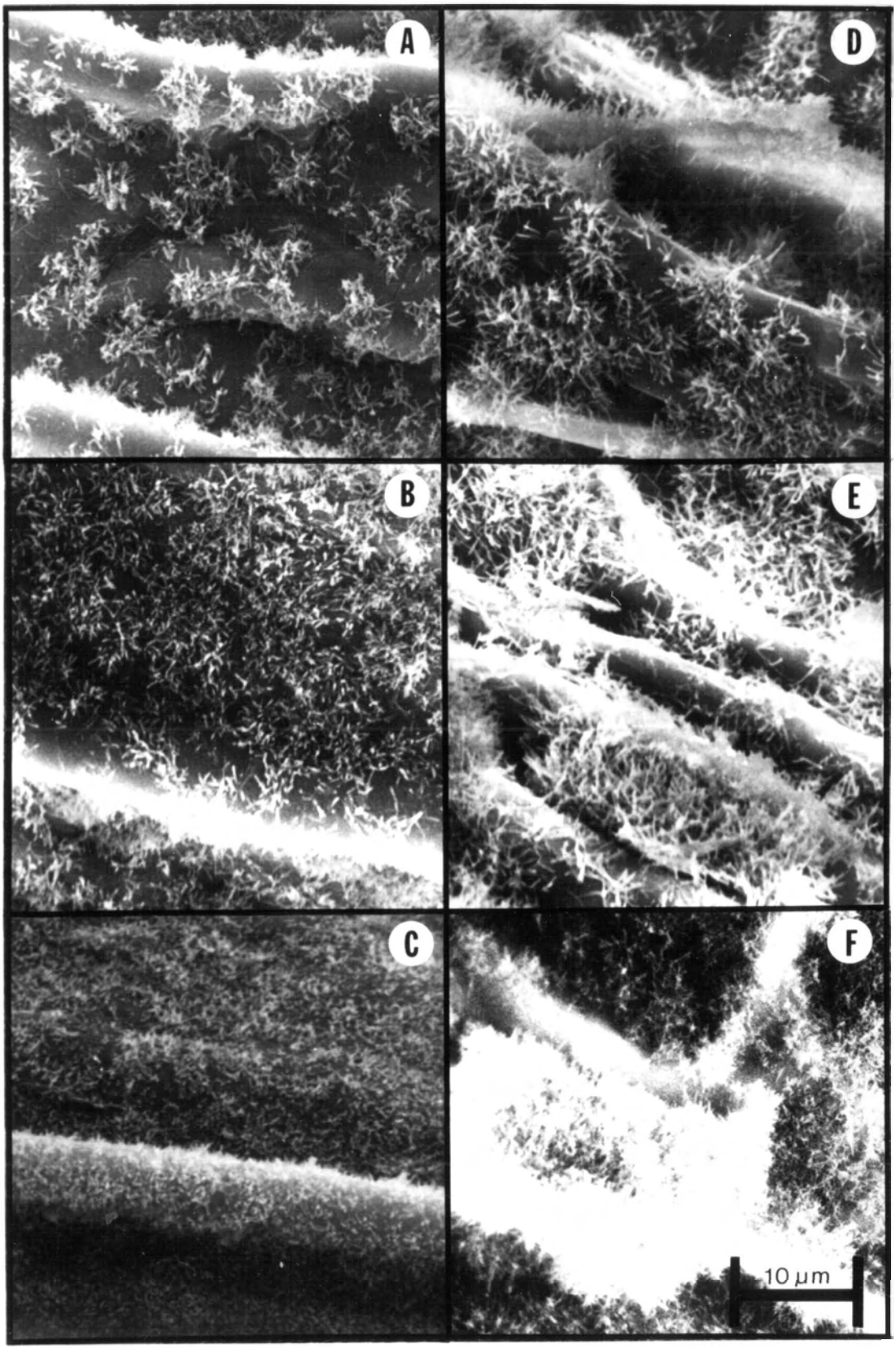
General epicuticular features of seedling needles from Douglas-fir have been described by Thair and Lister [1975]. Wax crystals on plantlet (Fig.6: A,B,D,E) and seedling (Fig.6: C,F) needles were cylindrical in shape, ranging from 0.5 to 3.0 µm in length. Crystals on plantlets, prior to transplantation, tended to occur in discontinuous clusters, particularly on the adaxial surfaces. Wax density was greater on abaxial surfaces of all plants, and was especially high around stomatal regions. New needles from 7-week-old transplants possessed more wax on adaxial and abaxial surfaces than did needles on plantlets prior to transplantation, but had slightly less wax than seedlings. Also, persistent (not shown) and new needles had similar features. These results suggest that while 7-week-old transplants had increased amounts of epicuticular wax

FIGURE 6. Scanning electron micrographs of needle epicuticular wax of plantlets and seedlings. Adaxial [*A*-*C*] and abaxial [*D*-*F*] surfaces.

A,*D*: Plantlet, prior to transplantation, showing discontinuous clusters of wax;

B,*E*: New needles from plantlet, 7 weeks after transplantation;

C,*F*: Seedling.


A
D
B
E
C
F
10 µm

relative to new plantlets, they were not fully acclimatized in this respect.

No differences in wax characteristics were detected among plantlets with varying shoot and root morphology. This was not surprising, as wax production appears to be controlled primarily by environmental conditions such as light intensity, temperature, and humidity [Whitecross and Armstrong 1972; Sutter and Langhans 1982].

SUMMARY AND CONCLUSIONS

Conditions which caused water stress in plantlets apparently did not have the same effect in seedlings. Plantlets were more susceptible to water stress as indicated by their relatively low deposition of epicuticular wax and high water potentials. However, root and shoot morphology were important factors in controlling net water loss in plantlets. In particular, the presence of a well-developed root system was a requirement for maintaining adequate internal water levels.

CHAPTER III

RELATIONSHIPS BETWEEN PLANTLET MORPHOLOGY, GAS EXCHANGE, AND VARIABLE CHLOROPHYLL *a* FLUORESCENCE IN DOUGLAS-FIR

INTRODUCTION

It has been shown that certain characteristics of root and shoot morphology of tissue-cultured Douglas-fir are associated with higher survival and growth during acclimatization, and that some of these features are linked to the control of net water loss [Chapter I, Mohammed and Vidaver 1990; Chapter II]. The present study correlated the photosynthetic ability of plantlets, according to important root and shoot features. These features were: root number, root surface area, length of the longest root, shoot height, needle surface area, and the ratio of needle surface area/root surface area. Plantlet performance was compared to that of conventionally-grown seedlings.

Techniques that were employed included conventional gas exchange in the determination of CO_2 uptake, dark respiration or root respiration, and variable chlorophyll *a* fluorescence. The fluorescence technique has emerged as a promising tool in the physiological assessment of quality of

plants, including gymnosperms [Krause and Weis 1984; Conroy et al. 1988; Öquist and Wass 1988; Toivonen and Vidaver 1988; Vidaver et al. 1990]. So far, it has been used only twice in assessing quality of micropropagated plantlets: with white spruce [Toivonen 1985], and with red raspberry [Donnelly 1984]. The instrument that was used in this study was first described in Toivonen and Vidaver [1984], and is unique in that it utilizes an integrating sphere as a measurement chamber, so that fluorescence results are obtained for an intact shoot rather than for individual needles as is more commonly done. The system is non-invasive and non-destructive, permits repeated sampling of the same plant, and provides information on plant size.

MATERIALS AND METHODS

Plantlets and seedlings were produced according to the general protocol outlined in Section B.

CO_2 uptake, variable chlorophyll *a* fluorescence, root respiration

One batch of plantlets was used to supply three samples of sixteen to twenty plantlets per sample. The first sample was tested and sacrificed at transplantation (Week 0). The second sample was tested and sacrificed at Week 1. The third

sample was tested at Weeks 3, 5, and 7, and sacrificed on the final testing date. Eight to nine seedlings were used as comparisons in gas exchange and fluorescence measurements. Plants were measured either in their transplant pots (assemblies inserted into appropriately-sized cuvettes) or following transfer to Kaputs containing water. The container was found not to influence results significantly during the measurement period of these experiments.

Net CO_2 exchange was measured using a Model 865 Beckman infra-red gas analyzer (IRGA), and an ambient CO_2 range of 338-374 $\mu l \cdot l^{-1}$. Plants were positioned to receive 366 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPFD from a GE Lucolox high pressure sodium lamp. Other conditions were 54-62% relative humidity, 23 $\pm$ 2°C temperature, and 97.6 $\pm$ 0.5 kPa atmospheric pressure. Two 3-minute readings were taken and averaged for each plant. Finally, plants were covered with black cloth to obtain readings for dark respiration. Gas exchange was measured at all sampling dates.

Variable chlorophyll *a* fluorescence was monitored with the integrating fluorometer, as described in Toivonen and Vidaver [1984], which was interfaced to a personal computer for data acquisition and processing. The fluorometer normalized raw fluorescence data to compensate for differences in plant size. Plants were dark-adapted for

20 min and measured at 95 $\mu mol \cdot s^{-1} \cdot m^{-2}$ from a filtered tungsten halogen lamp (EFP 12V 100W). Atmospheric conditions were similar to those for gas exchange. Fluorescence was monitored at all sampling dates.

Root respiration was measured after transferring each plant to a Kaput. Apiezon Q black sealing compound was applied around the base of the stem and Kaput opening to seal off the shoot, thereby exposing only the roots to the gas chamber. The chamber was then covered with two layers of black cloth during measurement. Root respiration was measured at Week 0 and Week 1 only.

At transplantation, the following plantlet features were recorded: root number, root surface area, length of the longest root, needle surface area/root surface area, and shoot height. Estimates of needle surface areas (refer to Section B) were made at the various sampling times for use in gas exchange calculations. Dry weights were obtained following oven-drying at 80°C and cooling in a desiccator. Relationships were sought between initial morphology and physiological performance.

CO_2 uptake under varying light levels

Two plantlets (at transplantation) from each of two root number categories, and two seedlings, were transferred from their containers to Kaputs containing 15 ml water. CO_2 uptake was measured as described above, except that various light levels ranging from 35 to 600 $\mu mol \cdot s^{-1} \cdot m^{-2}$ were used. Light variation was achieved by adjusting the distance between the plant and the light source. Dark respiration was measured after the complete range had been tested. CO_2 exchange was calculated on a needle surface area basis. This experiment was repeated twice.

CO_2 uptake under stress

Two plantlets (at transplantation) from each of three root number categories, and two seedlings, were transferred from their containers to Kaputs containing 15 ml water. These were set out on a laboratory bench with atmospheric conditions of 54-62% R.H. and 23 $\pm$ 2°C. Lighting was a combination of natural (through window) and artificial (fluorescent ceiling lights) to give a total PPFD of approximately 32 $\mu mol \cdot s^{-1} \cdot m^{-2}$. CO_2 uptake was measured as described above, at 2-h intervals over a 6-h period. Dark respiration was measured at each time. CO_2 exchange was calculated on a needle surface area basis. This experiment was repeated once.

RESULTS AND DISCUSSION

CO_2 uptake

In comparison to seedlings, plantlets at Week 0 had significantly lower net rates of CO_2 uptake. The average rate for seedlings was 7.0 ± 0.5 mg·h^{-1}·dm^{-2}. Net uptake rates among plantlets, including a random assortment of morphologies, averaged 1.8 mg·h^{-1}·dm^{-2} at Week 0, decreased slightly to 1.1 a week later, then gradually increased over subsequent weeks to reach 2.7 mg·h^{-1}·dm^{-2} by Week 7. At Week 0, net CO_2 uptake ranged from -0.5 to 3.8 mg·h^{-1}·dm^{-2}. The rate was positively related to root number, root surface area, shoot height, needle surface area and needle dry weight (Table 7). At Week 0, no relationship was identified between CO_2 uptake and the ratio of needle surface area/root surface area (NSA/RSA), or the length of the longest root. (The 5-week results are presented in Table 8. Similar patterns were identified at 3 and 7 weeks after transplantation.) Uptake was positively related to initial shoot height, needle surface area, root number, and root surface area. It was inversely related to NSA/RSA, and was unrelated to the length of the longest root.

Little previous research has been done on CO_2 uptake in tissue-cultured gymnosperms. In white spruce, Toivonen [1985] showed that CO_2 uptake competence (on a chlorophyll

Table 7. Plantlet morphology and net CO_2 uptake, at transplantation.

Morphological feature [x]	Net CO_2 uptake [Range 0.5-3.8 $mg \cdot h^{-1} \cdot dm^{-2}$] [U]	R^2
Shoot height (mm) [Range 15-32 mm]	U=0.079x	0.85
Needle surface area [0.07-0.15 dm^2]	U=16.5x	0.83
Needle dry weight [35-70 mg]	U=0.036x	0.81
Root number [1-36]	$U=0.63\sqrt{x}$	0.76
Root surface area [0.001-0.27 dm^2]	U=-0.74 log(x)	0.72

Note: n=20, $p \leq 0.01$.

Table 8. Relationship between initial plantlet morphology and gas exchange, 5 weeks after transplantation.

Morphological feature [x]	Net CO_2 uptake [Range 0.5-5.3 $mg \cdot h^{-1} \cdot dm^{-2}$] [U]	Dark respiration [0.15-2.1mg $CO_2 \cdot h^{-1} \cdot dm^{-2}$) [DR]
Shoot height [Range 29-36 mm]	U=0.075x (R^2=0.72)	not related
Needle surface area [0.13-0.15 dm^2]	U=16.7x (0.72)	not related
Root number [2-22]	U=0.72$\sqrt{x}$ (0.75)	DR=2.96/$\sqrt{x}$ (0.87)
Root surface area [0.002-0.13 dm^2]	U=15.9$\sqrt{x}$ (0.7)	DR=0.1/$\sqrt{x}$ (0.84)
Needle surface area/root surface area [8-68]	U=6.9/$\sqrt{x}$ (0.74)	DR=0.98 log(x) (0.85)
Length of longest root [6-46 mm]	not related	DR=1.1/log(x) (0.82)

Note: n=19, $p \leq 0.01$.

basis) of plantlets at transplantation was comparable to seedlings, but over the following month, a serious physiological deterioration occurred in plantlets. It was suggested that roots, which were produced in agar, may have been non-functional and, hence, were poor sinks for photosynthate. The findings of the present study, which used peat:perlite as opposed to agar as a rooting substrate, did not reflect a deterioration in performance but, rather, an improvement. Previous work on Douglas-fir has indicated the unsuitability of agar as a rooting substrate [Mohammed et al. 1989].

Other studies, on cultures of angiospermous, have reported lower photosynthetic rates than controls [Grout and Aston 1978; Donnelly and Vidaver 1984b]. However, Smith et al. [1986] reported that in birch [*Betula platyphylla*], photosynthetic activity was enhanced following rooting and was then comparable to that of seedlings. It was not known whether this resulted from the rooting environment or the presence of roots.

When comparing coniferous plantlets to seedlings, a certain degree of caution must be exercised, because of differences in needle age and origin. (For example, it has been reported that in many species of *Pinus*, *Picea* and *Abies*, as foliar age increases, net photosynthesis decreases [Daniel et al. 1979].) In the present study, plantlet

shoots which were compared to seedlings possessed some needles which had been present for 20 weeks or longer, including the initial shoot elongation period prior to the rooting treatment. Seedling needles were less than half that age. Thus, the lower CO_2 uptake of plantlets may be partly accounted for by the higher proportion of older needles. Also, 7-week-old transplants consisted of persistent and new needles. These needles might be expected to vary in photosynthetic efficiency [Donnelly and Vidaver 1984b] depending on origin, but, of course, this would not be evident in an integrated measurement.

It has been suggested that whether or not plantlets are photosynthetically competent in the initial weeks of acclimatization may be a secondary consideration in survival [Wainwright and Scrace 1989]. This is because the acclimatization environment, like the *in vitro* culture environment [Neuman and Bender 1987; Pospisilova et al. 1988; Solarova 1989], probably limits photosynthesis as a consequence of low availability of CO_2. The primary requirement might then be a sufficient store of carbohydrate reserves to nourish plantlets until new needles are produced and plantlets can be transferred to more normal conditions.

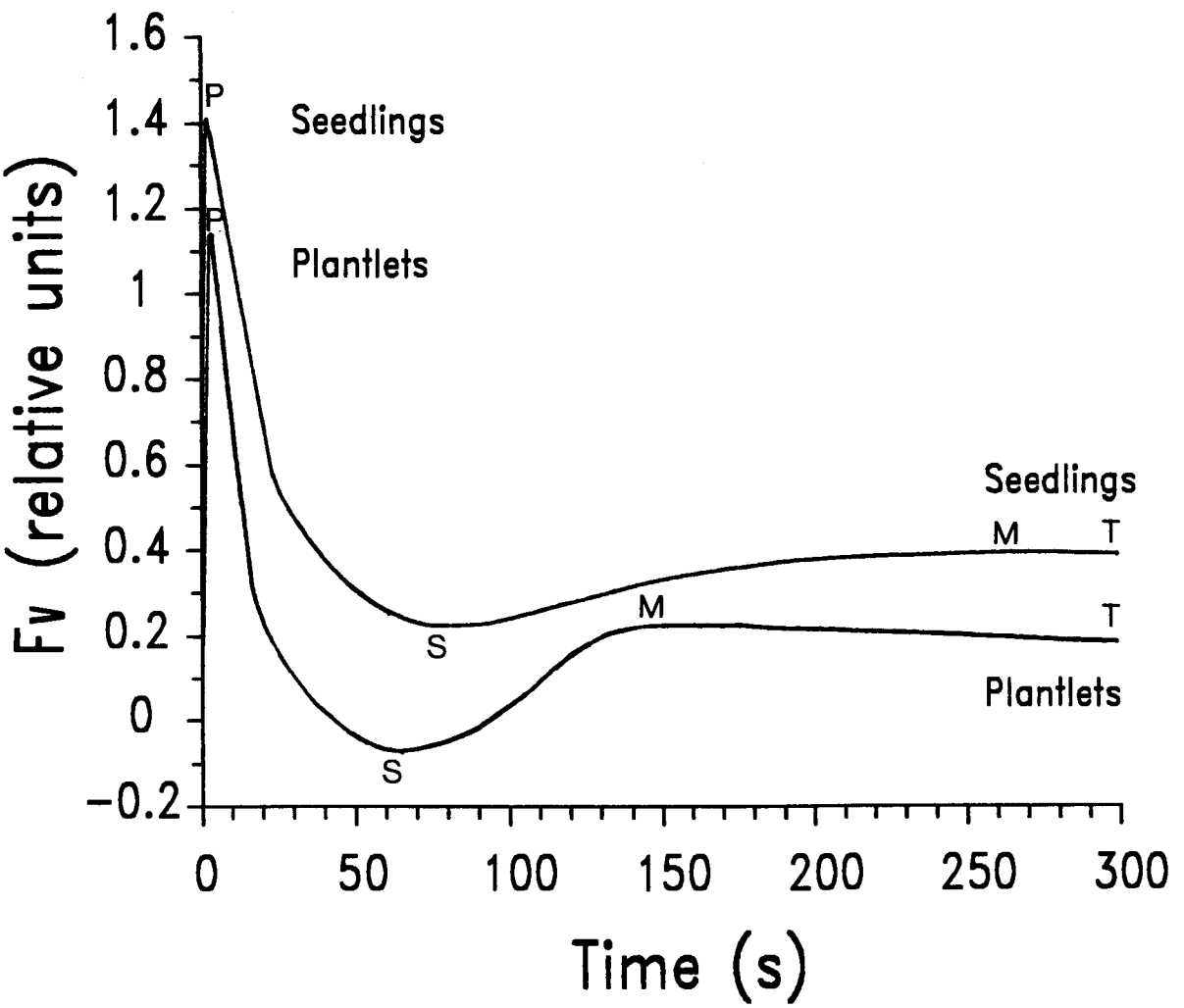
Dark respiration

The rate of dark respiration increased during the 3 weeks following transplantation, going from 0.8 ± 0.2 to 2.1 ± 0.2 mg $CO_2 \cdot h^{-1} \cdot dm^{-2}$. Subsequently, the rate decreased and was 1.7 ± 0.2 $mg \cdot h^{-1} \cdot dm^{-2}$ at 7 weeks. Prior to the 3rd week, dark respiration was not associated with morphological features. However, from Weeks 3-7, the rate was inversely related to initial root number, root surface area, and the length of the longest root (Table 8, 5-week results). It was positively related to NSA/RSA. No associations with initial shoot height or needle surface area were found.

Variable chlorophyll *a* fluorescence

Fluorescence induction curves for seedlings and plantlets at Week 0 are shown in Figure 7. The typical features of such curves, known as 'Kautsky curves' have been described in Krause and Weis [1984]. 'P' is taken as the distance in relative units from the x-axis to the maximum peak, 'S' is the distance from the x-axis to the mimumum point, 'M' is the vertical rise from the minimum point to the next peak, and 'T' is the distance from the x-axis to the point on the curve at 300 s. The data have been normalized by the measurement apparatus to compensate for differences in O-level. Values for P, S, and T were significantly different between the two plant groups.

Figure 7. Variable chlorophyll a fluorescence of seedlings and plantlets.



Note: n=9 seedlings, 20 plantlets.

Features of plantlet curves were tested for any relationships to initial morphology. Similar patterns were identified throughout the sampling period. Results from Week 5 are representative and have been presented in Table 9. P increased with initial root number, root surface area, and the length of the longest root. It was inversely related to NSA/RSA, and unrelated to initial shoot height or needle surface area. P is considered to be an indicator of water splitting activity [Krause and Weis 1984; Vidaver et al. 1989]. It is not surprising that water splitting activity was higher in plantlets with good root systems, since it was shown [Chapter II] that such plantlets are better able to maintain internal water levels.

M was positively related to initial root number, root surface area, and shoot height, inversely related to NSA/RSA, and unrelated to needle surface area or the length of the longest root (Table 9). In white spruce, lower M values have been attributed to reduced CO_2 uptake [Toivonen and Vidaver 1988; Vidaver et al. 1988; Vidaver et al. 1989]. This interpretation was supported by the present findings, whereby net CO_2 uptake (y) in $mg \cdot h^{-1} \cdot dm^{-2}$ was related to M according to the equation:

$$y=14.33(M),\ R^2=0.73,\ p<0.01 \text{ (5-week results).}$$

CO_2 uptake was unrelated to other features of the curve.

In order to maintain the validity of the general

Table 9. Relationship between initial plantlet morphology and chlorophyll *a* fluorescence parameters, 5 weeks after transplantation.

Morphological feature [x]	Parameter [y] P[1] [Range 0.45-1.4]	M[2] [0.06-0.22]	(P-S)/time (min) [0.4-1.1]
Shoot height [Range 29-36 mm]	not related	$y=0.005x$ ($R^2=0.92$)	not related
Root number [2-22]	$y=0.26\sqrt{x}$ (0.93)	$y=0.16 \log(x)$ (0.94)	$y=0.2\sqrt{x}$ (0.96)
Root surface area [0.002-0.13 dm^2]	$y=5.6\sqrt{x}$ (0.86)	$y=\sqrt{x}$ (0.82)	$y=4.4\sqrt{x}$ (0.89)
Needle surface area/root surface area [8-68]	$y=2.5/\sqrt{x}$ (0.92)	$y=0.46/\sqrt{x}$ (0.91)	$y=1.9/\sqrt{x}$ (0.97)
Length of longest root [6-46 mm]	$y=0.69 \log(x)$ (0.92)	not related	$y=0.15\sqrt{x}$ (0.93)

Note: n=19, $p \leq 0.01$.

[1]Distance from x-axis to the maximum peak on the curve.
[2]Vertical rise from the minimum point S to the next peak.

statement that CO_2 uptake increases with M, it had to be applied within a population. For example, M for seedlings was lower than that for plantlets (Figure 7), although seedlings had a much higher rate of net uptake. Therefore, judging the photosynthetic ability of plantlets and seedlings simply by comparing M-values would lead to incorrect conclusions.

The rate of decline between P and S was used as a marker of quenching rate. Quenching occurs through electron transport as well as other processes [Hipkins and Baker 1986], and its presence generally indicates normal photosynthetic functioning. Quenching rate was positively correlated with initial root number, root surface area, and the length of the longest root. It was inversely related to NSA/RSA, and unrelated to shoot height or needle surface area (Table 9).

High root numbers and root surface areas were, therefore, consistently linked with photosynthetic efficiency. The length of the longest root was apparently associated with water splitting activity and quenching rate. Shoot height appeared to be linked primarily to net CO_2 uptake. Needle surface area did not appear to be correlated to photosynthetic parameters, although such relationships have been identified by gas exchange as discussed above.

Root respiration

Rates of root respiration in 1-week-old transplants ranged from 0 to 32.2 mg $CO_2 \cdot h^{-1} \cdot dm^{-2}$, on a root surface area basis. At Week 0, rates were considerably higher, ranging from 5.3 to 142 $mg \cdot h^{-1} \cdot dm^{-2}$. Seedling rates were uniformly low at 4.0 ± 0.3 $mg \cdot h^{-1} \cdot dm^{-2}$. At Week 0 and Week 1, plantlet rates were inversely related to initial root number, root surface area, and the length of the longest root (Table 10). They were positively related to NSA/RSA, and unrelated to shoot height, needle surface area, or needle dry weight. These relationships reflected those obtained for dark respiration at Weeks 3-7.

There are several possible explanations for the lower respiration rates observed in larger root systems. For example, it is possible that metabolic activity is being channelled into the production of other compounds (e.g. amino acids, nucleotides, pigment precursors) at the expense of CO_2 production. Roots would then serve as efficient sinks for photosynthate and would also support higher photosynthetic activity in the shoot through the production of useful compounds. In measuring CO_2 evolution, this study assumed that complete oxidation of respiratory substrates was taking place. Since this is not always true [Salisbury and Ross 1985], CO_2 evolution can be only a measure of apparent respiration. Also, Johnson-Flanagan and Owens

Table 10. Relationship between plantlet morphology and root respiration.

Morphological feature [x]	Root respiration (mg $CO_2 \cdot h^{-1} \cdot dm^{-2}$) [y]	
	At time of transplantation (n=16) [Range 5.3-142]	1 week after transplantation (n=36) [0-32.2]
Root number [Range 3-18]	$y=215/x$ ($R^2=0.90$)	$y=27.5/\sqrt{x}$ (0.65)
Root surface area [0.003-0.37 dm^2]	$y=0.15/x$ (0.96)	$y=1/\sqrt{x}$ (0.73)
Needle surface area/root surface area [6-78]	$y=-3.2+1.4x$ (0.91)	$y=0.76x$ (0.73)
Length of longest root [5-35 mm]	$y=424/x$ (0.82)	$y=124/x$ (0.74)

Note: $p \leq 0.01$.

[1986] indicated that elongating vs. absorbing roots of white spruce may undergo different amounts of alternative pathway respiration. Similar processes may be operating in plantlets of Douglas-fir.

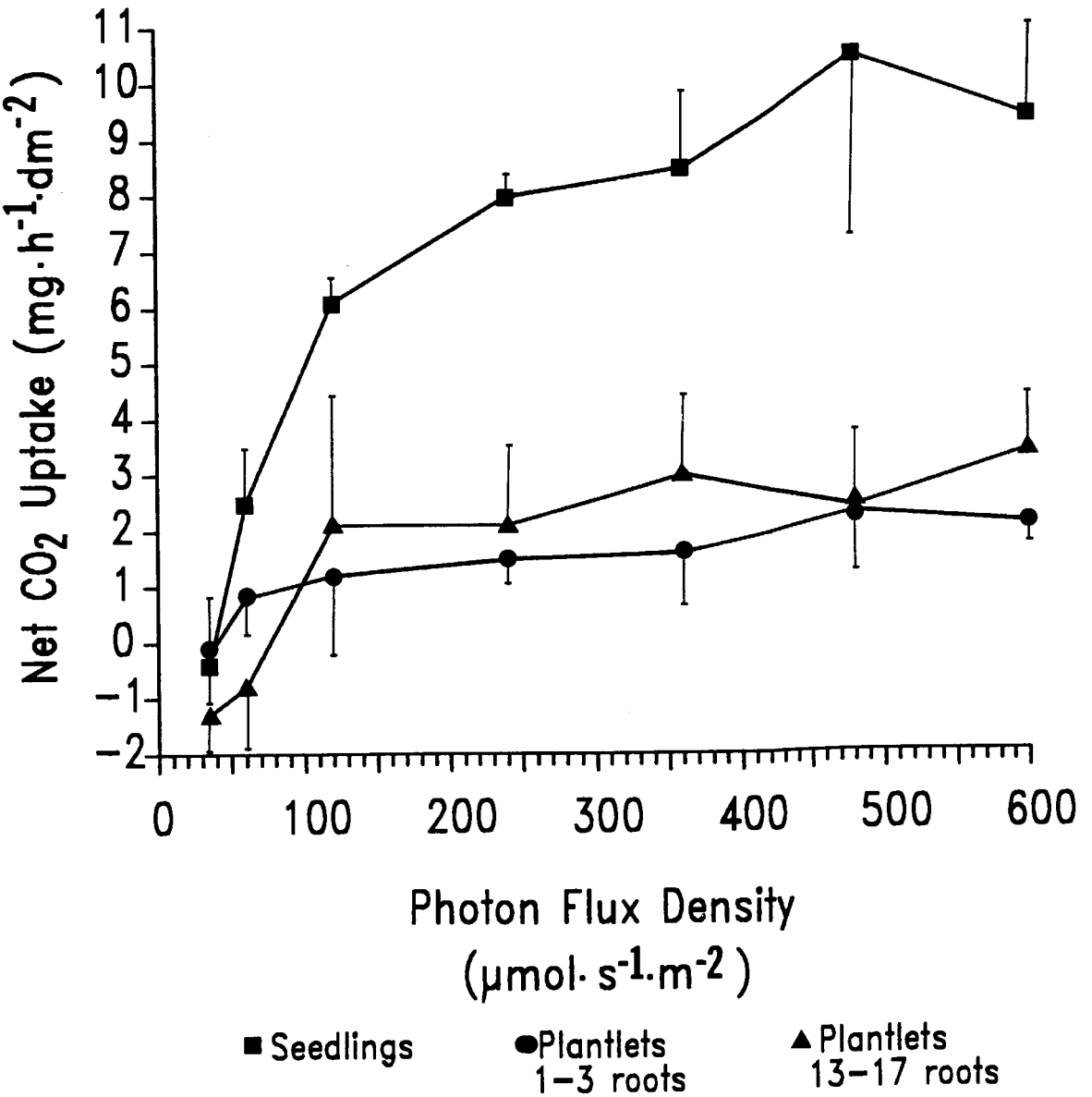
CO_2 uptake under varying light levels

Net CO_2 uptake by seedlings increased substantially with photon flux density (Figure 8). The highest rate was 10.5 $mg \cdot h^{-1} \cdot dm^{-2}$, which was recorded at a PPFD of 480 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Plantlets were much less responsive than seedlings, showing only slight increases in net uptake. Plantlets with 13-17 roots had slightly higher uptake rates than those with 1-3 roots. The light compensation point for seedlings was 40 $\mu mol \cdot s^{-1} \cdot m^{-2}$, while that for plantlets was 40 (few roots) or 75 (many roots) $\mu mol \cdot s^{-1} \cdot m^{-2}$.

In a similar study by Donnelly [1984], it was shown that net CO_2 uptake by plantlets of red raspberry changed little with increasing light intensity, unlike that of field-grown controls which showed marked increases. This was partly attributed to lack of anatomical specialization (e.g. less developed palisade layers, reduced xylem area) in cultured leaves [Donnelly and Vidaver 1984a].

The inability of plantlets to utilize relatively high light levels for photosynthesis renders them highly susceptible to photodamage, especially under conditions of

Figure 8. Effect of light on net CO_2 uptake by plantlets and seedlings.



Note: n=2, standard errors shown as bars.

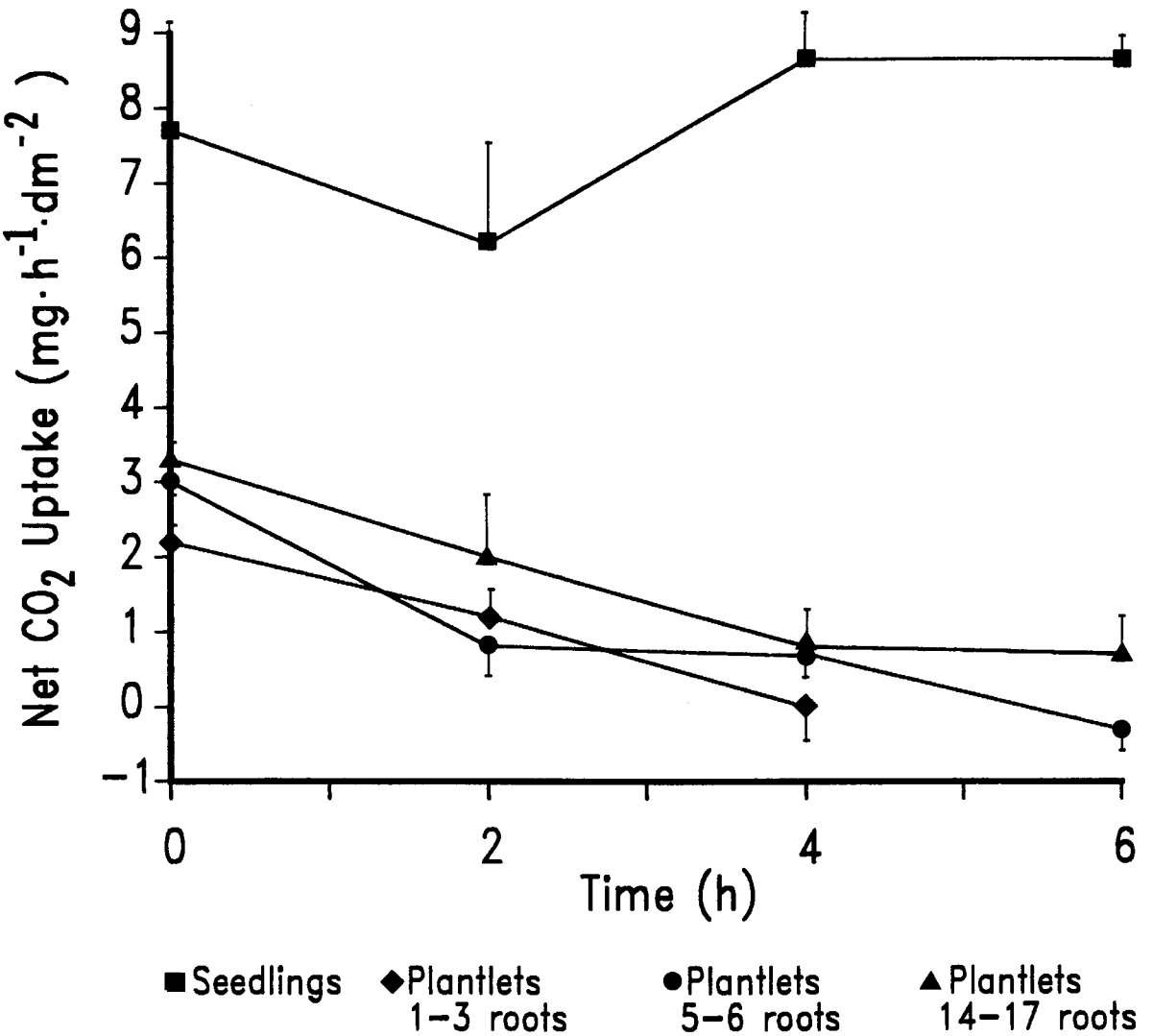
environmental stress. Photodamage results from the formation of reactive oxygen species, with consequent inactivation of chlorophyll reaction centers and ultimate photodestruction of pigments [Anderson 1986]. Hence, it is important either to keep light levels low during the initial stages of acclimatization, or to modify the culture protocol to produce plantlets which are anatomically and physiologically capable of functioning under relatively intense light. The former approach is usually followed.

CO_2 uptake under stress

Under imposed stress, net CO_2 uptake initially decreased for all plant types (Figure 9). However, uptake in seedlings increased after 2 h, and by 4 h, had recovered to normal levels (compare Figure 8, 360 $\mu mol \cdot s^{-1} \cdot m^{-2}$). Meanwhile, uptake rates among plantlets remained depressed. Among the three root number categories, plantlets with 14-17 roots were the only group with net positive rates after 6 h and beyond (not shown). These plantlets also showed indications of stabilization of CO_2 uptake after about 4 h.

In Chapter II, it was shown that plantlets with many roots can better adjust for net water loss by more efficient water uptake. Therefore, these plantlets would be less vulnerable to water stress and would be expected to suffer less impairment of photosynthetic function than plantlets

Figure 9. Net CO_2 Uptake of plantlets and seedlings under stress.



Note: n=2, standard errors shown as bars.

with few roots. This was observed to be the case. The differences in CO_2 uptake were not dramatic, but even slight differences might be sufficient to prevent photodamage.

SUMMARY AND CONCLUSIONS

In general, plantlets at all stages had lower apparent photosynthetic activity than seedlings. This included lower net CO_2 uptake, less responsiveness to increased PPFD, and depression of CO_2 uptake under mechanical disruption and water stress. Results obtained with gas exchange were correlated to those obtained with variable chlorophyll *a* fluorescence. Plantlets also had substantially higher rates of root respiration.

These features could all contribute to poorer performance of transplants relative to seedlings. However, important correlations were identified between plantlet morphology at transplantation and subsequent performance. High root number, high root surface area, and low NSA/RSA were associated with higher CO_2 uptake, a correlate of water splitting activity, and quenching rate, and were also associated with lower rates of dark and root respiration. Shoot height and needle surface area were positively correlated to CO_2 uptake. The length of the longest root was positively related to water splitting and quenching

rate, and was inversely correlated to dark and root respiration.

Chlorophyll fluorescence, a promising analytical tool in this study, is essentially a qualitative method. Results can be calibrated against data obtained by conventional physiological methods such as gas exchange, and subsequently applied in non-destructive quality assessments. This is the subject of intensive investigation by Simon Fraser University's Ecophysiology Research Group (W.E. Vidaver and Associates). The present study suggests a future role for this technique in non-disruptive assessment of root system quality, since fluorescence parameters were strongly linked to features such as root number, root surface area, and the length of the longest root (representative of root system length).

CHAPTER IV

MORPHOLOGICAL COMPARISONS BETWEEN SEEDLINGS AND PLANTLETS OF DOUGLAS-FIR

INTRODUCTION

Morphological comparisons between gymnosperm seedlings and micropropagated plantlets have shown many important differences [Mohammed and Vidaver 1988]. These differences pertain primarily to root systems. For example, seedling root systems of loblolly pine had ten times as many lateral roots as plantlets after 6 weeks in the greenhouse, and plantlet roots were short with large diameters [McKeand and Wisniewski 1982; McKeand and Allen 1984]. Therefore, the surface area available for nutrient absorption was reduced in plantlets. Poorly-developed root systems may even be implicated in early maturation [Franclet et al. 1980]. Early maturation is a common phenomenon in plantlets of radiata pine [Smith et al. 1982], maritime pine [*Pinus pinaster* Sol.] [Franclet et al. 1980], loblolly pine [Frampton et al. 1985; McKeand 1985], and Douglas-fir [Timmis and Ritchie 1984; Ritchie and Long 1986].

This study investigated the morphology of seedlings and plantlets of Douglas-fir, with two objectives:

(i) to identify morphological differences which may help to account for the poor ability of plantlets to photosynthesize and withstand water stress; and (ii) to determine how plantlets compared with seedlings after a number of weeks in the greenhouse. Other findings from this study allude to why tall shoots may be better able to survive under stress, as was shown in Chapter I.

MATERIALS AND METHODS

Morphological assessments

Thirty-six plantlets, representing a random collection of morphologies, were produced according to the general method outlined in Section B. One-half of the plantlets were sacrificed for morphological assessments at the end of the rooting treatment, and the other half were acclimatized according to the general protocol and sacrificed at 7 weeks following transplantation. Prior to transplanting, shoot height and total root length were recorded. Eight seedlings were sacrificed at the same time as the 7-week-old transplants.

The following morphological features were recorded for all plants: whole plant dry weight (DW), needle DW, stem DW, shoot DW/root DW, shoot height, number of axillary shoots, needle surface area (NSA)/shoot height, number of

needles/shoot height, average needle length, root number, number of roots≥1 mm long, number of lateral roots, length of the longest root, total root length, total root length/root DW, root surface area (RSA)/root DW, NSA/RSA, average length of primary roots, and average length of lateral roots. In addition, original (present at transplantation) and new tissue from 7-week-old transplants were separated and the following features were assessed: stem DW, needle DW, number of needles/shoot height, average needle length and NSA/shoot height. For dry weights, plants were oven-dried at 80° for 24 h and cooled in a dessicator.

Morphologies were compared among the three groups of plants. Also, the possible relationships between initial root number or shoot height and final features were investigated.

Stem anatomy

To compare the internal anatomy of transplant and seedling stems, stem segments were excised from five 7-week-old transplants and five seedlings. Segments from transplants were taken from original and new tissue, at 1 cm below the start of new growth and at 1 cm below the tip of new growth, respectively. These were fixed for 24 h in FAA (50 ml ethanol:10 ml formalin:5 ml acetic acid:35 ml water). The tissue was then dehydrated in a tertiary butanol series

and embedded in Paraplast[R] (Fisher Scientific) [Sass 1958; O'Brien and McCully 1981]. Ten-µm sections were cut with a rotary microtome, stained for 20 min in a mixture of 0.5% safranin O, 0.2% basic fuschin, and 0.2% crystal violet in 50% ethanol, then counterstained for 4-5 s in 0.5% fast green FCF in absolute ethanol [Yeung and Peterson 1972].

RESULTS AND DISCUSSION

The morphological features of plantlets and seedlings are summarized in Table 11. Compared to seedlings and 7-week-old transplants, new transplants had relatively poor root systems. This was shown in the higher NSA/RSA, shoot DW/RDW, and lower lateral root number, root length, and RSA/RDW. Poorer development of roots relative to shoots could partly explain the susceptibility to water stress and the low photosynthetic activity that were described in Chapters II and III. For example, roots are a source of growth regulators and other compounds which are essential for photosynthesis [Boote 1977].

Relative to seedlings, new and 7-week-old transplants had higher shoot DW/RDW, and the length of the longest root was lower. These plus other differences indicate that, even after 7 weeks, plantlet roots were still not as well developed in all aspects as seedling roots. However, both

Table 11. Comparative morphology of seedlings and plantlets.

Morphological feature	Plantlets at transplant (n=18)	Plantlets 7 weeks after transplant (n=18)	Seedlings (n=8)
Whole plant DW	75.9 a	237.6 b	70.1 a
Needle SA/SH[1]	37.2 b	37.9 b	27.4 a
# needles/SH[1]	3.7 b	3.2 b	1.6 a
Needle length[1] (mm)	11.9 a	14.7 b	20.8 c
Needle DW/SH[1]	2.3 c	1.6 a	1.9 b
Stem DW/SH[1]	0.3 b	0.6 c	0.1 a
Total needle SA/ total root SA	26.1 b	1.4 a	0.6 a
Total shoot DW/ total root DW	51.8 c	8.1 b	2.6 a
Root number	7.9 a	32.9 b	16.9 a
# primary roots	7.9 b	10.1 b	1.0 a
# lateral roots	0.0 a	22.9 b	15.9 b
Length (cm) of longest root	1.4 a	10.6 b	21.7 c

Over/

Table 11: Continued

Total root length (cm)	5.6 a	53.8 b	45.5 b
Total root length (cm)/total root DW	2.8 a	2.5 a	2.5 a
Total root SA/ total root DW	31.4 a	59.0 b	62.0 b
Length (cm) primary roots	0.8 a	4.9 b	21.7 c
Length (cm) lateral roots	-	0.2 a	1.6 b

Abbreviations: DW=dry weight (mg), SH=shoot height (mm), SA=surface area (mm^2).

Note: Results within a row followed by the same letter were not significantly different, according to t-tests, or Duncan's multiple range test. Needles which were not fully expanded were considered to be part of the stem. Root measurements were taken from the root collar. Except for whole plant DW, shoot parameters for seedlings do not include hypocotyl. Plantlets represented a random sample of morphological types.

[1] Determined for a dominant shoot (central or axillary).

groups did not differ significantly with respect to lateral root number, root length, RSA/RDW, and NSA/RSA.

The morphological features of original and new shoot segments from 7-week-old transplants are listed in Table 12. Original portions had a higher stem DW/SH, lower NDW/SH, and shorter needles, but similar NSA/SH. They also had higher (but not significantly higher) needle number/SH. The higher stem DW/SH can be attributed to a greater amount of secondary xylem in original portions (Figure 10A, 10B). Original stem portions also had more secondary xylem than seedling stems (Figure 10C).

Of special interest is the discovery of lower NDW/SH. This value was 2.3 $\pm$ 0.07 $mg \cdot mm^{-1}$ for needles at transplantation, but only 1.1 $\pm$ 0.05 $mg \cdot mm^{-1}$ for persistent needles of 7-week-old transplants. Two possible explanations could account for this reduction in needle dry weight. First, needles may have died under the relatively harsh, new conditions. If this were the case, we would not expect to find a great difference in the NDW/SH value among individual plantlets, since new conditions would have affected all of them equally. The second possibility is that the original needles were acting as nutrient reserves which were utilized and depleted over time. Under this hypothesis, we might expect to find differences in the NDW/SH among plantlets according to the degree of new

Table 12. Plantlet[1] morphology of original and new shoot segments, 7 weeks after transplantation.

Morphological feature	Shoot segment	
	Original	New
Stem dry weight (mg)/ shoot height (mm)	0.7 b	0.4 a
Needle dry weight (mg)/ shoot height (mm)	1.1 a	2.6 b
# needles/shoot height (mm)	3.7 a	2.6 a
Needle length (mm)	11.5 a	17.3 b
Needle surface area (mm^2)/ shoot height (mm)	37.2 a	37.9 a

Note: n=18. Results within a row followed by the same letter were not significantly different, according to t-tests. Results were taken from a dominant shoot (central or axillary).

[1]Plantlets were 7-week-old transplants described in Table 11.

FIGURE 10. Stem anatomy of seedlings and 7-week-old transplants.

A: Plantlet, original portion of stem, with considerable amount of secondary xylem;

B: Plantlet, new growth;

C: Seedling.

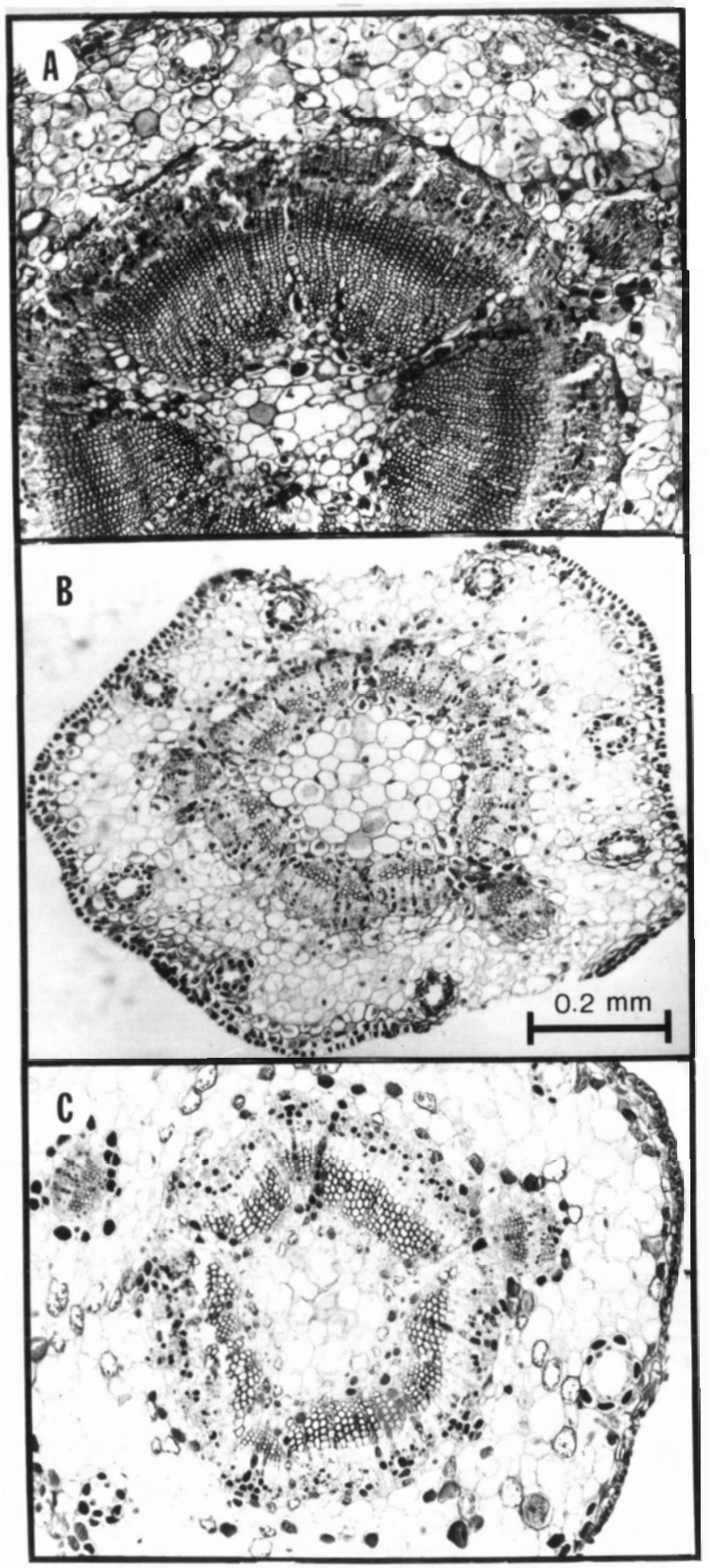


FIGURE IO

growth.

Various relationships between NDW/SH of old needles and the morphology of 7-week-old transplants were tested. NDW/SH was found to be inversely correlated to new root length/old root length according to the equation:

$$NDW/SH = 2.3/\sqrt{(NRL/ORL)},\ R^2=0.89,\ p<0.01,$$ range of NDW/SH=0.9-1.7, NRL/ORL=1-31.

It was positively correlated to new shoot length/old shoot length according to the equation:

$$NDW/SH = 1.6\sqrt{(NSL/OSL)},\ R^2=0.95,\ p<0.01,$$ range of NSL/OSL=0.2-1.4.

Therefore, as the proportion of new root growth increased, NDW/SH was diminished. This effect did not occur if considerable new shoot growth took place. This finding indicates that the second hypothesis may be true, and that old needles were acting as nutrient reserves to support new root extension in the absence of shoot activity. This result is consistent with reports for seedlings of Douglas-fir, which state that new root growth is normally dependent on current photosynthate [Zaerr et al. 1973], but under planting stress, it may depend on stored reserves [Van den Driessche 1987].

SUMMARY AND CONCLUSIONS

Early in growth, seedlings and plantlets differed considerably with regard to morphology. Among other differences, new transplants had highly-reduced root systems, which might account for their overall poor control of water loss as well as their lower photosynthetic activity. By 7 weeks after transplantation, plantlets and seedlings had a number of features in common.

The study also suggested that plantlets utilized old needles to support new root growth. This apparently was not true where shoots grew also. The implication is that, in the absence of shoot growth (possibly because of depressed photosynthetic levels), sufficient reserves must be present to maintain root extension. This could explain the importance of initial shoot height on survival during stress, which was reported in Chapter I. Taller shoots possess more needles which would provide a larger nutrient reservoir.

CHAPTER V

THE CONTROL OF ADVENTITIOUS ROOT PRODUCTION AND PLANTLET MORPHOLOGY IN TISSUE-CULTURED DOUGLAS-FIR

INTRODUCTION

Plantlet morphology has been shown to be an important element in post-transplantation performance of tissue-cultured Douglas-fir [Chapters I through IV; Mohammed and Vidaver 1990]. Root and shoot morphology were related to the survival, growth, net water loss, and photosynthetic activity of plantlets. This study investigated the possibility of controlling morphology to obtain desirable traits. Since the most challenging aspect was the control of root system morphology, this was the main subject of this study.

The rooting stage has presented a major obstacle to the successful micropropagation of Douglas-fir [Boulay 1979; Timmis and Ritchie 1984], and some other gymnosperms [Mohammed and Vidaver 1988; Horgan and Holland 1989; Stiff et al. 1989]. However, we have reported 87% rooting success with Douglas-fir [Mohammed and Patel 1989], using a modification of the medium for conifer morphogenesis [Bornman 1983]. This modified treatment was used as the

standard protocol in Chapters I through IV.

Generally in micropropagation, researchers have concentrated simply on achieving an acceptable rooting percentage. Few reports have attempted to link the rooting protocol to subsequent root features. Some exceptions were: Horgan and Aitken [1981] on radiata pine, who found that roots produced in agar withered and died following transplantation; Skolmen and Mapes [1978] on *Acacia koa*, who found that agar roots lacked root hairs and fully-developed vascular systems; and Poissonnier et al. [1980] on redwood [*Sequoia sempervirens* Lamb.], who found that the rooting substrate was an essential determinant of root elongation and secondary root development.

Sucrose concentration during rooting is usually kept lower than that used for shoot production [Mohammed and Vidaver 1988]. However, this may be detrimental, considering the metabolic roles of sucrose during rooting [Thorpe 1982; Driver and Suttle 1987; Gaspar and Coumans 1987]. Boron has been found to increase root number in cuttings of mung bean [*Phaseolus aureus* Roxb.] [Middleton et al. 1978; Jarvis et al. 1984], but the potential benefit of this micronutrient has not been investigated for tissue-cultured conifers.

The objective of this study was to determine whether root system morphology could be manipulated by:

(i) the height of shoots selected for rooting; (ii) the concentration of sucrose in the medium; (iii) the concentration of boric acid in the medium; and (iv) the root elongation medium and/or substrate used.

Treatment effects were evaluated according to root system morphology and rooting percentage. Root number was taken to be an acceptable indicator of root surface area, as was reported in Chapter II. The significance of the treatments for shoot morphology and acclimatization performance has been included in some instances.

MATERIALS AND METHODS

Shoots were produced according to the general protocol outlined in Section B.

Shoot height

Shoots, measuring 1, 2, or 3 cm from the cut base to the apex, were rooted according to the general protocol. Thirty to forty shoots of each height were used. Rooting percentage, root number, shoot health, and shoot height were observed after 7 and 10 weeks.

Sucrose

The general rooting treatment was used with various

concentrations of sucrose in the root induction medium. Concentrations were 1, 2, 3, 4, 6, 8, and 10%. At each concentration, two levels of $FeSO_4 \cdot 7H_2O$ (5.56 or 27.8 $mg \cdot l^{-1}$) were tested. These levels represented 1/5- and full-strength concentrations, respectively, of that reported in the medium for conifer morphogenesis [Bornman 1983]. The concentration of the chelating agent, Na_2EDTA, was adjusted accordingly. Twenty-four shoots were used per treatment. Rooting percentage, root number, shoot tip activity, and shoot health were observed after 7 and 10 weeks.

Twelve to thirty healthy, green plantlets from 1% sucrose (and low iron) and from 6, 8 and 10% sucrose (and high iron) were transplanted to 100 ml pots containing a 3:1 volume ratio of peat:vermiculite. Fine gravel was spread over the surface of the substrate. Pots were held in a tray which was covered with a clear plastic lid. Plantlets were incubated under the following high stress regime: 50% relative humidity, a day:night temperature of 30:28°C, and 27 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPFD from a combination of incandescent (Lumiline II 60W, 115-125V) and cool white fluorescent (Sylvania F20 T12) bulbs. The daylength was 16 h. Plantlets were hand-misted once per day and watered once per week. The lid was removed after 1 week. Two weeks after transplantation, survival was recorded.

To compare the internal anatomies of low-sucrose and

high-sucrose plantlets, stem, needle, and root segments were excised from the treatments involving 1% and 6% sucrose (three plantlets each). Segments were taken at the end of the rooting treatment. These were fixed for 24 h in FAA (50 ml ethanol:10 ml formalin:5 ml acetic:35 ml water), using gentle aspiration. The tissue was then dehydrated in a tertiary butanol series and embedded in Paraplast. Ten-µm sections were cut on a rotary microtome, then stained in safranin, and counterstained in fast green FCF [Sass 1958; O'Brien and McCully 1981].

Boron

Shoots were rooted using the general method, except that sucrose was 4% during root induction and ferrous sulphate was 27.8 $mg \cdot l^{-1}$. Eight concentrations of boric acid were tested during the root induction and elongation treatments: 0, 0.3, 1.5, 3, 4.5, 6, 7.5 and 9 $mg \cdot l^{-1}$. Eighteen to twenty shoots were included per treatment. Rooting percentage and root number were observed after 7 and 10 weeks.

Root elongation

After 7 weeks on the general rooting treatment, shoots were transferred, *in vitro*, to the following MCM-based root elongation media:

A: 1/5-strength salts, full iron and organics, 1% sucrose, 0.1% activated charcoal; in vermiculite.

B: Same as A, except substrate was peat:perlite (60:40 by volume, Sunshine Mix No.4).

C: 1/2-strength salts, full iron, no organics or charcoal; in vermiculite.

D: Same as C, except substrate was peat:perlite.

E: 1/5-strength salts and organics, full iron, 3% sucrose, 0.1% activated charcoal; in vermiculite.

F: Same as E, except substrate was peat:perlite.

Note: Medium F is part of the general protocol.

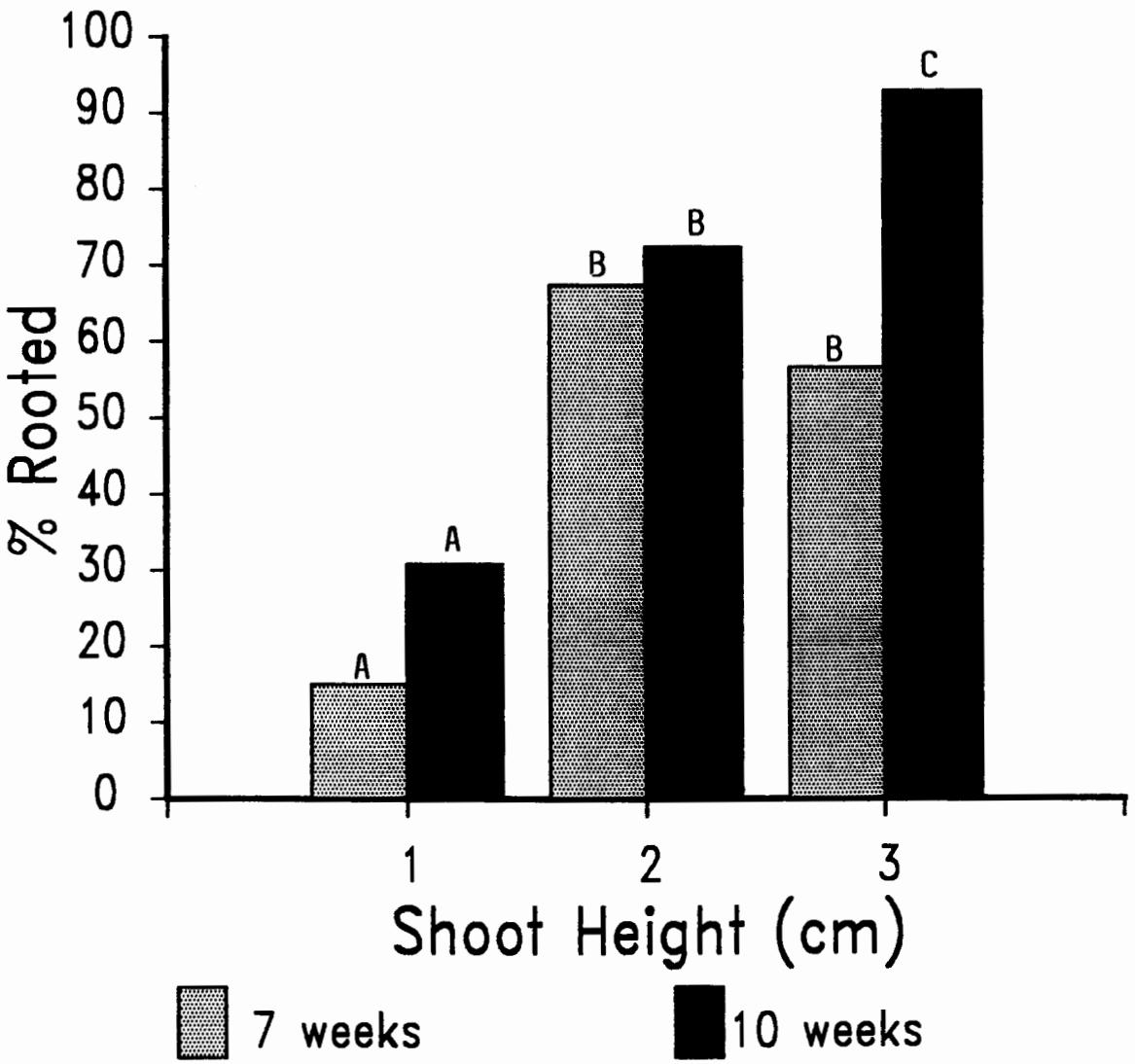
Twenty to thirty-three plantlets were used per treatment, and results were recorded after 21 d. Root elongation, average root length, root number, the presence/absence of root hairs, the proportion of white root tips, and apical shoot activity were observed.

RESULTS

Shoot height

Shoot height was found to influence both the rooting percentage and root number. Rooting percentages, prior to the root elongation treatment, were significantly higher for 2-cm- and 3-cm-tall shoots than for 1-cm-tall shoots (Figure 11). Following the root elongation treatment, 93% of the

Figure 11. Rooting percentage of 1, 2 or 3-cm-tall shoots after 7 or 10 weeks.



Note: n=30 to 40. At 7 weeks, shoots were transferred to the root elongation medium. Within each time, results with the same letter were not significantly different according to the chi-square test.

3-cm-tall shoots rooted. Results were significantly lower for shoots of other heights.

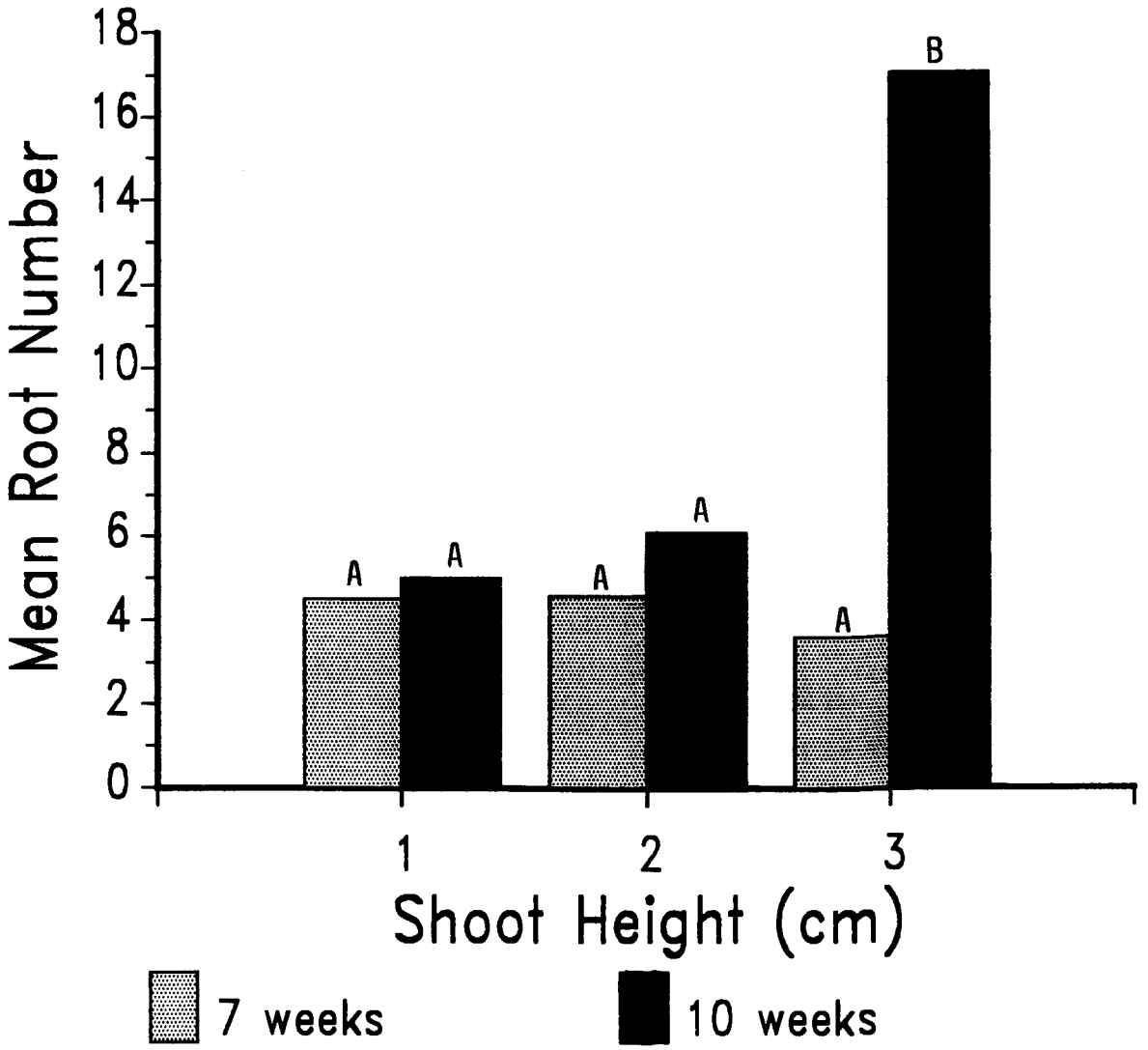
At the end of the rooting treatment, 3-cm shoots had the highest number of roots (Figure 12). Prior to the root elongation treatment, the average root number did not exceed five for any shoot height. After 10 weeks, plantlets from 1-cm shoots had either 1-3 or 4-10 roots, with roughly equal distribution between the two classes (Figure 13). Plantlets from 2-cm shoots had mainly 4-10 roots. Eighty-five percent of those from 3-cm shoots had more than 10 roots.

Some problems were noted in the health of tall shoots during rooting (Table 13). For example, there were higher frequencies of brown needles, and twisted apical needles. The occurrence of these traits was neglibible with shoots of other heights.

Sucrose and iron

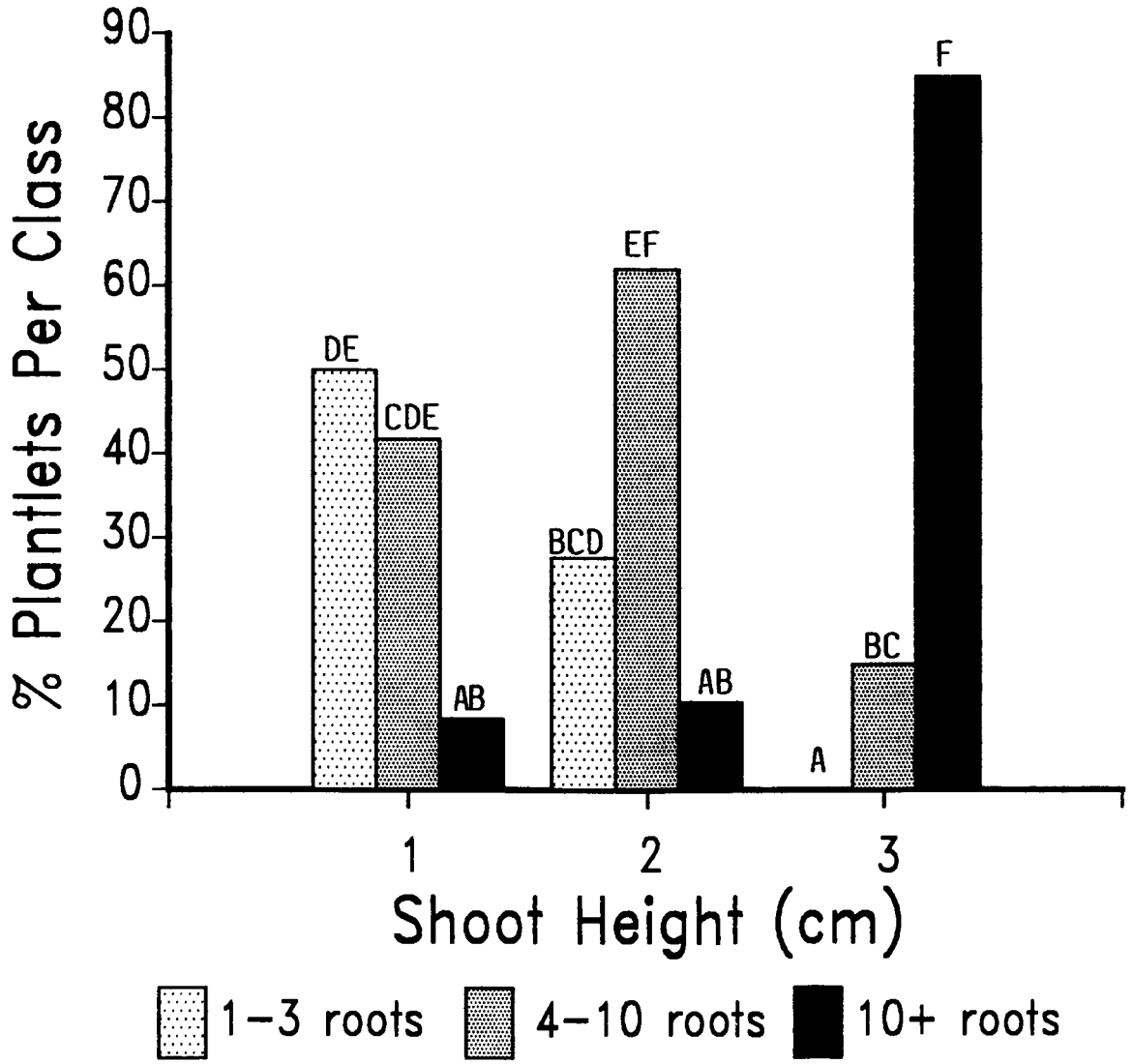
Shoots on the high iron concentration were healthier, with less paling of shoot tips, than those on low iron (Tables 14 and 15). On both iron levels, browning of needles was more frequent at 6% or higher sucrose. At these concentrations, shoots also tended to produce apical scales and to cease further growth. These effects were pronounced on the low iron concentration, where rooting percentage decreased as sucrose concentration increased (Table 16).

Figure 12. Root number of plantlets from 1, 2 or 3-cm-tall shoots, after 7 or 10 weeks.



Note: n=6 to 28. At 7 weeks, shoots were transferred to the root elongation medium. Within each time, results with the same letter were not significantly different according to the chi-square test.

Figure 13. Percentage of plantlets from 1, 2 or 3-cm-tall shoots with 1-3, 4-10, or 10+ roots, after 10 weeks.



Note: n=12 to 28. At 7 weeks, shoots were transferred to the root elongation medium. Bars with the same letter(s) were not significantly different according to the chi-square test.

Table 13. Relationship between the height of shoots selected for rooting and morphology during the rooting treatment.

Morphological characteristics	Shoot height (cm)					
	1	2	3	1	2	3
	(7 weeks)			(10 weeks)		
Shoot height (mm)	22.8a	29.8b	37.4c	24.9a	33.9b	37.8c
% shoots with green colour	100.0b	100.0b	23.3a	84.6b	87.5b	20.7a
% shoots with at least 3 brown needles	0.0a	0.0a	27.6b	10.3ab	7.5a	27.6b
% shoots with twisted apical needles	0.0a	0.0a	13.3b	0.0a	0.0a	17.2b

Note: n=30 to 40. At 7 weeks, shoots were transferred to the root elongation medium. For each time, results followed by the same letter(s) within a row were not significantly different according to Duncan's multiple range test for shoot height, or the chi-square test for other features.

Table 14. Morphological characteristics of shoots on seven sucrose concentrations from 1 to 10% and 5.56 $mg \cdot l^{-1}$ ferrous sulphate, after 7 weeks on the rooting treatment.

Morphological characteristics [y]	Sucrose (%) [x]						
	1	2	3	4	6	8	10
% shoots with pale tips	92	92	46	100	68	88	76
			(not related)				
% shoots with at least 3 brown needles	0	0	25	0	40	24	44
			$y=-3.5+4.6x$ ($R^2=0.64$)				
% shoots with resting apical bud	0	0	0	0	44	88	68
			$y=-22.1+10.4x$ ($R^2=0.81$)				

Note: n=24, $p \leq 0.03$.

Table 15. Morphological characteristics of shoots on seven sucrose concentrations from 1 to 10% and 27.8 $mg \cdot l^{-1}$ ferrous sulphate, after 7 weeks on the rooting treatment.

Morphological characteristics [y]	Sucrose (%) [x]						
	1	2	3	4	6	8	10
% shoots with pale tips	0	8	0	16	20	25	0
			$y=5.5x-0.47x^2$ ($R^2=0.72$)				
% shoots with at least 3 brown needles	0	4	0	0	16	13	20
			$y=3.7+2.3x$ ($R^2=0.79$)				
% shoots with resting apical bud	0	0	0	0	16	8	52
			$y=-12.4+4.8x$ ($R^2=0.68$)				

Note: $n=24$, $p \leq 0.04$.

Table 16. Rooting percentage on seven concentrations of sucrose from 1 to 10% and two concentrations of ferrous sulphate, after 7 weeks on the rooting treatment.

Shoot response to rooting treatment [y]	Sucrose (%) [x]						
	1	2	3	4	6	8	10
	(5.56 mg·l^{-1} ferrous sulphate)						
% shoots rooted or nodular	88	68	54	76	32	20	8
	$y=91.3-8.6x$ ($R^2=0.88$)						
% rooted	52	48	50	24	24	0	4
	$y=59-6.2x$ ($R^2=0.88$)						
	1	2	3	4	6	8	10
	(27.8 mg·l^{-1} ferrous sulphate)						
% shoots rooted or nodular	56	60	88	96	68	71	44
	$y=34.5x-3.1x^2$ ($R^2=0.95$)						
% rooted	28	24	64	48	36	29	0
	$y=20.9x-2.1x^2$ ($R^2=0.92$)						

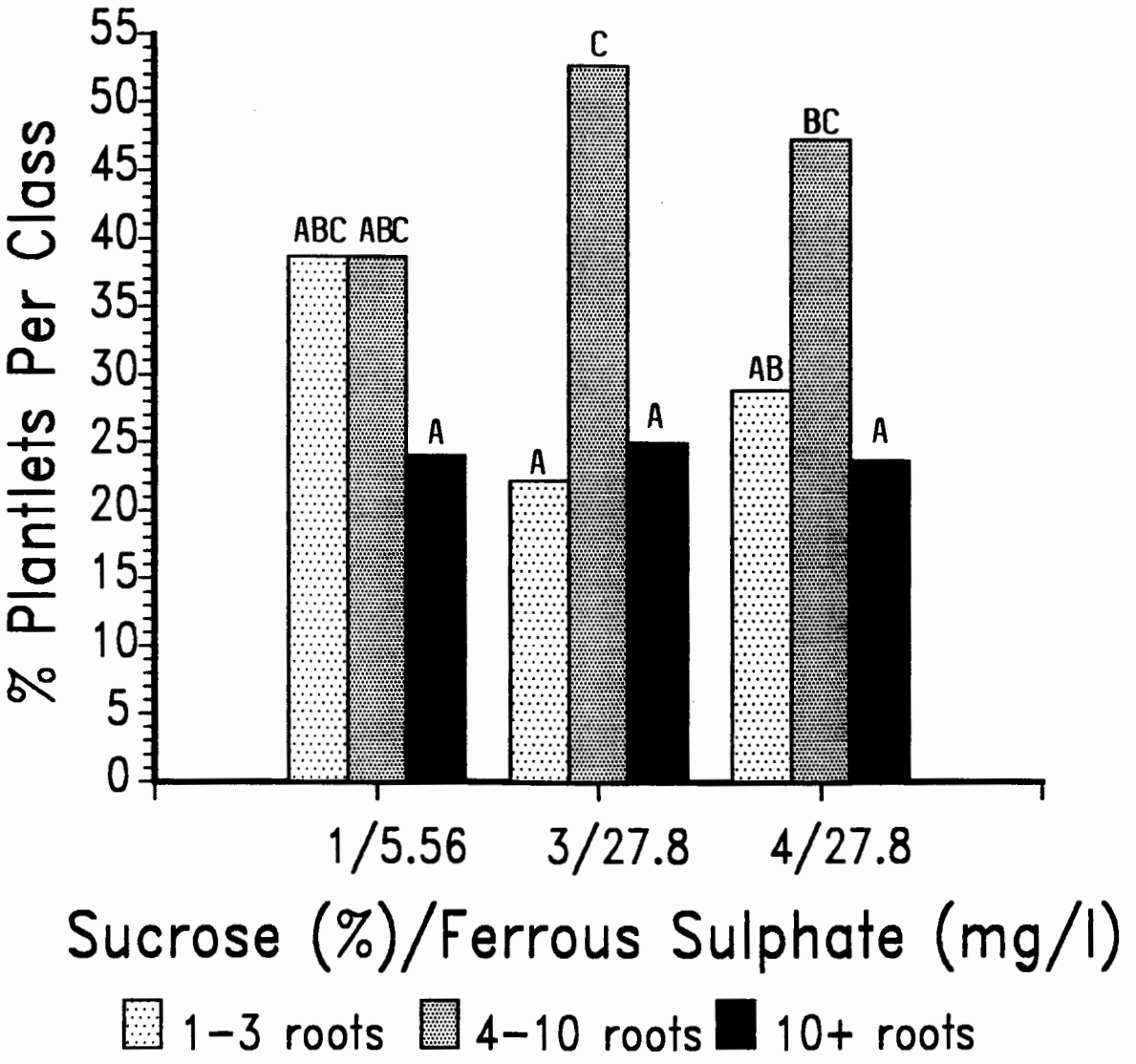
Note: n=24, $p \leq 0.01$.

The highest rooting percentage on low iron was 52%, which was obtained with 1% sucrose. On the high iron concentration, rooting peaked (64%) with 3% sucrose. At 3-8% sucrose, rooting was greater on high iron, while low iron was better with 1-2% sucrose. At very high sucrose concentrations, rooting was poor.

The production of nodular or rooted shoots showed a similar trend (Table 16). On low iron, 88% of the shoots were rooted or nodular. This percentage declined with increasing sucrose. On high iron, the percentage increased until it reached 96% on 4% sucrose, then dropped. The distribution of plantlets among three root number classes is shown in Figure 14. Results from the three best treatments have been presented. Approximately 50% of the plantlets from 3-4% sucrose and high iron had 4-10 roots. The majority of the plantlets from 1% sucrose and low iron were equally distributed between the 1-3 or 4-10 root number classes. Only a quarter of the plantlets from any of the three treatments had more than 10 roots.

Plantlets produced on very high sucrose apparently were better able to withstand stress following transplantation. Survival from the 1% and 6% sucrose treatments was 30% and 20%, respectively, and these results were not statistically different. However, survival on 8% and 10% sucrose was significantly higher at 75% and 92% respectively. At the

Figure 14. Percentage of plantlets from 3 rooting media, with 1–3, 4–10, or 10+ roots, after 10 weeks.



Note: n=36. At 7 weeks, shoots were transferred to the root elongation medium. Bars with the same letter(s) were not significantly different according to the chi–square test.

end of the rooting treatment, plantlets from 6-10% sucrose looked and felt more woody than those from low sucrose. Anatomically, these plantlets had considerably greater development of secondary vascular tissue (Figure 15). No marked differences were present among needle or root segments from low or high sucrose.

Boron

On 3 $mg \cdot l^{-1}$ boric acid, 100% of the shoots rooted after 10 weeks (Table 17). The average root number on this treatment was 11.4, and 50% of the plantlets had more than 10 roots (Table 18). High rooting percentages were also observed on higher concentrations of boric acid, but root number declined above 4.5 $mg \cdot l^{-1}$. Results for the production of nodular or rooted shoots after 7 weeks often provided a close estimate of 10 week results (Table 17). This was true for concentrations of 0.3, 1.5, 4.5 and 6 $mg \cdot l^{-1}$ boric acid.

On concentrations of 3 $mg \cdot l^{-1}$ or higher, 10-20% of shoot tips became pale with brown spots developing after 10 weeks on the rooting treatment. When these plantlets were transplanted, up to 75% of the affected shoot apices died within 3 weeks, and axillary shoots took over. Analysis of foliar minerals (Norwest Labs, Langley, B.C.), conducted on a sample of affected plantlets at the end of the rooting treatment, revealed a deficiency in zinc and iron.

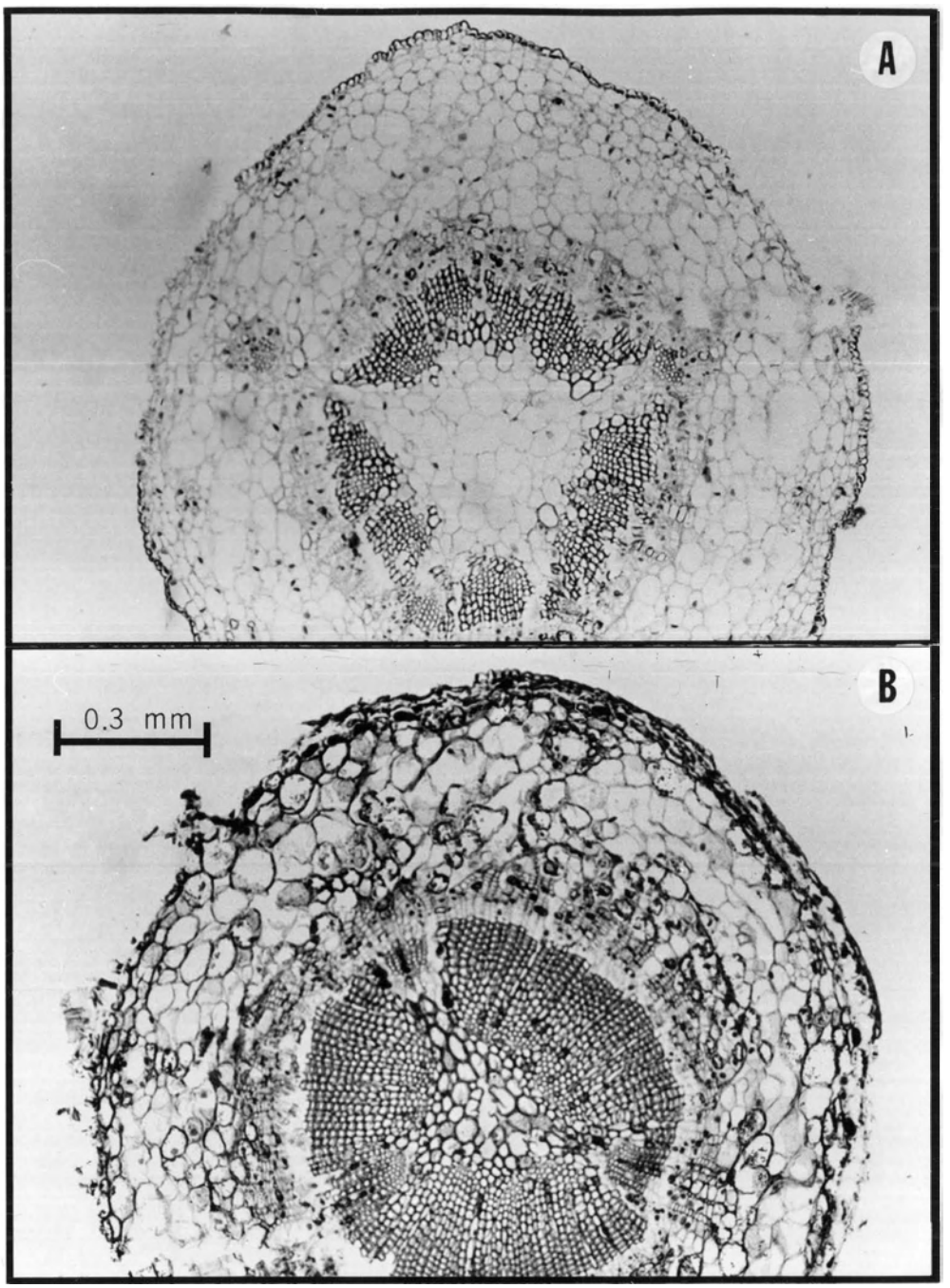


FIGURE 15. Anatomy of plantlet stems on different concentrations of sucrose and ferrous sulphate.

A: 1% sucrose and 5.56 mg·l^{-1} $FeSO_4 \cdot 7H_2O$;

B: 6% sucrose and 27.8 mg·l^{-1} $FeSO_4 \cdot 7H_2O$.

Note: greater amount of secondary xylem.

Table 17. Rooting percentage on eight concentrations of boric acid from 0 to 9 $mg \cdot l^{-1}$, with 4% sucrose and 27.8 $mg \cdot l^{-1}$ ferrous sulphate.

Shoot response to rooting treatment [y]	Boric acid ($mg \cdot l^{-1}$) [x]							
	0	0.3	1.5	3.0	4.5	6.0	7.5	9.0
% shoots rooted or nodular after 7 weeks	35	55	65	75	90	70	74	55
			$y=42.9+16.3x-1.7x^2$ ($R^2=0.85$)					
% rooted after 7 weeks	15	15	5	50	40	40	58	35
			$y=16.7+3.9x$ ($R^2=0.49$)					
% rooted after 10 weeks	65	55	55	100	90	70	95	90
			$y=57.4+11.6\sqrt{x}$ ($R^2=0.46$)					

Note: n=18 to 20, $p \leq 0.06$. At 7 weeks, shoots were transferred to the root elongation medium.

Table 18. Root number of plantlets on eight concentrations of boric acid from 0 to 9 $mg \cdot l^{-1}$, with 4% sucrose and 27.8 $mg \cdot l^{-1}$ ferrous sulphate.

Root number after 10 weeks [y]	Boric acid ($mg \cdot l^{-1}$) [x]							
	0	0.3	1.5	3.0	4.5	6.0	7.5	9.0
% plantlets with 1-3 roots	38	45	27	5	6	21	6	39
				$y=44.1-15.7x+1.6x^2$ ($R^2=0.76$)				
% plantlets with 4-10 roots	38	54	64	45	50	50	56	33
				(not related)				
% plantlets with 10^+ roots	23	0	9	50	44	29	39	28
				$y=15.5x-1.4x^2$ ($R^2=0.86$)				
Mean root number	5.9	4.7	6.2	11.4	10.3	8.5	9.5	6.6
				$y=4.9+2.2x-0.2x^2$ ($R^2=0.74$)				

Note: n=11 to 20, $p \leq 0.03$. At 7 weeks, shoots were transferred to the root elongation medium.

Root elongation

Media E and F (F being the standard treatment) resulted in the greatest percentage of plantlets with root elongation, and the highest percentage of elongated roots (Table 19). Average root length and the percentage of active root tips were best on Media B, E and F. The proportion of plantlets with 10^{+} roots was highest on Medium F. None of the media significantly affected shoot height or shoot apical activity.

Prior to the root elongation treatment, plantlets had no root hairs. Subsequently, root hairs were more prevalent on vermiculite substrate (media A, C and E).

DISCUSSION

This study has identified various factors which affect root production and development in Douglas-fir. Final shoot height, rooting percentage, and root number were high using tall shoots. But since tall shoots tended to produce many roots, the demand for nutrients may have exceeded the supply. For example, twisted apical needles may be a symptom[1] of calcium and/or boron deficiency [Salisbury and

[1]This symptom has not been observed on plantlets which have been produced from 1.5-2 cm tall shoots, using the general protocol.

Table 19. Influence of root elongation treatment on *in vitro* morphology of plantlets.

Morphological feature	Medium					
	A	B	C	D	E	F[1]
% plantlets with active main shoot apex	95a	90a	90a	90a	92a	85a
% plantlets with root elongation	48a	57a	57a	40a	87b	94b
% original roots elongated	57a	71ab	80b	63a	95c	97c
Average root length (mm)	3.9a	5.8b	4.4a	3.8a	6.5b	5.9b
% plantlets with 10^{+} roots	0a	0a	0a	0a	0a	12b
% root tips that were white (active)	84a	86a	96b	86a	100c	95b
% plantlets with root hairs	33cd	0a	24bc	5ab	62d	0a

Note: n=20 to 33. Results within a row with the same letter(s) were not significantly different according to the chi-square test (for percentages) or Duncan's multiple range test (for average root length). The standard root induction treatment was used.

[1] Standard medium.

Ross 1985]. Developing roots are known to have a special requirement for these nutrients [Haissig 1986; Haussling et al. 1988; Kuiper et al. 1989]. While the symptom was not linked to any deterioration in plantlet health during acclimatization, it may signify a decreased nutrient reservoir in the shoot for the subsequent sustenance of root growth.

The concentration of boric acid influenced both the rooting percentage and root number. Best results were obtained with 3 $mg \cdot l^{-1}$. It has been proposed that boron promotes the oxidation of auxin [Middleton et al. 1978; Jarvis et al. 1984]. It has also been suggested that a deficiency of boron results in poor lignification and xylem differentiation [Lewis 1980]. Philbeam and Kirby [1983] and Ali and Jarvis [1988] have summarized the apparent roles of this nutrient. Boron influences: (i) growth and differentiation at the whole plant level; (ii) membrane permeability and sugar transport; and (iii) enzymes involved in the metabolism of carbohydrates, phenolics, lignin, auxins, and nucleic acids. The formation of roots in the absence of supplied boron was observed here as well as in another study [Middleton 1977], although Middleton suggests that boron was probably present as a contaminant from water sources or glassware.

In subsequent acclimatization trials with the plantlets

from high boron, the deficiency symptoms that were observed were linked to a greater frequency of dieback of central shoot tips. An axillary shoot then became dominant. (This process occurred in approximately 75% of the affected plantlets. Survival was not affected.)

Biochemically, root initiation is a high-energy process, requiring a continuous supply of free sugars from the medium [Greenwood and Berlyn 1973; Thorpe 1980, 1982; Gaspar and Coumans 1987]. Sucrose is also necessary for maintaining an osmotic balance. Thus, it may be detrimental to reduce its concentration during rooting of conifer tissue cultures, as is the common practice [review, Mohammed and Vidaver 1988]. With respect to the good responses from tall shoots on low sucrose, these shoots may possess relatively substantial carbohydrate reserves. This aspect needs further study.

Sucrose requirement was related to the concentration of ferrous sulphate in the medium. Sucrose and iron are both essential for respiration, which has been shown to increase during rooting [Ooishi et al. 1978]. Therefore, maintaining a balance between these two nutrients would be important for ensuring adequate rates of respiration for root production. Furthermore, increasing sucrose alone may upset osmotic processes to produce the visible symptoms of brown needles and apical buds. With adequate iron in the medium, elevated

sucrose was associated with a greater production of nodules on shoot bases. Nodules typically are root precursors [Mohammed et al. 1989].

Also, higher sucrose was found to enhance the production of secondary vascular tissue. Increased lignification under high (4%) sucrose has been reported for other woody species [Driver and Suttle 1987]. In that study, like the present one, plantlets from high sucrose were apparently better able to withstand acclimatization stress.

It is possible that increased concentrations of sucrose had a conditioning effect on plantlet water potential, by lowering osmotic potential. Consequently, plantlets might have had sufficiently negative water potential to avoid losing water to the acclimatization substrate or atmosphere. A beneficial osmotic adjustment seemed to occur only at moderately high sucrose concentrations. At very high levels (e.g. 8%, 10%) shoot health and rooting responses were adversely affected.

The root elongation treatments which were tested in this study represent a subset of a much wider range which has been tested in the past. Recall that previous results have shown that agar is unsuitable for rooting of Douglas-fir [Mohammed and Patel 1989]. All of the present treatments showed some degree of success. Special

considerations during root elongation are: (i) the requirement for sufficient aeration; (ii) moisture retention [Poissonnier et al. 1980; Griffis et al. 1983]; and (iii) minimal microbial contamination [Bluhm 1978]. Either vermiculite or peat:perlite containing the standard root elongation nutrients is acceptable; however, there may be an advantage to using vermiculite because it induces root hairs. This result may have been due to more aeration in vermiculite (particles or vermiculite were larger and more loosely packed).

SUMMARY AND CONCLUSIONS

This study has shown that it is possible to control plantlet morphology by manipulating certain factors in the rooting stage. Useful new information has been obtained on the control of root number, root elongation, shoot height, and shoot health. Further research is necessary to eliminate the apical foliar deficiency symptoms on high boron without sacrificing root system morphology. The symptoms which were observed on high boron were later found to be more serious than those on plantlets from tall shoots because the former were associated with dieback of central shoot tips and subsequent axillary dominance during acclimatization. The ultimate effect could be laterally-oriented shoot growth, which would be unacceptable in a lumber species. Therefore, until the boron supplementation treatment is improved, the use of tall shoots is recommended for the production of morphologically superior plantlets.

D. GENERAL SUMMARY AND CONCLUSIONS

The first part of this work sought to identify whether there was a link between initial plantlet morphology and performance during acclimatization. It was found that certain morphological features could help to offset the adverse effects of water stress during this stage. Tall shoots and many roots were beneficial for both survival and shoot growth during the first 7 weeks following transplantation. Other beneficial features were: (i) upright needles (for survival), and (ii) active shoot tips, white root tips, and greater than 1 cm of root system length (for growth). Features which did not have an effect on performance included root thickness, diameter of the shoot:root junction, and slight paling of shoot tips.

Plantlets were then assessed with respect to their physiological performance. Performance was analyzed according to morphology. The features which formed the focus for these studies were root number, shoot height, root surface area, needle surface area, the ratio of needles surface area/root surface area, and the length of the longest root. This last feature was meant to be a measure of root system length. Surface area calculations were included because of the possibility that root number or shoot height were simply indicators of surface area. By including surface area better quantitative information was sometimes obtained. Responses according to root number were

taken as representative of those responses according to root tip colour, shoot tip activity, or needle form, because of the strong correlations between these traits [Tables 2 and 5, Chapter I].

Plantlets were generally far more susceptible than seedlings to detrimental water loss during stress. Low deposition of epicuticular wax would have contributed to this problem. (Stomatal conductance, which was not investigated here, may be another contributing factor to water loss.) An important finding was that relative water content and the rate of water uptake were highest in plantlets with low needle surface area/root surface area ratios. This indicates that a balance between the shoot and root system is an essential requirement. The importance of shoot:root ratios in the performance of tissue-cultures has not been reported previously. The other features mentioned above were also significantly related to water loss.

Plantlets generally showed lower apparent photosynthetic activity than seedlings. However, two points need to be considered. First, it must be noted that CO_2 uptake rates were measured for whole shoots, including old and new needles on transplants. It is highly likely that uptake rates varied considerably among different zones on the shoot, as has been documented for red raspberry by Donnelly and Vidaver [1984b]. The intent of this study was

not to differentiate between zones, but to relate morphology to overall CO_2 uptake. Second, there is the question of whether or not plantlets have to achieve the same uptake rates as seedlings. Quantitative targets for CO_2 do not exist; therefore, seedling rates can provide only a comparison.

High root number, high root surface area, and low needle surface area/root surface area were associated with higher CO_2 uptake, water splitting activity, and quenching rate. These same features, as well as the length of the longest root, were also linked to lower rates of dark respiration and root respiration. CO_2 uptake increased with shoot height and needle surface area. Water splitting and the rate of quenching increased with the length of the longest root. Finally, the ability of plantlets to utilize high photon flux densities and to photosynthesize under water stress may be enhanced by the presence of many roots.

It should not be surprising that plantlets with good root systems also have higher apparent photosynthetic activities. The root system provides the shoot with growth regulators and other metabolic products which are used in photosynthesis [Dickson 1979; Pate 1980]. Roots are also important sinks for photosynthate.

The study on morphological changes following transplantation indicated that persistent needles may be

used to support new root growth. Thus, the beneficial effects of tall shoots may arise partly from the relatively large nutrient reserves which they can provide. Wainwright and Scrace [1989] suggested that nutrient reserves might even supersede photosynthetic activity in determining survival during the early stages of acclimatization.

The final chapter in this work showed that it is possible to control root and shoot morphology by culture manipulation at the rooting stage. For example, tall shoots produced many roots and had high rooting percentages. Similar results could be obtained with shorter shoots on a medium containing extra boric acid, sucrose, and ferrous sulphate. High concentrations of sucrose in the rooting medium resulted in greater development of secondary vascular tissue, and increased survival under water stress.

Several topics for future research emerge from these studies. They include: (i) the anatomy and biochemistry of tall versus short shoots, to be studied with respect to vascular development, the efficiency of water transport, and the availability of nutrient reserves; (ii) the biochemical nature of respiratory activity in different root systems, e.g. relative production of CO_2 compared to other products; and, (iii) the potential role of chlorophyll *a* fluorescence in indicating root system morphology.

In conclusion, plantlet morphology can serve as a

useful indicator of physiological function and, thus, overall plantlet quality. From a practical perspective, plantlet morphology can be assessed easily, quickly, and non-destructively. Thus, setting morphological targets appears to be a reasonable approach to improving plantlet quality. The likely targets which have emerged from this work are: 41-60 mm final shoot height, more than 10 roots per plantlet, greater than 1 cm root system length, and a low needle surface area/root surface area ratio.

The findings of these experiments have several potential applications. First, the improvements in root production and acclimatization success are relevant to any future effort to commercially micropropagate Douglas-fir. Micropropagation is a labour-intensive, and hence costly, procedure. Costs of production increase considerably with low rooting frequency and/or high plantlet mortality [Hasnain et al. 1986]. The present results should help to minimize commercial costs for this species. Second, the information on root production may be applicable to the rooting of conventional cuttings, since both systems involve adventitious root production. Pertinent information might be derived from the results on shoot height selection and medium constituents. Finally, the correlations which have been identified between plantlet morphology and physiological performance under water stress could help to

advance existing knowledge about cuttings or seedlings. Morphological features which are associated with stress resistance are likely to be similar in different stock types. These features could be targets for improvements in stock quality.

APPENDIX

MEDIUM FOR CONIFER MORPHOGENESIS

[Bornman 1983]

COMPOUND	CONCENTRATION ($mg \cdot L^{-1}$)
KNO_3	2000.0
$Ca(NO_3)_2 \cdot 4H_2O$	500.0
$(NH_4)_2SO_4$	400.0
$MgSO_4 \cdot 7H_2O$	250.0
KH_2PO_4	270.0
KCl	150.0
Urea	150.0
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.3
$ZnSO_4 \cdot 7H_2O$	3.0
H_3BO_3	1.5
KI	0.25
$Na_2MoO_4 \cdot 2H_2O$	0.25
$MnSO_4 \cdot 4H_2O$	0.22
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Inositol	90.0
Nicotinic acid	1.7
Pyridoxine·HCl	1.2
Thiamine·HCl	1.7
Folic acid	1.1
Pantothenate	0.5
Glycine	2.0

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