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To determine whether shading increases drought-induced root mortality by changing the chemical composition of roots, I conducted an experiment in which high and low light treatments in combination with high and low soil moisture treatments were applied to seedlings of Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco). Because synthesis of suberin (a water-repellent compound found in roots) is metabolically expensive, I hypothesized that suberin synthesis might be reduced in roots of a shade-stressed tree and thereby, susceptibility to desiccation would be increased. As expected, the drying treatments increased root mortality for the shaded seedlings, however, no differences in suberization were found. I measured starch and sugar concentrations of the roots and found that carbohydrate reserves of

roots in the shaded treatments were nearly exhausted. I concluded that root mortality induced by shading and drought was probably caused by depletion of carbohydrate reserves in the root rather than by differences in resistance to desiccation.

I then set up an experiment to determine whether accumulation and depletion of carbohydrate reserves were indeed the physiological mechanisms underlying fine root turnover. Douglas-fir seedlings were grown at soil temperatures of 10°C, 20°C, and 30°C, at uniform air temperatures, with and without light. It was necessary to develop a starch extraction procedure using 35% perchloric acid in order to consistently extract all starch from the tissue. Sequential harvests were weighed and analyzed for starch and sugars. Starch reserves were exhausted in the roots of the dark-grown seedlings at all temperatures and were also exhausted in the roots of the light-grown seedlings at 20°C and 30°C. In the light-grown seedlings from the 10°C treatment, however, significant accumulations of starch were observed in association with pulses of root growth. Based on this study and on others from Sweden and from Oregon, I suggest that root lifetimes may be determined by the amount of starch with which a newly grown root is endowed and the rate at which this starch supply is respired. A simple means of estimating root biomass production and

turnover based on root starch and soil temperature is described.

Physiological Control of Fine Root Turnover in Douglas-fir

by

John D. Marshall

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TABLE OF CONTENTS

INTRODUCTION	1
DROUGHT-INDUCED ROOT MORTALITY CAUSED BY DEPLETION OF STARCH AND SUGAR IN DOUGLAS-FIR SEEDLINGS	5
Abstract	6
Introduction	7
Methods and Materials	9
Results	13
Discussion	14
Literature Cited	25
PREDICTION OF FINE ROOT PRODUCTION AND TURNOVER FROM ACCUMULATION AND DEPLETION OF STARCH	29
Abstract	30
Introduction	31
Seedling Experiment	34
Results	39
Discussion	44
Literature Cited	67
PERCHLORIC ACID EXTRACTION OF STARCH FROM DOUGLAS-FIR TISSUE	71
Abstract	72
Introduction	73
Methods and Materials	74
Results	77
Discussion	77
Literature Cited	80
BIBLIOGRAPHY	82
APPENDIX I. EXTRACTION AND MEASUREMENT OF SUGAR AND STARCH	88
Preparation of Samples for Analysis	89
Ethanol Extraction	89
Removal of Interfering Substances from Ethanol Extract	90
Perchloric Acid Extraction of Starch	91
Colorimetric Determination of Carbohydrates Using Anthrone	92
Iodine Precipitation of Starch from Perchlorate Extract	94
Enzymatic Hydrolysis of Iodine Precipitate	96
Reagents	97
Literature Cited	98

LIST OF FIGURES

FIGURE		PAGE
I.1	Correlation between root tip mortality against root mortality by weight.	21
I.2	Fine root mortality by weight percentage for shade and drought treatment combinations.	22
I.3	Root tip mortality for shade and drought treatment combinations.	23
I.4	Starch and sugar concentrations of fine roots for shade and drought treatment combinations. Shaded areas represent starch concentrations.	24
II.1	Potential starch and sugar dynamics of a fine root, showing differences in lifespan associated with root temperature.	57
II.2	Starch concentrations of roots without secondary thickening from seedlings grown at specified soil temperatures and exposed to light or maintained in darkness.	58
II.3	Starch concentrations of roots with secondary thickening from seedlings grown at specified soil temperatures and exposed to light or maintained in darkness.	59
II.4	Starch concentrations of shoots of seedlings grown at specified soil temperatures and exposed to light or maintained in darkness.	60
II.5	Starch concentrations of roots without secondary thickening at specified temperatures and with shoots exposed to light or maintained in darkness.	61
II.6	Relative growth rate of fine roots can be predicted from net changes in starch concentration.	62

II.7 Starch + sugar concentrations in shoots of seedlings maintained in darkness. Data from all root temperatures were combined. The regression model was significantly improved by separating pre-budset from post-budset periods. 63

II.8 Regression of fine root turnover rates as measured by Santantonio (1982) against estimated maintenance respiration rates of fine roots. 64

II.9 Predicted and measured fine root biomass from data for control plots of Ericsson and Persson (1980). 65

II.10 Predicted and measured fine root biomass from data for irrigated and fertilized plots from Ericsson and Persson (1980). 66

III.1 Labile polysaccharides extracted by 35% perchloric acid from Douglas-fir tissue before and after prolonged dark-treatment. Shaded areas represent iodine-precipitable carbohydrate, which is almost entirely starch. 79

LIST OF TABLES

TABLE		PAGE
I.1.	Starch and sugar concentrations (mg/g d.w.) of live and dead fine roots from combinations of shade and drought treatments.	20
II.1.	Tests of significance of F-tests from analysis of variance for starch concentrations of seedling components.	53
II.2	Tests of significance of F-tests from analyses of variance for weights of seedling components.	54
II.3	Weights (mg) of various components harvested at selected dates following seedling exposure to specified light and soil temperature conditions.	55
II.4	Regression equations describing depletion of starch and sugar in seedlings maintained in darkness. Slopes are estimates of maintenance respiration rates.	56

PHYSIOLOGICAL CONTROL OF FINE ROOT TURNOVER IN DOUGLAS-FIR

INTRODUCTION

The importance of the smallest elements of the root system, the fine roots, has been discovered by accounting for all biomass produced by trees throughout a year (Harris *et al.* 1978, Agren *et al.* 1980, Grier *et al.* 1980). Though the fine roots may constitute less than 1% of the biomass of a mature tree, they may account for as much as two-thirds of annual biomass production (Grier *et al.* 1980). This represents a substantial drain on the pool of photosynthate available for above-ground growth.

Maintenance requirements of fine roots are not high relative to other tissues (Agren *et al.* 1980). The high photosynthate requirement of fine roots is attributable to the frequency with which they are replaced. Fine roots may be replaced (turn over) two to three times per year (Persson 1979, Santantonio 1982), or perhaps even more (Head 1973, Persson 1978). Moreover, the turnover rate varies depending on site conditions (Santantonio 1982).

Because fine root turnover is so costly for the tree, determining the physiological mechanisms underlying differences in turnover would contribute greatly to our

understanding of forest productivity. It might also provide a means by which fine root turnover could be estimated without the extensive excavation and sorting that are now required.

An experimental approach was needed in order to isolate physiological causes. I therefore chose to work with 2-0 Douglas-fir seedlings, first in a raised nursery bed, then in a growth room. I assumed that seedling roots are similar to those of large forest trees. I was able to validate this assumption by comparing predictions based on the results of the seedling experiments against data collected in forest stands.

I began by assuming root growth is correlative--that trees need a particular quantity of fine roots for uptake of water and nutrients and that trees maintain an optimal balance between shoot and root by growing roots when necessary (Loomis 1953). The standing biomass of fine roots would thus be controlled by tree size and availability of water and nutrients. Moreover, dead roots would be replaced as necessary. The control of fine root turnover would then rest with the causes of fine root death.

The first chapter of this thesis deals with the hypothesis that fine root death is caused by root desiccation during periods of drought. Because root death had been observed at water potentials ranging from -0.02 to

less than -1.5 MPa, however, I further hypothesized that roots varied in susceptibility to desiccation, perhaps due to differences in suberization. As expected, differences in susceptibility to drought were observed, however, differences in suberization were not. Rather, root mortality appeared to be more closely associated with depletion of sugar and starch reserves.

In the second chapter, the relationships between sugar and starch reserves, root growth, and root death are examined. I found a simple pattern: a single pulse of starch deposition followed by gradual depletion to meet maintenance respiration requirements, and finally, by root death. Because maintenance respiration was temperature-dependent, so were root longevity and turnover. These results were tested against field data of Santantonio (1982) and were found to explain differences in turnover rates. I was also able to predict fine root production for a Scots pine stand in Sweden using starch and soil temperature data (Ericsson and Persson 1980).

To obtain these results, it was necessary to develop a method of starch extraction that insured complete starch removal from conifer tissue. The third chapter describes the extraction method and demonstrates that the procedure extracts large quantities of starch and relatively small amounts of carbohydrates other than

starch. The amounts of "background carbohydrates" remained unchanged despite large differences in starch concentration, indicating that they do not serve as reserve materials. The appendix provides detailed instructions and explanations for the starch and sugar extraction procedures.

CHAPTER I.

DROUGHT-INDUCED ROOT MORTALITY CAUSED BY DEPLETION OF STARCH AND SUGAR IN DOUGLAS-FIR SEEDLINGS

by

J.D. Marshall

ABSTRACT

To determine whether shading increases drought-induced root mortality by changing the chemical composition of roots, I conducted an experiment in which high and low light treatments in combination with high and low soil moisture treatments were applied to seedlings of Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco). Because synthesis of suberin (a water-repellent compound found in roots) is metabolically expensive, I hypothesized that suberin synthesis might be reduced in roots of a shade-stressed tree and thereby, susceptibility to desiccation would be increased. As expected, the drying treatments increased root mortality for the shaded seedlings, however, no differences in suberization were found. I measured starch and sugar concentrations of the roots and found that carbohydrate reserves of roots in the shaded treatments were nearly exhausted. I concluded that the direct cause of root mortality induced by shading and drought was depletion of carbohydrate reserves in the root rather than desiccation. Although water stress increased root mortality, its effect appeared to be indirect.

INTRODUCTION

In recent studies attempting to account for all biomass produced by trees throughout a year, the smallest elements of the root system, the fine roots, have been demonstrated to be extremely important in terms of the photosynthate allocated to them (Agren *et al.* 1980, Harris *et al.* 1978, Grier *et al.* 1980). Although the fine roots may constitute less than 1% of the biomass of a mature tree, they may account for 66% of annual biomass production (Grier *et al.* 1980).

The maintenance requirements of living fine roots are not exceptionally high relative to other tissues (Agren *et al.* 1980). Their high photosynthate requirement can be attributed to the frequency with which they are replaced, or turnover rate. Fine roots may be replaced (turn over) two to three times per year (Persson 1979, Harris et al. 1978, Santantonio 1982) or perhaps even more (Head 1973, Persson 1978). Moreover, the turnover rate varies greatly depending on site conditions (Persson 1979, Santantonio 1982). Because fine root turnover is so costly for the tree, determination of the physiological controls underlying differences in turnover would contribute greatly to our understanding of forest productivity.

I have approached the question of root turnover by

concentrating on the causes of root death. The most frequently suggested cause of fine root death is drought (Persson 1980, Deans 1979, Santantonio 1982). Deans (1979) observed that fine root mortality of Sitka spruce in Scotland began whenever soil water potentials fell below -0.02 MPa. Santantonio (1982) reported increasing rates of fine root turnover in Douglas-fir on progressively drier sites in the Oregon Cascades. In contrast, it has been reported that fine roots can survive and even grow in soil at water potentials well below the conventional wilting point (-1.5 MPa) (Leshem 1965, Teskey and Hinckley 1981). Consequently, factors other than soil water potential must contribute to root mortality.

Root carbohydrate depletion has also been suggested as a possible cause of fine root death (Lyr and Hoffman 1967, Reynolds 1970, Persson 1980). Root starch and sugar contents associated with changes in fine root biomass have been examined in Sitka spruce forests in Scotland (Ford and Deans 1977) and in Scots pine stands in Sweden (Ericsson and Persson 1980), but no clear patterns were identified in either of these studies. Nonetheless, carbohydrate depletion is implicated as a possible cause of root death by studies showing increased root mortality following defoliation (Redmond 1959).

Fine root turnover might also result from an inter-

action between carbohydrate status and drought. Carbohydrate status could regulate suberization, the process whereby a layer of water-repellent material (suberin) is deposited within the root. Suberization has been shown to reduce water loss from plant roots (Robards _et al._ 1979) and from potato tubers (Kolatukuddy and Dean 1974), and might therefore be expected to reduce water loss from roots into dry soil. In fact, rates of suberization have been observed to increase in dry soil (Leshem 1965, Lyr and Hoffman 1967), and it has been suggested that this represents a mechanism of surviving drought (Wilcox 1964, Leshem 1965). Due to its chemical structure, however, suberin would be metabolically expensive for the plant to synthesize (McDermitt and Loomis 1980). Since synthesis of metabolically expensive compounds is often curtailed under conditions of low carbohydrate status (Loomis 1953), I hypothesized that shading might curtail suberization, increasing susceptibility of fine roots to subsequent drought, and might thereby increase fine root turnover.

METHODS AND MATERIALS

Two-year-old seedlings of Douglas-fir were obtained from the D.L. Phipps state nursery at Elkton, Oregon in February 1982. The seed had been collected on the east side of the Oregon Coast Range at an elevation of

approximately 300 m. The seedlings showed low rates of mycorrhizal infection but some _Thelophera terrestris_ (Ehrh.) Fr. was present. In March 1982, seedlings were planted into two-liter cardboard milk cartons containing washed river sand.

So that watering could be controlled, the seedlings were placed under clear plastic frames to exclude natural rainfall. Half the seedlings were covered with shade cloth, reducing light levels by 95%. Seedlings were watered about every five days. No nutrients were provided during the course of the experiment in order to favor root growth (Ingestad 1979).

After five months the drought treatments were begun. Water was withheld from half the seedlings in the unshaded and half the seedlings in the shaded treatments. Seedlings were harvested after leaf conductance measurements with a null-balance porometer (Beardsall _et al._ 1972) showed that stomates were barely opening and foliage had begun to wilt. The drought treatment lasted 22 days.

Harvested seedlings were divided into roots and shoots at the point midway between the first branch and the first lateral root. This compensated for differences in depth of planting. The root systems were carefully washed and all fine roots ($\leq$2 mm in diameter) were separated from the coarse roots (>2 mm in diameter).

Fine roots were cut into segments no longer than 10 cm and laid in a numbered tray. Root segments were selected at random for determination of mortality. Estimates of root tip mortality were based on subsamples of at least 1000 root tips from each seedling, counting individual root tips on a single mycorrhizal short root as separate tips. Root mortality estimates by weight were obtained from the same subsamples. The subsamples on which mortality was determined averaged 13.9% (SE=1.6%) of the total fine root weight of each seedling. Dead roots were identified by their darkened, wrinkled appearance and, if there was doubt about whether a root was dead, the root was broken and the vascular cylinder was examined. Discoloration of the vascular cylinder was assumed to indicate that the root was dead (Santantonio 1982). The live root subsample and the dead root subsample, as well as the remaining fine roots, the coarse roots, the foliage, and the stem were dried in a forced-draft oven at 70°C. Dried samples were weighed and ground to pass a 40-mesh sieve in preparation for chemical analysis. Specific leaf area (cm^2 projected leaf area/g dry wt.) was measured for year-old and current-year's foliage using a LICOR-3100 surface area meter.

Samples were first extracted with 100% acetone to remove chlorophyll and other pigments (W.D. Loomis, pers. comm., 1984). Sugars were then extracted with hot 80%

ethanol three to four times, until the extract was colorless. This procedure is similar to the Soxhlet extraction (AOAC Methods 1980). The ethanol extract was cleared first with 100 mg insoluble polyvinylpyrrolidone and then with 100 l saturated lead acetate solution (Sanderson and Perera 1966). Sugars were determined colorimetrically by the anthrone reaction (Yemm and Willis 1954, Hansen and Moller 1975). Labile polysaccharides were extracted from the ethanol-extracted residue using 35% perchloric acid (Hansen and Moller 1975) on an orbital shaker for sixteen hours. "Apparent starch" was measured colorimetrically with anthrone (Hansen and Moller 1975). I have called this "apparent starch" because the perchloric acid also extracts approximately 40 mg/g d.w. of non-starch carbohydrates, probably pectic materials (see Chapter III). Therefore, apparent starch concentrations should be adjusted downward by approximately 40 mg/g d.w. to obtain true starch concentrations.

Suberin concentrations were determined using the acid-detergent method of Goering and Van Soest (1970), which was developed for analysis of lignin and cutin. Because the various parts of the suberin polymer resemble either lignin or cutin (Kolatukuddy 1980), it was expected that this procedure would give a good estimate of suberin concentration of the root. Ash concentrations

of the residue were determined by loss-on-ignition (Goering and Van Soest 1972).

The experiment was analyzed as a randomized block design; the shading treatments were treated as blocks, the watering treatments as treatments, and each combination was represented by four individual trees. Means were compared using the Student-Newman-Keuls test (Steel and Torrie 1980). One tree was excluded from the analysis of root mortality following changes in procedures and one tree was excluded from the starch and sugar analyses because the sample was lost.

RESULTS

Percentage of dead root tips was closely correlated with percentage of dead root weight (Fig. I.1). A significant interaction between shading and watering was found in an analysis of variance of root mortality by weight. The analysis of variance for root tip mortality revealed that both shading and watering had significant effects but no significant interaction was found. Both measures of mortality showed a significant increase only when the shade and drought treatments were combined (Figs. I.2 and I.3).

When live and dead fine roots were not separated, I found no differences in suberin concentrations of the fine root systems among any of the treatment combinations. Apparent starch concentrations differed

among treatments, however, being significantly higher in the unshaded and watered treatment (Fig. I.4). Sugar concentrations were significantly higher in the unshaded treatments than in the shaded treatments, independent of watering. No significant differences in weight of any seedling component were demonstrated, partly because of high variability. When ash-corrected fine root weight was normalized by dividing by leaf area, however, the resultant ratio was significantly higher in the unshaded treatments (120 ± 6 gm^{-2}) (mean $\pm$ standard error) than in the shaded treatments (80 ± 7 gm^{-2}).

When the subsamples of live and dead fine roots were analyzed separately, suberin concentrations were significantly higher in the dead roots (43.7 ± 1.2%) than in the live roots (32.7 ± 0.7%). Apparent starch and sugar concentrations of the live and dead roots are presented in Table I.1. Although I had insufficient plant material to replicate the chemical analyses of the live and dead root subsamples, the data resemble those from the whole fine root systems presented earlier.

DISCUSSION

There are a number of problems in identifying dead roots. The classification of live vs. dead roots is unavoidably subjective. It is not known how long after root death the discoloration of the vascular cylinder

develops. If discoloration proceeds slowly, roots that have died only recently would not be recognized as dead. Another problem with the method is that decay and fragmentation of dead roots could lead to underestimation of root mortality. Decay may have caused the higher suberin concentrations of the dead roots, since suberin is resistant to microbial decomposition (Kolatukuddy 1980) and would become more concentrated as roots decompose. The increase in the suberin concentration of the dead roots may also have resulted from the loss of the cortex (Head 1973). In any case, if mortality was underestimated, it was probably underestimated to the same extent in all treatments because the pulse of root death induced by the drought treatment occurred simultaneously in the shaded and unshaded treatments.

The hypothesis that drought is the direct cause of root mortality must be rejected because of the lack of a significant increase in root mortality when the drought treatment was applied to unshaded seedlings. The significance of the interaction between drought and shade suggests that drought is somehow involved in root mortality, but that other factors must also be considered. I also hypothesized that shading might decrease the plant's ability to suberize its roots, making them more susceptible to desiccation, however, no differences in suberin concentration were found. The

third hypothesis, that fine roots die from starvation due to the inability to meet maintenance respiration requirements, finds some support in these data. This is particularly true if one remembers that the starch and sugar analysis procedures used here measure materials in addition to starch and sugar in the extracts. In fact, if the apparent starch concentrations presented in Table 1 are corrected by subtracting 40 mg g $d.w.^{-1}$ of non-starch carbohydrate (see Chapter III), true starch concentrations are approximately zero in the dead roots and in live roots from all treatments except the unshaded, watered treatment. Furthermore, the sugar concentrations of the dead roots are approximately equal to those of roots forced to exhaust their starch and sugar reserves (see Chapter II). Therefore, we may conclude that the roots in the shaded treatments were unable to meet their maintenance requirements due to exhaustion of starch and sugar reserves.

Exhaustion of reserve carbohydrates might also have been implicated as a cause of root mortality due to inability of the roots to maintain positive turgor. Conversion of starch to sugar would certainly decrease osmotic potential of the roots, however the differences in sugar concentrations observed here would account for a difference of little more than 0.1 MPa, assuming the sugar was sucrose and 1.5 g symplastic water per g d.w.

tissue (Joly 1984) (see Noble 1974 for calculations). This would not account for the survival of the roots in the unshaded, unwatered treatment because the soil was much drier than this amount of osmotic adjustment could have offset.

It was noted above that drought had some indirect effect on root mortality that led to significance of the drought X shade interaction. I have suggested that this effect did not involve desiccation of the roots, however, drought could have other, less direct effects. First, drought would tend to increase soil temperatures. I measured a 7°C increase in soil temperature in the dry pots as compared with the watered pots in the mid-afternoon at the end of the drought treatment. This temperature difference would sharply increase respiration rates. Second, drought would, by forcing stomatal closure, inhibit photosynthesis. If the fine roots were meeting their maintenance requirements directly from incoming photosynthate, cessation of photosynthesis would eventually cause them to die.

If root mortality occurs whenever the supply of stored photosynthate is exhausted and import from the shoot is curtailed, then roots might be expected to die even when soil moisture levels are high (Deans 1979). On the other hand, roots would be able to survive periods of extreme drought, as has been frequently observed (Leshem

1965, Teskey and Hinckley 1981), provided they were sufficiently stocked with stored carbohydrate.

Drought apparently has little effect upon root mortality, probably because water losses from moist roots to drier soil are extremely limited (Molz and Peterson 1976). Electron micrographs of suberized barley roots show the endodermis contains many plasmodesmata, valve-like pores that allow water to flow inward along a water potential gradient, but close whenever the potential gradient favors water flow outward into the soil (Clarkson and Robards 1975). Douglas-fir roots probably have similar mechanisms to impede root desiccation. In this study, suberization was observed in all Douglas-fir roots, independent of plant carbohydrate status. This suggests that suberization is unaffected by growing conditions and that all roots are equally resistant to water loss.

Hermann (1964) has discussed reduced survival of planted seedlings after exposure of the root systems to dry air. It must be remembered, however, that even at a soil water potential of -5.0 MPa (-50 bars), equilibrium relative humidity is above 96% (Nobel 1974). Therefore, evaporative demand is much lower in the soil than in the atmosphere and the results of exposure studies cannot be assumed to apply to conditions in the soil.

I conclude that drought is not directly responsible

for death of fine roots. I found no massive die-off of roots in response to drought unless starch and sugar reserves were nearly depleted. The physiological controls over fine root mortality were therefore concluded to be more closely related to the root's ability to continue to respire, drawing from its starch and sugar reserves. In a subsequent paper (Chapter II), I examine the relationship between root starch and root mortality and the means by which patterns of starch deposition and depletion relate to fine root turnover. These results begin to explain observed differences in fine root turnover rate from site to site. Their inclusion will improve physiological models of forest growth.

Table I.1. Starch and sugar concentrations (mg g d.w.$^{-1}$) of live and dead fine roots from combinations of shade and drought treatments.

Sample	Apparent starch	Sugar	Total
	(mg g d.w.$^{-1}$)		
Shaded, unwatered, dead roots	33	23	56
Shaded, unwatered, live roots	31	30	61
Shaded, watered, live roots	44	34	78
Unshaded, unwatered, live roots	40	98	138
Unshaded, watered, live roots	78	61	139

Figure I.1. Correlation between root tip mortality against root mortality by weight (● unshaded, watered; ○ unshaded unwatered; ■ shaded, watered; □ shaded unwatered).

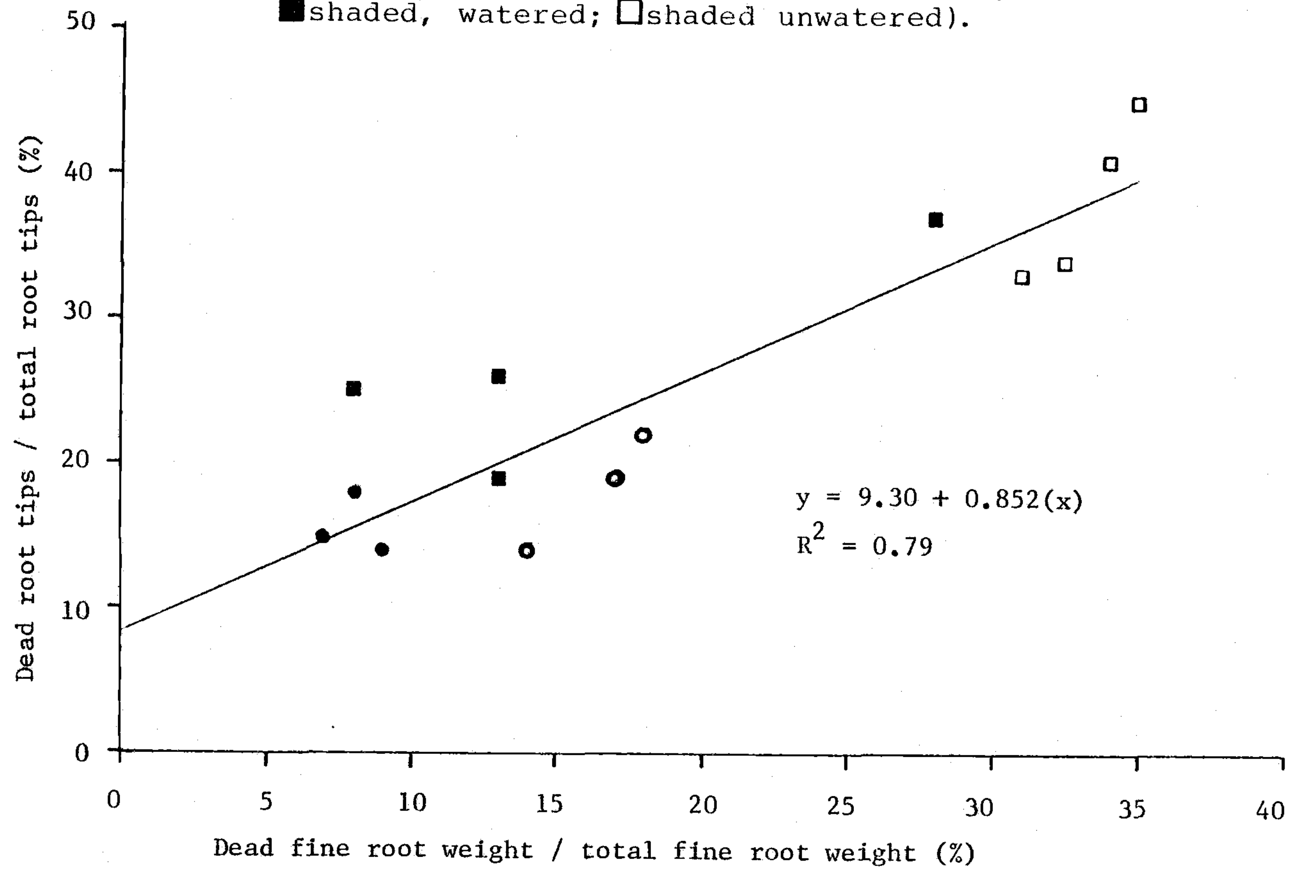


Figure I.2. Fine root mortality by weight percentage for shade and drought treatment combinations.

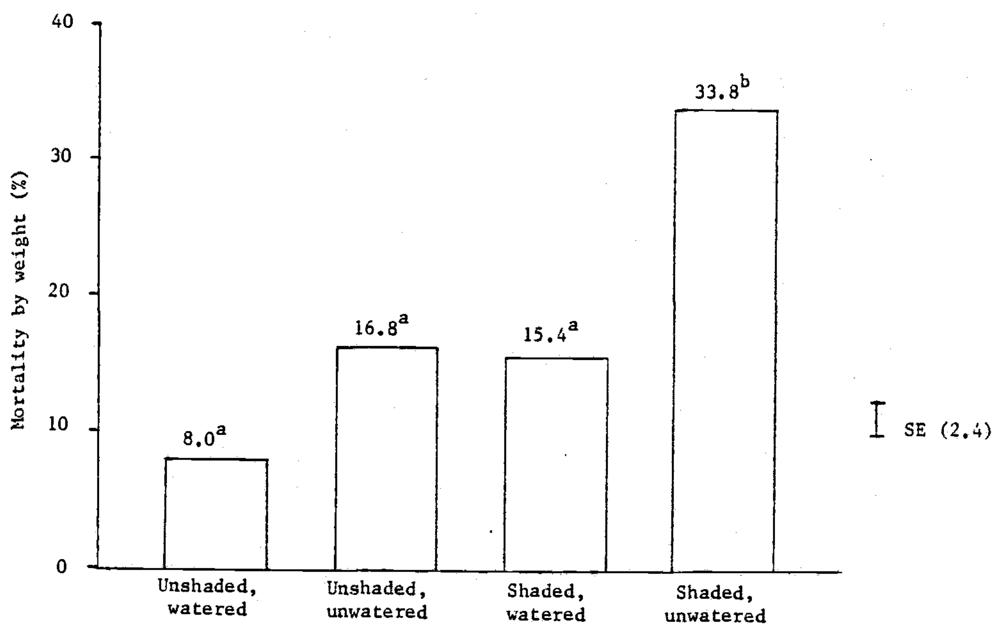


Figure I.3. Root tip mortality for shade and drought treatment combinations.

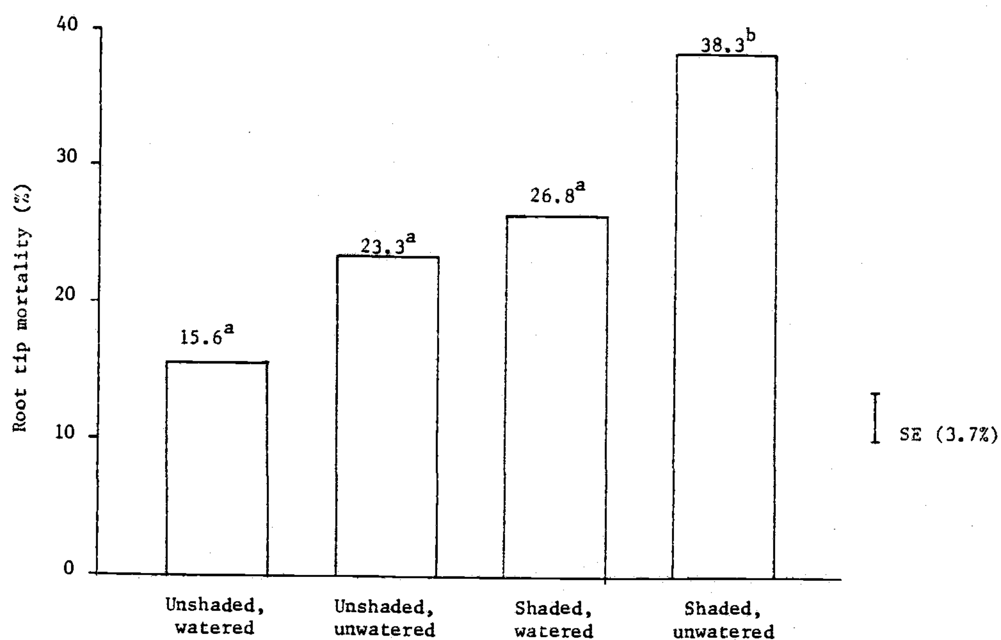
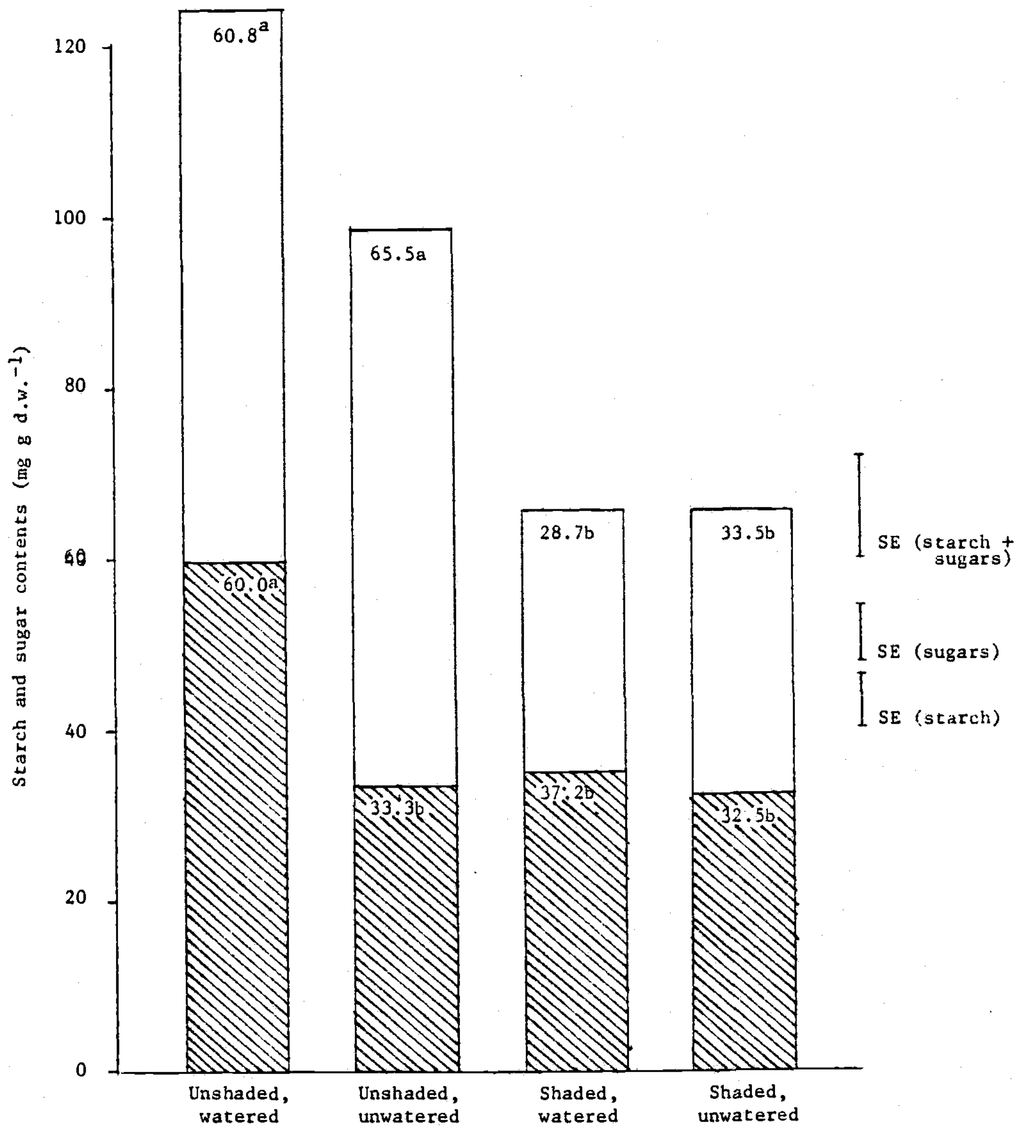


Figure I.4. Starch and sugar concentrations of fine roots for shade and drought treatment combinations. Shaded areas represent starch concentrations.



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CHAPTER II.

PREDICTION OF FINE ROOT PRODUCTION AND TURNOVER FROM ACCUMULATION AND DEPLETION OF STARCH

by

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ABSTRACT

To determine how longevity of fine roots (those without secondary thickening) is controlled, Douglas-fir seedlings were exposed to light or covered with aluminum foil to exclude light and roots were maintained at temperatures of 10°C, 20°C, and 30°C. Root maintenance respiration rates, estimated from rates of starch and sugar depletion in the covered seedlings, ranged from 0.83 to 3.25 mg starch g d.w.$^{-1}$ day^{-1}. Although the uncovered seedlings showed substantial root growth, root starch depletion was similar in the covered and uncovered seedlings at 20°C and 30°C. On the other hand, at 10°C, starch was deposited in the roots of the uncovered seedlings whenever the root systems grew. Based on these results, I suggest that starch deposition in a fine root is limited to the time when the fine root is growing and that the starch is subsequently respired to meet maintenance requirements. A simple means of estimating root biomass production and turnover based on root starch and soil temperature is described and compared to field data.

INTRODUCTION

It has long been known that fine roots, or feeder roots, are responsible for the majority of nutrient and water uptake by trees (Lyr and Hoffman 1967, Trappe and Fogel 1977). The costs of maintaining a functioning fine root system are, however, remarkably high (Keyes and Grier 1981, Agren *et al.* 1980, Grier *et al.* 1980). Although fine roots may constitute less than 1% of the total biomass of a mature tree, they may account for as much as two-thirds of total annual biomass production (Grier *et al.* 1980). This can be attributed largely to their short lifetimes; fine roots may be replaced (turn over) two to three times each year (Persson 1979, Santantonio 1982), or perhaps even more (Head 1973, Persson 1978). These findings are important to foresters and production ecologists because below-ground production, coming from a finite supply of photosynthate, substantially reduces potential above-ground production. Keyes and Grier (1981) describe two stands with equal total production, but in one stand allocation belowground was twice that in the other. Increased belowground production was counterbalanced by a loss in above-ground productivity.

What determines how long fine roots live is a key physiological question of ecological significance.

Drought and carbohydrate exhaustion have been suggested as two probable causes of fine root death (Persson 1979,1980, Santantonio 1982). In a previous study, I found that the life span of fine roots was unaffected by the normal range of drought conditions *per se*, but that root mortality followed closely upon depletion of starch and sugar (see Chapter I).

Survival of a seedling (Etter 1969) or of a single root (Vartanian 1981) may depend on its starch reserves. Even when a tissue is not growing, enzymatic machinery and membrane transport systems must be maintained (Penning de Vries 1975). The energy used in these maintenance processes is derived from "maintenance respiration" (Penning de Vries 1972). Maintenance respiration rates are exponentially related to temperature (Penning de Vries 1975). Respiration rates of actively growing tissue are much higher than the maintenance respiration rate because additional CO_2 is released in biosynthetic processes associated with growth. The respiratory cost of a given unit of tissue, termed "growth respiration", is not related to temperature and depends only on the chemical composition of the tissue (Penning de Vries *et al.* 1974, McDermitt and Loomis 1980). Growth can, of course, be curtailed, but a tissue must continuously meet its maintenance requirements or it will die.

Seedling physiologists have long been aware of the importance of starch reserves for supporting maintenance respiration (Winjum 1963). Nursery seedlings are commonly stored under cold, dark conditions for several months prior to planting. Loss of reserves during storage has been demonstrated (Ronco 1973, Ritchie 1982) and these losses have been implicated in poor root regeneration and poor seedling survival following planting (cf. Ritchie 1982).

Reserves are used for growth as well as maintenance. Shoot growth in conifers has been clearly demonstrated to utilize reserve foods (Gordon and Larson 1970). Similarly, two recent studies have concluded that reductions in root starch concentrations are inversely correlated with root growth (Ford and Deans 1979, Ericsson and Persson 1980), and therefore, that starch is the substrate from which new roots are synthesized. These latter studies, however, did not clearly distinguish whether starch reserves were depleted from all roots or only those of larger diameter with secondary thickening.

In this study I test the following hypotheses: 1) that growth of fine roots is accompanied by starch accumulation rather than depletion, 2) that a grown fine root meets its maintenance requirements wholly from its starch and sugar reserves, and 3) that the root dies when its starch and sugar reserves are exhausted (Fig. II.1).

A corollary of these hypotheses is that soil temperature controls fine root turnover through its effect on maintenance respiration. To evaluate these hypotheses, I conducted an experiment in which light and soil temperature were controlled and starch and sugar concentrations were monitored in fine roots. From results of this experiment, I developed a model and used it to predict fine root production and turnover in two field studies, the first with Douglas-fir forests growing over a range of soil moistures regimes (Santantonio 1982) and the second with a young Scots pine stand growing in the boreal climate of Sweden (Ericsson and Persson 1980).

SEEDLING EXPERIMENT

Two-year-old Douglas-fir (*Pseudotsuga menziesii* Mirb. (Franco)) seedlings, grown from seed collected in 1981 near Vernonia, Oregon (latitude 46°N, longitude 123°W) were used as experimental material. Mycorrhizal infection was low at the beginning of the experiment. In March 1983, the seedlings were carefully washed free of soil and transplanted into washed river sand in 550 cm^2 plastic tubes. A small amount of root growth had already begun prior to the replanting. The seedlings were then grown in a cold frame until treatments began.

In early April, as buds were beginning to swell, the seedlings, in their plastic tubes, were transferred into

metal boxes filled with sand. The boxes were placed into water baths circulating water at temperatures of 10, 20, and 30° $\pm$0.5^{o}C in order to alter maintenance respiration rates of the root systems. The growth chamber was maintained at an average temperature of 21.0($\pm$0.5)^{o}C and relative humidity varied between 70 and 100%. Light levels, provided by fluorescent light supplemented by 300W Sylvania incandescent bulbs, ranged from 90 to 120 einsteins m^{-2} sec^{-1}.

At the time of transferral into the growth chamber, half the seedlings in each temperature treatment were covered by an aluminum foil sleeve of about 500 cm^{3} volume. All foliage was tucked up into the sleeve and the sleeve was pushed down to ground level, effectively excluding all light.

Seedlings were not fertilized during the experiment in order to favor root growth and starch accumulation (Etter 1969, Ariovich and Cresswell 1983). Seedlings were watered whenever dry to two cm below the soil surface; watering schedules were adjusted as deemed necessary after each seedling harvest. Average watering intervals varied from 21 days for the covered seedlings at 10°C, to 12 days for the uncovered at 10°C, 11 days for the covered at 20°C, 10 days for the uncovered at 20°C, and 9 days for the covered and the uncovered seedlings growing at 30°C. In the 10°C and 20°C treat-

ments, soil was always moist at harvest so watering was presumably adequate. In the 30°C treatments, however, soils dried quickly and watering intervals were necessarily decreased as the experiment progressed.

Harvest intervals were chosen to match estimated rates of starch depletion based on pre-budbreak starch concentrations measured by Ericsson and Persson (1980) and fine root respiration rates presented by Agren *et al.* (1980). The first harvest was made just before seedlings were transferred into the growth room.

At the start of the experiment only 10 seedlings were analyzed. These 10 seedlings represented initial conditions in separate analyses of variance for each of the soil temperature treatments. Although none of the seedlings had yet been shielded from light, the seedlings were arbitrarily divided into five covered and five uncovered so the first harvest could be included in the analyses of variance. As the experiment progressed, the sampling intervals were adjusted to accommodate differences in growth rates and surprisingly long life under dark conditions. At 10°C, the last harvest was made after 140 days whereas at 20°C and 30°C final harvests were made after 120 and 60 days, respectively.

Five covered and five uncovered seedlings were chosen at random from each water bath at each harvest date. Because seedlings had not been planted at constant

depths, I divided the plant into root and shoot sections immediately above the first lateral root. Root systems were carefully washed; most of the sand was easily removed, except on the growing tips. Roots were then divided into three classes: 1) Coarse roots; those roots showing evidence of secondary thickening, specifically, shreds of the periderm overlying a lighter, woodier bark, 2) Old fine roots; those roots without evidence of secondary thickening that appeared to have been present before planting, and 3) New fine roots. Old fine roots were separated from new fine roots by their darker color, lack of root hairs, more basipetal position, and smaller diameter than the new fine roots.

Samples were dried in a forced-draft oven at 70°C, weighed, and ground to pass a 40-mesh sieve. Samples were first extracted with 100% acetone to remove chlorophyll and other pigments (W.D. Loomis, personal communication). Sugars were then extracted with hot 80% ethanol three to four times, until the extract was colorless. This procedure is similar to the Soxhlet extraction (AOAC Methods 1980). The ethanol extract was cleared first with 100 mg insoluble polyvinylpyrrolidone and then with 100 1 saturated lead acetate solution (Sanderson and Perera 1966). Sugars were determined colorimetrically by the anthrone reaction (Yemm and Willis 1954, Hansen and Moller 1975). Labile

polysaccharides were extracted from the ethanol-extracted residue using 35% perchloric acid (Hansen and Moller 1975) on an orbital shaker for sixteen hours. Quantities of labile polysaccharides (mostly starch, see Chapter III) were determined colorimetrically with anthrone (Yemm and Willis 1954, Hansen and Moller 1975).

The experiments were analyzed as nine separate comletely randomized designs, one for each combination of tissue type (shoots, coarse roots, and old fine roots) and temperature (10°C, 20°C, and 30°C). Harvest and light treatment were analyzed as treatments in a factorial design (Steel and Torrie 1980). Because new fine root samples were composited for starch analysis, they were excluded from the analysis of variance for starch concentrations. Comparisons among subsequent harvests and between dark-grown and light-grown seedlings within a harvest were made using the least significant difference (LSD) (Steel and Torrie 1980).

Maintenance respiration rates were calculated by observing the decrease in total carbohydrate reserves (sugar and starch) in covered seedlings exposed to different root temperatures. I assumed that in the covered seedlings, no further carbohydrate translocation into the root system was occurring. The first harvest was excluded because respiration rates often respond to sudden changes in temperature (Smakman and Hofstra 1982).

The last harvest was excluded because depletion of starch and sugar was generally already complete. Regression lines were fitted to the starch and sugar depletion data and slopes and intercepts were compared using covariance analysis (Neter and Wasserman 1974).

With regression analysis, I predicted fine root growth from net changes in fine root starch concentration for the roots from the 10°C treatment. Net daily rates of change in starch concentration were calculated by comparing mean starch contents of subsequent harvests and dividing by the interval between the harvests. I compared these predictions with measured changes in root relative growth rates obtained from differences in mean weights between subsequent harvests (Evans 1972).

RESULTS

Analyses of variance of starch concentrations and weights measured the importance of light, harvest date, and the interaction between light and harvest date for each combination of plant components and root temperatures. In the analysis of starch concentrations (Table II.1), interactions between light level and harvest date were significant (α=0.01) for shoots and fine roots at all temperatures. The significance of the interaction for the coarse roots was demonstrated at 10°C but not at 20°C or 30°C. At 20°C and 30°C, the effect of light alone was not significant for either the fine or

coarse roots. For roots at $10^{o}C$, however, as well as for the shoots at all temperatures, light effects were very highly significant (α=0.001).

The above results can be interpreted by examining the interaction means, shown in Figs. II.2, II.3, and II.4. The significance of the interactions for the old fine roots at $20^{o}C$ and $30^{o}C$ is due to the covered and uncovered seedlings being alternately higher than one another as the experiment progressed (Figs. II.2a and II.2b). This is probably attributable to inconsistent separation of the root fractions from one harvest to the next. The differences between covered and uncovered seedlings are inconsistent and are small relative to the decline in starch content over time. At $10^{o}C$ however, starch concentrations of the roots were definitely affected by light conditions (Figs. II.2c, II.3c, and II.5c). Starch concentrations in old fine roots of the covered and uncovered seedlings declined in parallel between harvests one and two (Figs. II.2c and II.5c). Between harvests two and three, however, the starch concentration of the fine roots increased in the light-grown plants. Starch concentrations of the coarse roots in the light did not fall significantly below pre-budbreak level until the final harvest (Fig. II.3c). Thus the significance of the light effect in the analysis of variance for fine and coarse roots at $10^{o}C$ (Table

II.1) was due to the consistently higher starch concentrations in the root systems of the light-grown trees.

Starch concentrations of shoots of the covered seedlings declined sharply over the first thirty days (Fig. II.4) from a maximum of 328 mg g d.w.$^{-1}$, then continued to decline, although much less sharply, until day 100, when all starch was depleted. Starch concentrations of the shoots of the light-grown seedlings declined less steeply and increased to over 200 mg g d.w.$^{-1}$, later to fall again.

I also calculated an analysis of variance on the weights of seedling components for each combination of light treatment and harvest date at the different soil temperatures (Table II.2). The light level by harvest date interaction was always significant for the shoots, coarse roots, and new fine roots. For the old fine roots, however, the light by harvest date interaction was never significant and the effects of light treatments were inconsistent.

In the covered seedlings, weights of all seedling components remained essentially unchanged, although there were downward trends in weights of all components except the new roots, probably as a result of starch depletion (Table II.3). Shoot weights of the uncovered seedlings increased significantly, but not until shoot elongation

had ceased and starch levels had begun to recover. Coarse root and new root weight in uncovered seedlings rose significantly following the increase in shoot weight. There were no consistent increases in weight of old fine roots in the light.

Relative growth rate was regressed against change in starch concentration for composited new and old fine roots grown at 10°C under light (Fig. II.6). As hypothesized, fine root growth was associated with starch deposition. Small decreases in average starch concentration may result from starch losses in maintenance respiration in excess of starch gains in the growing roots.

Maintenance respiration rates of each of the seedling components were calculated by linear regression of mean starch and sugar concentration against time for each temperature treatment, using only the covered seedlings. There were no differences among the slopes and intercepts of the starch and sugar depletion curves for the shoots; therefore shoots from all root temperature treatments were combined. When the pre-budset and post-budset periods (before and after 30 days) were separated by including a dummy variable in the regression, the regression equation improved with the R^2 changing from 0.66 to 0.96 (Figure II.7). The regression equations for the pre-and post-budset periods are

significantly different. The slopes and intercepts were not all significantly different for the roots at different temperatures, however, the separate equations are presented because the slopes are my best estimates of maintenance respiration rates (Table II.4). The regression equation predicting maintenance respiration rates (R_m) from root temperatures (T), where maintenance respiration is in mg CH_2O g^{-1} day^{-1} and temperature is in ^{o}C, is:

$$R_m = 0.408e^{0.0685T(^{o}C)} \qquad (1)$$

The R^2 of this equation is 0.99. The Q_{10} value of R_m is 1.98.

In the shoots and in roots at 30°C, covering the seedlings significantly reduced sugar concentrations, but only after all starch had been depleted. Starch depletion is here defined as 40 mg g $d.w.^{-1}$ perchlorate extractable carbohydrate remaining (see Chapter III). Sugar concentrations in the shoots declined, after starch exhaustion, from 65.5(4.9) mg g $d.w.^{-1}$ to 29.3(4.2) mg g $d.w.^{-1}$; this difference is significant at the 0.01 level. In roots from the 30°C treatment, sugar concentrations declined, after starch exhaustion, from 59.7(3.6) mg g $d.w.^{-1}$ to 23.0(2.0) mg g $d.w.^{-1}$, also significant at the 0.01 level. In the roots growing at 10°C and 20°C, differences in sugar concentration after starch depletion

were not significant, however, there were significant delines in sugar concentration shortly after seedlings were covered. These roots declined from 59.5(3.5) mg g d.w.$^{-1}$ at the first harvest to 27.9(1.7) mg g d.w.$^{-1}$ afterward. This difference was significant at the 0.001 level.

By the date of the final harvest, all seedlings maintained in darkness had died. Though the year-old foliage remained green and apparently unwilted under the aluminum sleeves, it quickly withered and browned upon exposure to light.

DISCUSSION

The hypothesized pattern of starch deposition and depletion presented in Fig. II.1 can be tested in light of the foregoing results. Fine root growth was associated with starch deposition at 10°C (Figs. II.1). Starch deposition was not observed at what appeared to be abnormally high root temperatures (Ericsson 1979, Santantonio 1982) On the other hand, the light levels under which the seedlings were grown were rather low. I expect that the pattern of starch deposition observed at 10°C is the normal one in the field.

Once deposited, starch is not augmented or replaced in the non-growing root. This is demonstrated by the non-significant light effect on the fine roots at high temperatures (Table II.1) and by the similarity in starch

depletion observed in the roots of covered and uncovered seedlings at 20 and 30°C (Figs. II.3 and II.4). If the sugars transported from the shoot to support root growth in the uncovered plants were also available to the non-growing roots, they would have been used for maintenance, reducing starch depletion in the uncovered plants relative to that in the covered plants. Also, light effects would have been significant in the analyses of variance (Table 1). Moreover, differences in starch concentrations between covered and uncovered seedlings would have been more pronounced and the starch concentrations of the uncovered plants would have been consistently higher than those of the covered plants as the experiment progressed. Since none of the above occurred (Figs. 2 and 3), I conclude that starch in non-growing fine roots is not augmented later from other sources. Rather, current photosynthate is used almost exclusively for growth of new fine roots and, if soil temperatures permit, for starch deposition in new fine roots.

Evidence against photosynthate being translocated out of fine roots into other organs rests in the constant slope of the starch depletion curves observed in the roots of covered plants (Table II.4, Figs. II.3 and II.4), despite near exhaustion of shoot starch during shoot elongation. If photosynthate was not translocated

from roots under such circumstances, it is unlikely that it ever is. I conclude, therefore, that starch deposited in fine roots remains to be depleted and is not translocated.

The final part of the hypothesis presented in Fig. II.1 is supported by my observations that seedlings died once starch and sugar were exhausted. Probably the same is true for individual roots. Further evidence for the above hypothesis, based on starch contents and turnover rates measured in field studies, will be presented shortly.

The starch and sugar depletion rates measured in this study are similar to the lowest maintenance respiration measurements in the literature, excepting those for dormant seeds. Woody tissues and storage tissues have much lower maintenance respiration rates than most other tissues (Penning de Vries 1972,1975). Further, gas exchange techniques used to measure root respiration tend to overestimates the process. Removal of a root from the soil disturbs the root and increases its respiration rate (Lawrence and Oechel 1983). Also, the O_2 concentration is likely to be much lower in the stagnant soil atmosphere than in a cuvette or a respirometer flask (Ledig *et al.* 1975). In a system in which the gas being measured is passed through a potted root mass (cf. Lawrence and Oechel 1983), microbial decom-

position of dead root biomass and of root exudates is included in the estimate of respiration. Finally, given the slow rates of photosynthate translocation in the root system (10 cm day^{-1}) measured by McLaughlin _et al._ (1977), the twelve-hour period allowed for roots to fall to a so-called maintenance respiration rate (Raper _et al._ 1978) is too short for trees, and would overestimate maintenance respiration rates. Thus one would generally expect maintenance respiration rates estimated from starch and sugar depletion data to be somewhat lower than those based on CO_2 exchange.

Another approach to measuring root respiration is to estimate root respiration as the difference between CO_2 uptake and net assimilation rate (Ledig _et al._ 1975). Such a procedure gave estimates, just prior to a period of respiratory weight loss in the roots, of 3.28 mg CH_2O g^{-1} day^{-1} at 32 ^{o}C in pine seedlings. This is similar to estimates obtained from starch and sugar depletion data in this study. Therefore, one may conclude that the requirements of maintenance respiration were being met almost entirely by starch and sugar depletion in this study. A similar conclusion was reached in studies by Ritchie (1982). These results support the hypothesis that starch and sugar in non-growing roots are used primarily to meet maintenance requirements.

The reason why starch accumulation in the root is

limited to the period immediately following growth may relate to the location of the starch reserves. In a root without secondary thickening, the starch is deposited exclusively in the cortex, which is the undifferentiated tissue surrounding the vascular cylinder (Esau 1977). As such a root ages, the cortex collapses (Head 1973). It seems reasonable to expect that collapse of the cortex might be associated with starch depletion. If so, replenishment of starch reserves would be impossible. A minority of fine roots experience a phase of secondary thickening which involves, among other things, the formation of a thick layer of phloem outside the cambium. In such roots, which no longer have a cortex, the starch supply is found in this newly formed ring of phloem (personal observation). The adaptive advantage to such a pattern of starch deposition and depletion lies in the plant's ability to 1) utilize current photosynthate exclusively for growth, and 2) to halt photosynthesis entirely without immediately starving the entire root system. Moreover, a plant is able to maintain and ramify those roots located in particularly favorable microsites by means of secondary thickening (Coutts and Phillipson 1976).

The relation between maintenance respiration and fine root turnover can be tested further using data collected in field studies. For example, the effect of

soil temperature on fine root turnover can be demonstrated from data of Santantonio (1982). He measured soil temperature and fine root biomass and turnover in three Douglas-fir stands on sites he classified by direct measurement as wet, moderate, and dry. While he attributed the differences in turnover he observed to differences in soil moisture, I have shown that soil moisture has little direct effect on root mortality (see Chapter I). I summarized Santantonio's soil temperature data (unpublished) and calculated average root respiration rates on each site using the prediction equation for maintenance respiration presented earlier. As shown in Figure II.7, the maintenance respiration rates predict rates of fine root turnover with an R^2 of 0.99. Using the maximum range of starch and sugar concentrations observed in this study (134 mg g $d.w.^{-1}$) and dividing it by estimates of maintenance respiration on each site, I estimate turnover rates of 1.87 to 2.08 yr^{-1}, similar to Santantonio's measurements (1.7 to 2.8 yr^{-1}). The greater range in turnover rates in Santantonio's data may be explained by the substantial damping of temperature fluctuations at 20 cm, which is the depth at which soil temperatures were measured. It may also have been caused by differences in initial starch concentrations on the different sites.

The non-significant light effect on accumulation of

root starch at 20 and 30°C in my growth room study indicated that little or no starch was deposited in these root systems as they grew. Moreover, starch concentrations of growing roots at 20 and 30°C never exceeded 70 mg g d.w.$^{-1}$ (Figs. II.5a and II.5b) and were not significantly different between covered and uncovered seedlings, even during periods of rapid growth. The extent to which fine root growth without starch deposition occurs in nature cannot be determined from this experiment. If it occurs in the field, it might result in roots living only two to three weeks (Head 1973), and should occur only when soil temperatures are high.

If one assumes that fine roots develop with the same initial starch concentration, it should be possible to estimate fine root biomass production from starch concentration and soil temperature data. Because I have shown that starch deposition occurs in fine roots only during growth, it is possible to estimate the starch concentration of any cohort of fine roots at any point in time by simply estimating the sum of maintenance respiration since ingrowth and reducing the root starch concentration by that amount. After deducting maintenance respiration from the initial starch concentration of each cohort, an overall mean starch concentration can be calculated. If the measured starch

concentration is higher than that predicted, the difference can be attributed to ingrowth of new roots. Root biomass of a given stand can then be estimated for a growing season using the maximum starch concentration observed, usually just before budbreak, as an estimate of new root starch concentration. I have done this using starch and root biomass data of Ericsson and Persson (1980). Root respiration rates were obtained from Agren et al.(1980). Root temperatures were estimated using air temperatures at 1 m (Ericsson 1979) increased by 12% to correct for diurnal temperature fluctuations (calculated from data of Halldin *et al.* 1980).

The equation was solved for each harvest after maximum starch concentrations had been reached. I used 290 mg g $d.w.^{-1}$ as a maximum value for new root starch concentration (Ericsson and Persson 1980). The roots were assumed still alive during the harvest at which they reached zero starch concentration, assuming that dead roots would not be recognized immediately. With the exception of one point on each figure (Figs. II.9 and II.10), the predicted biomasses fall within the standard errors of the biomass measurements. The lack of agreement at the two points might be attributable to measurement error, particularly given the high variability around the third point in Figure II.9. If the short-lived increase in root biomass shown in Figure

II.9 is real, it might be attributed to root growth without starch deposition.

Additional studies are needed 1) to determine the extent of root growth without starch deposition, 2) to compare starch dynamics of mycorrhizal to non-mycorrhizal roots, 3) to examine detailed patterns of starch accumulation and depletion in the various tissues of roots using ^{14}C-labeling, and 4) to field-test the ideas presented here with temperature and starch data collected specifically for that purpose.

I conclude from my work and from others' that initial starch concentration and soil temperature are key variables in estimating fine root turnover and fine root biomass. By separating growth from maintenance respiration and by knowing the initial amount of starch in new roots, it is possible to estimate turnover rates and biomass of fine roots within the resolution of present field measurements. Future field studies should include measurements of these variables because of their importance in explaining and predicting fine root growth and turnover.

Table II.1. Tests of significance* of F-tests from analyses of variance for starch contents of seedling components.

Root temperature	Light	Harvest	Light X Harvest
	Shoots		
30°C	***	***	***
20°C	***	***	***
10°C	***	***	***
	Coarse roots		
30°C	n.s.	***	n.s.
20°C	n.s.	***	n.s.
10°C	***	***	***
	Old fine roots		
30°C	n.s.	***	***
20°C	n.s.	***	***
10°C	***	***	***

* n.s. = not significant at 0.01 level, ** = significant at 0.01 level but not at 0.001, *** = significant at 0.001 level.

Table II.2. Tests of significance* of F-tests from analyses of variance for weights of seedling components.

Root temperatures	Light	Harvest	Light X Harvest
	Shoots		
30°C	***	**	***
20°C	***	**	**
10°C	***	n.s.	***
	Coarse roots		
30°C	n.s.	**	**
20°C	***	***	***
10°C	***	***	**
	Old fine roots		
30°C	n.s.	**	n.s.
20°C	n.s.	***	n.s.
10°C	**	***	n.s.
	New fine roots		
30°C	***	***	***
20°C	***	***	***
10°C	***	***	***

*n.s.= not significant at 0.01 level, ** = significant at 0.01 level but not at 0.001 level, *** = significant at 0.001 level.

Table II.3. Weights (mg) of various components harvested at selected dates following seedling exposure to specified light and soil temperature conditions. Letters compare adjacent harvests at = 0.01, asterisks compare light and dark treatments within a harvest at = 0.01.

Harvest date	Shoots		Coarse roots		Old fine roots		New fine roots	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light
10°C								
0	1640a	1650a	229a	175a	629a	716a	35a	27a
28	1110a	*2040a	251a	309a	412a	467b	43a	47a
60	1330b	*2620b	283a	436b	372b	*593b	87a	*271b
98	1290b	*2670b	337a	472b	485b	511b	96a	*324b
140	845b	*2730b	248a	*482b	336c	485b	122a	*640c
20°C								
0	1640a	1650a	229a	175a	629a	716a	35a	27a
17	1420a	1720a	248a	287b	538a	537b	64a	86a
32	1250a	*2000a	298a	339b	486a	569b	88a	121a
48	1300a	*2090a	350a	408b	426a	552b	128a	217a
62	1210a	*2530b	233b	*493b	397a	435b	123a	*347b
120	1500a	*2560b	296b	*571b	549b	435b	90a	*416b
30°C								
0	1640a	1650a	229a	175a	629a	716a	35a	27a
8	1500a	1590a	321a	233a	575a	393b	76a	69a
16	1400a	1610a	262a	286a	577a	397b	56a	102a
25	1360a	1570a	277a	243a	520a	398b	52a	103a
40	1470a	*2140b	313a	368a	519a	464b	63a	*158a
60	1150a	*2210b	260a	*452a	411a	454b	77a	*278b

Table II.4. Regression equations that describe depletion of starch and sugar in seedlings maintained in darkness. Slopes are estimates of maintenance respiration rates.

		Intercept mg/g dw	Slope mg CHO/g day	R^2	n
Coarse roots	10°C	191a	-2.53a	0.93	4
	20°C	183a	-2.10a	0.91	4
	30°C	176a	-1.03b	0.98	3
Fine roots	10°C	217a	-3.25a	0.86	4
	20°C	189a	-1.53a	0.96	4
	30°C	165a	-0.83b	0.95	3
Shoots before budset		338a	-7.87a	0.94	5
Shoots after budset		162b	-1.07b	0.94	6

Figure II.1. Potential starch and sugar dynamics of a fine root, showing differences in life-span associated with root temperature.

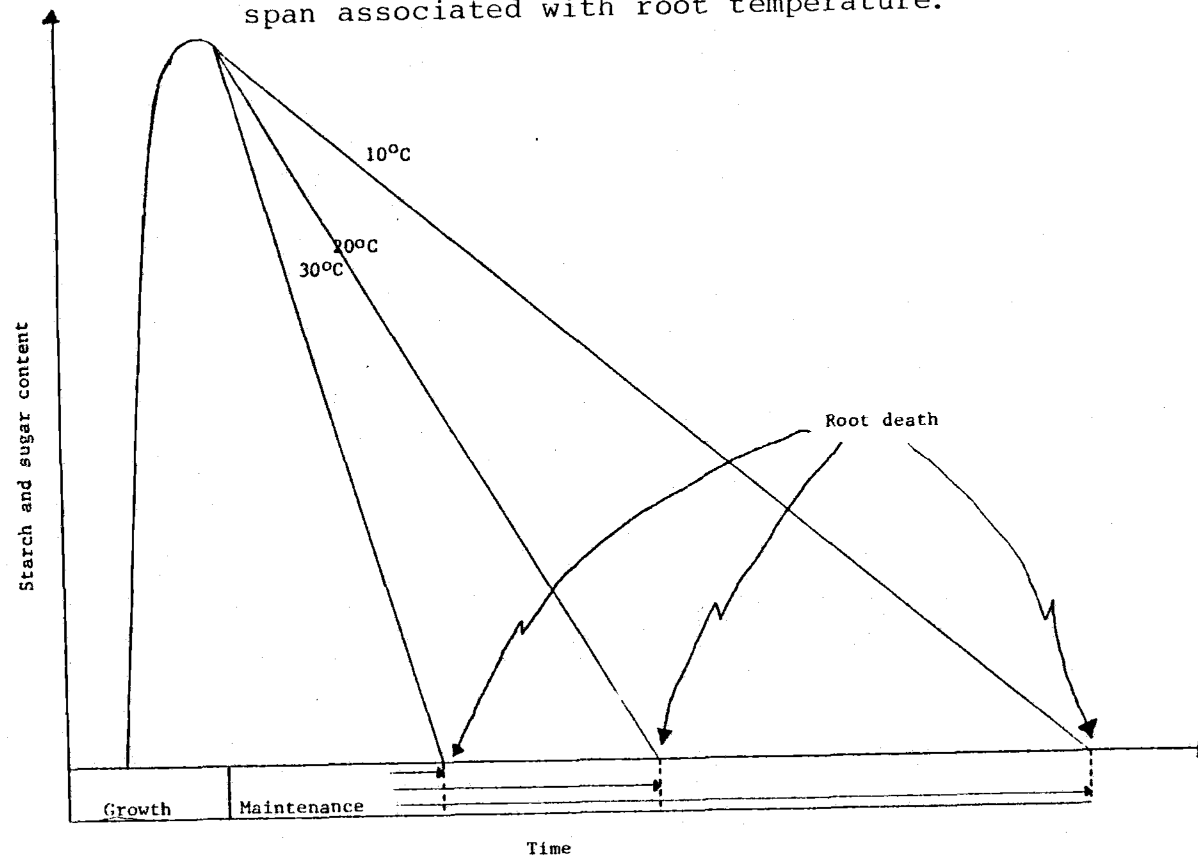
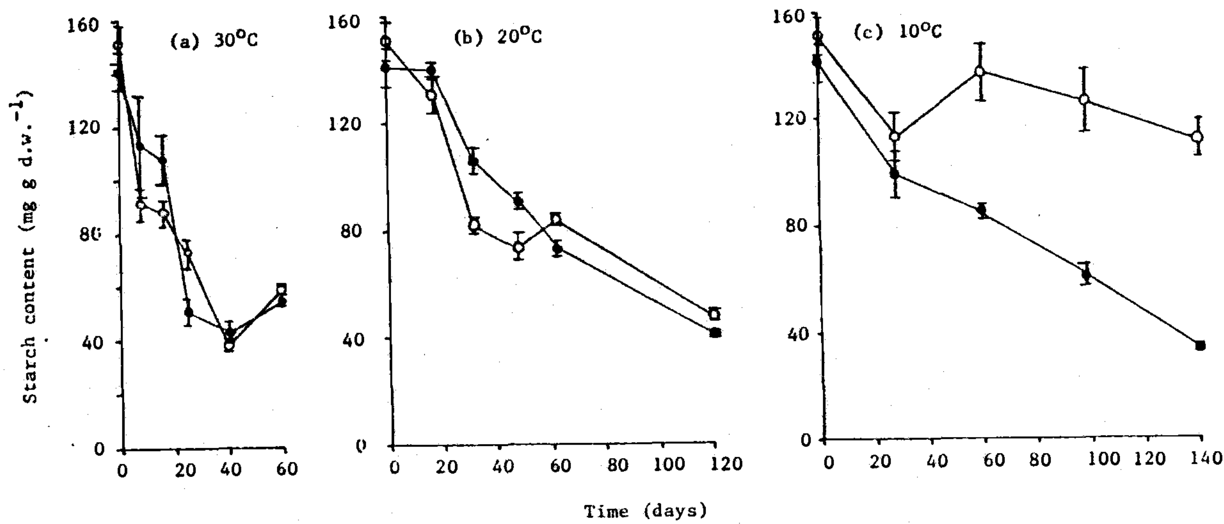


Figure II.2. Starch concentrations of roots without secondary thickening from seedlings grown at specified soil temperatures and exposed to light (○ ± SE) or maintained in darkness (● ± SE).



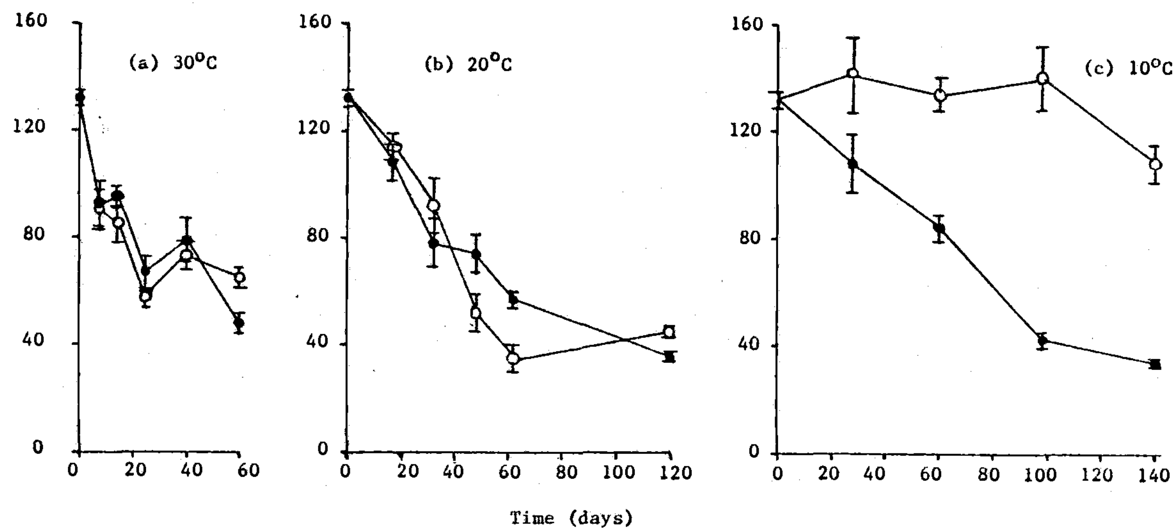


Figure II.3. Starch concentrations of roots with secondary thickening from seedlings grown at specified soil temperatures and exposed to light (○ ± SE) or maintained in darkness (● ± SE).

Figure II.4. Starch concentrations of shoots of seedlings grown at specified soil temperatures and exposed to light (○ ± SE) or maintained in darkness (● ± SE).

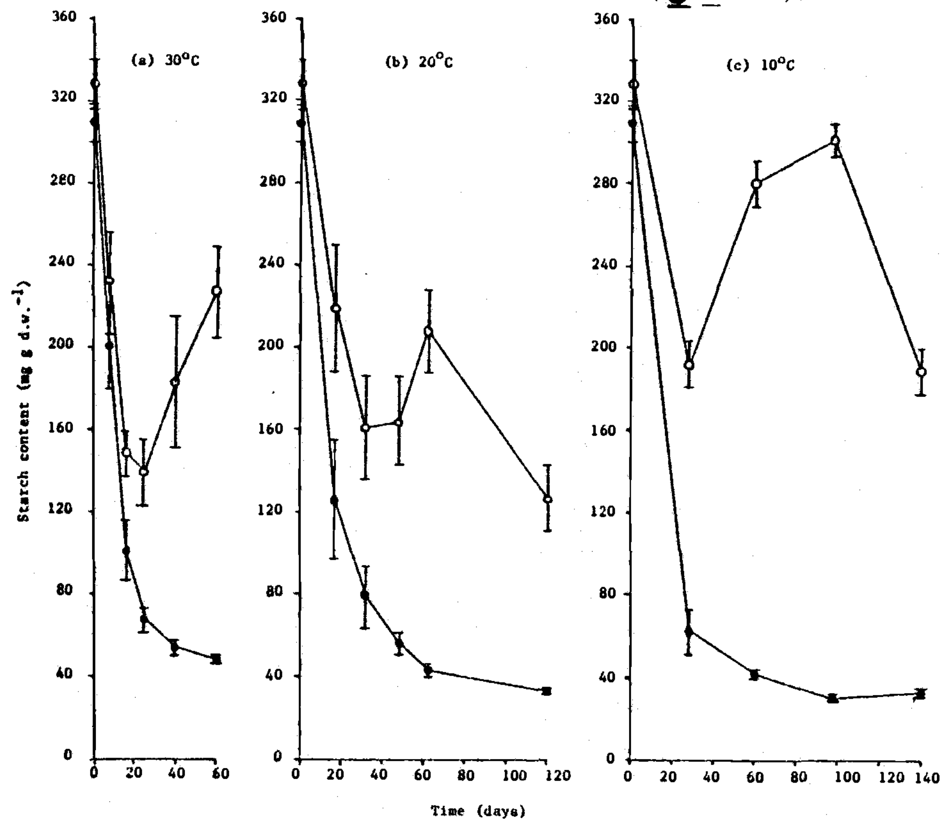


Figure II.5. Starch concentrations of roots without secondary thickening at specified temperatures and with shoots exposed to light or maintained in darkness.

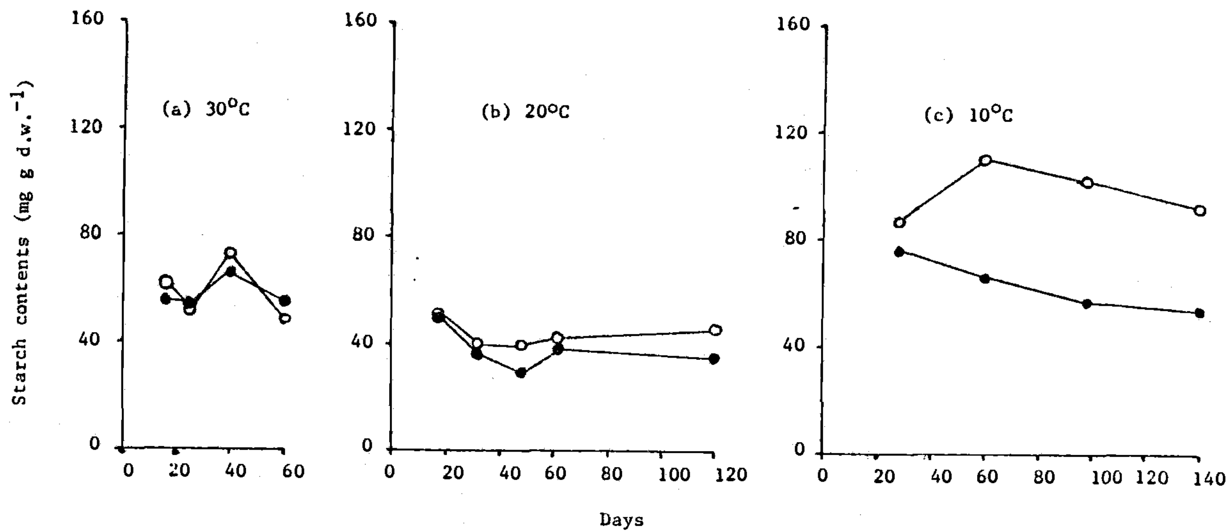


Figure II.6. Relative growth rate of fine roots can be predicted from net changes in starch concentration.

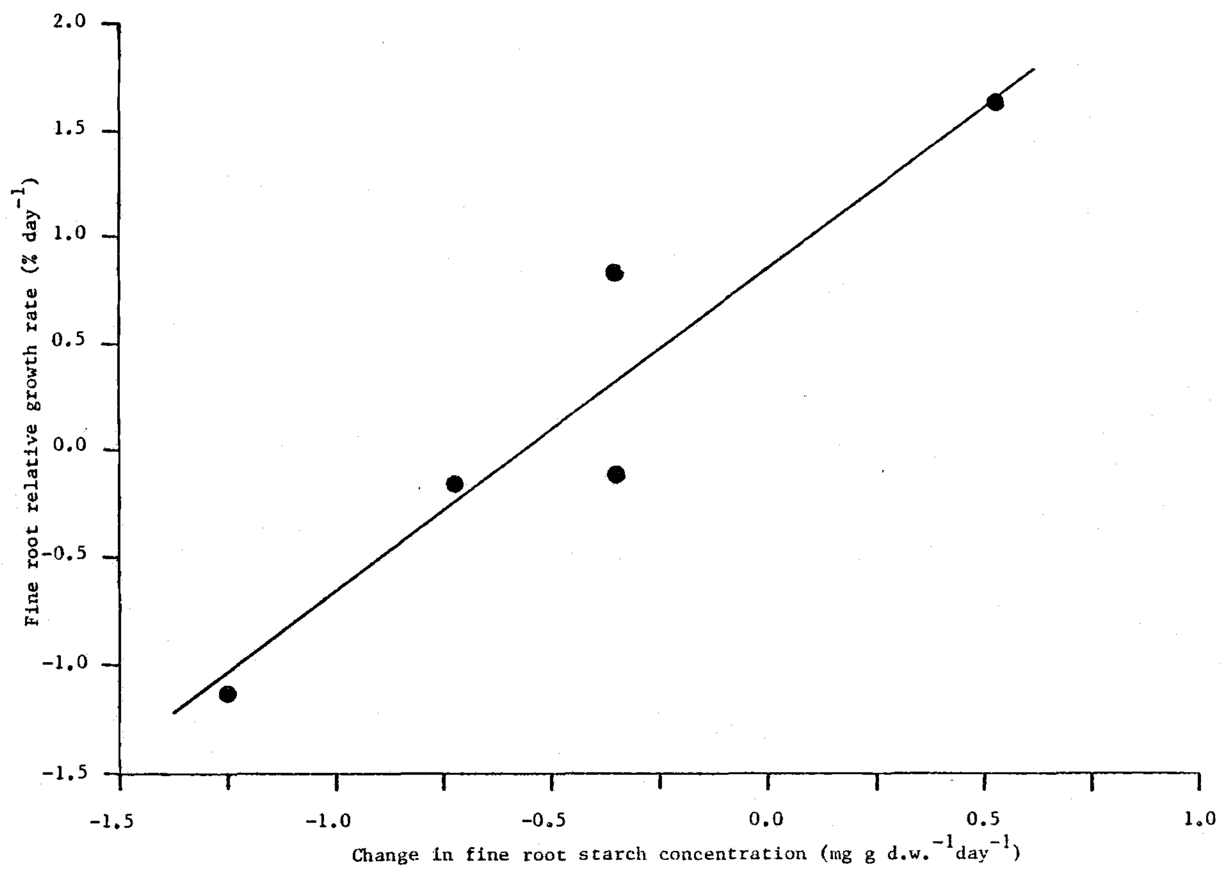


Figure II.7. Starch + sugar concentrations in shoots of seedlings maintained in darkness. Data from all root temperatures were combined. The regression model was significantly improved by separating pre-budset from post-budset periods.

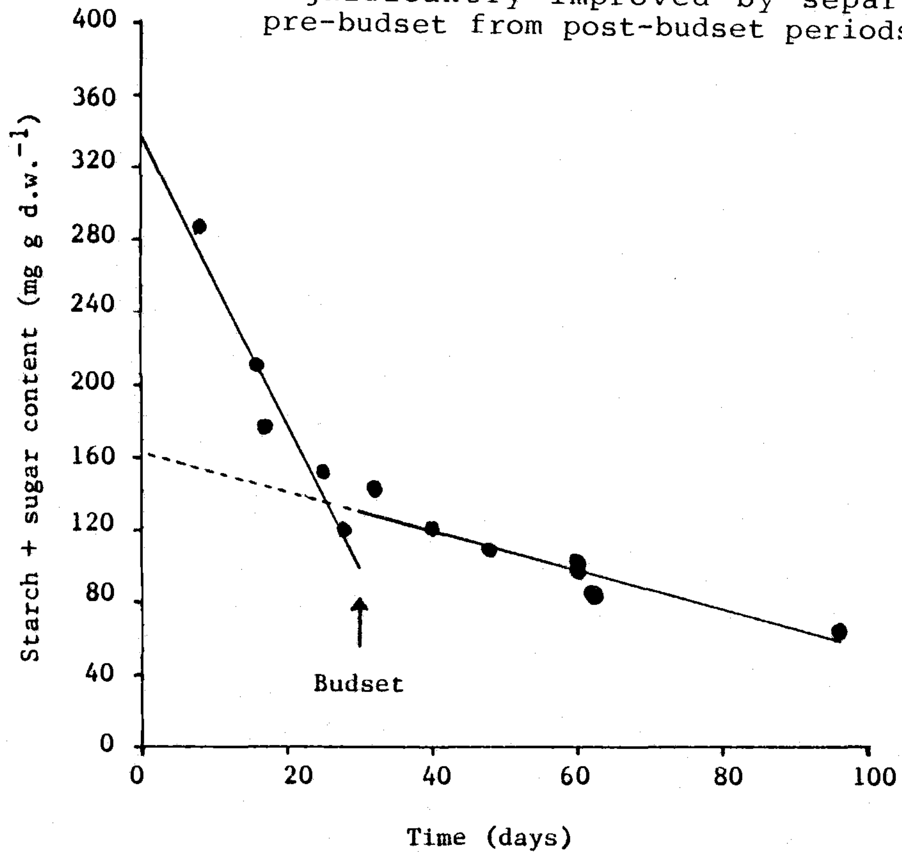


Figure II.8. Regression of fine root turnover rates as measured by Santantonio (1982) against estimated maintenance respiration rates of fine roots.

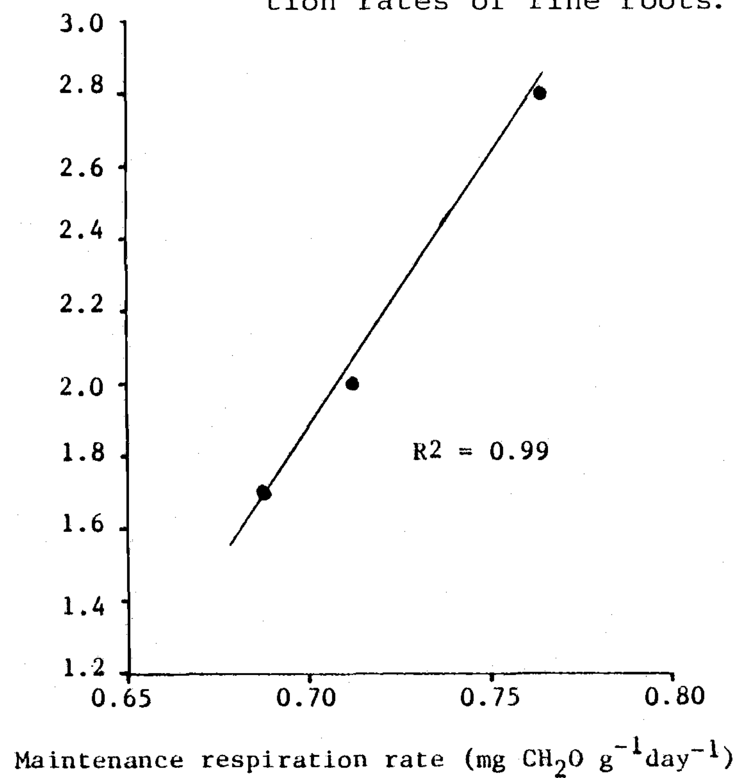


Figure II.9. Predicted and measured fine root biomass from data for control plots of Ericsson and Persson (1980).

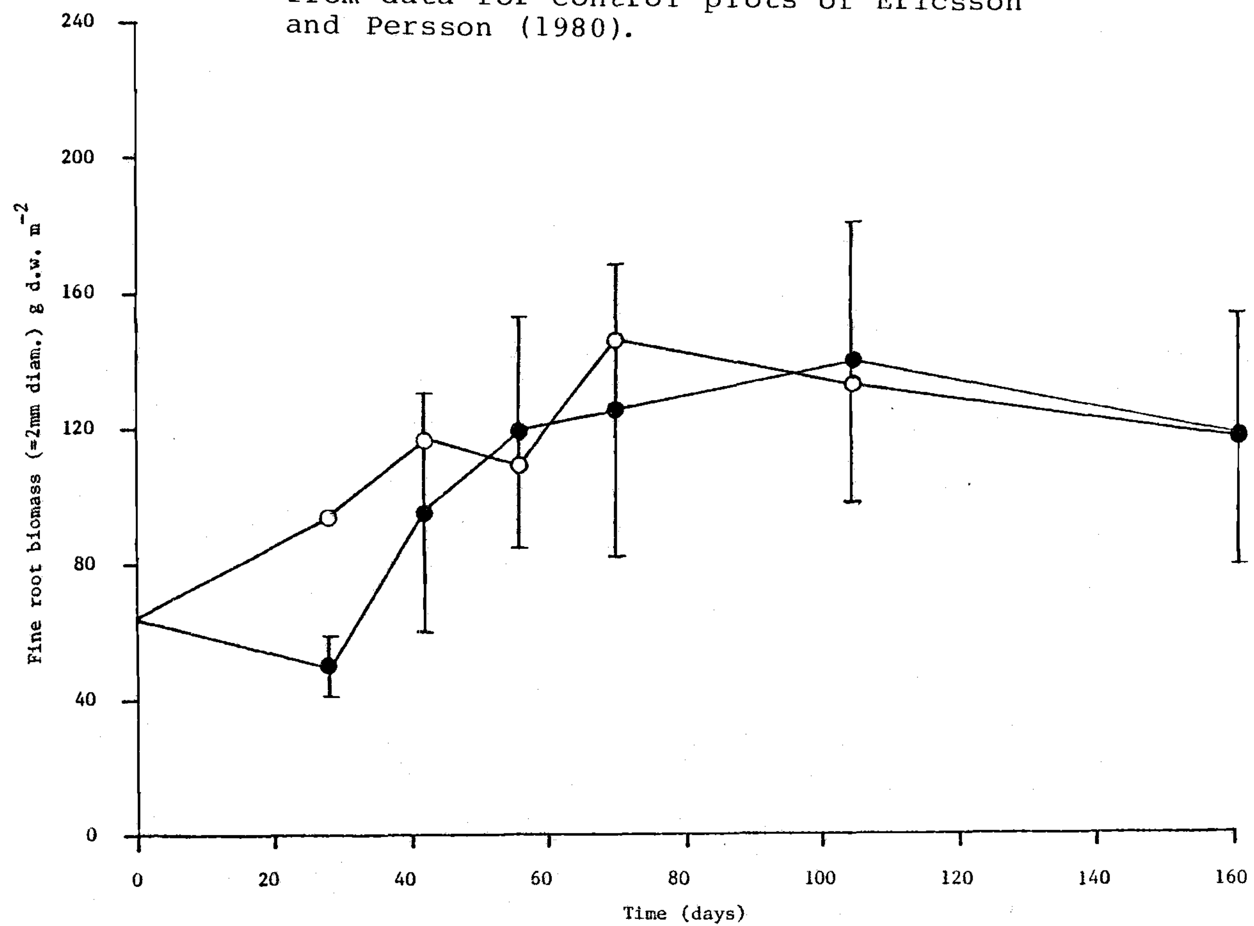
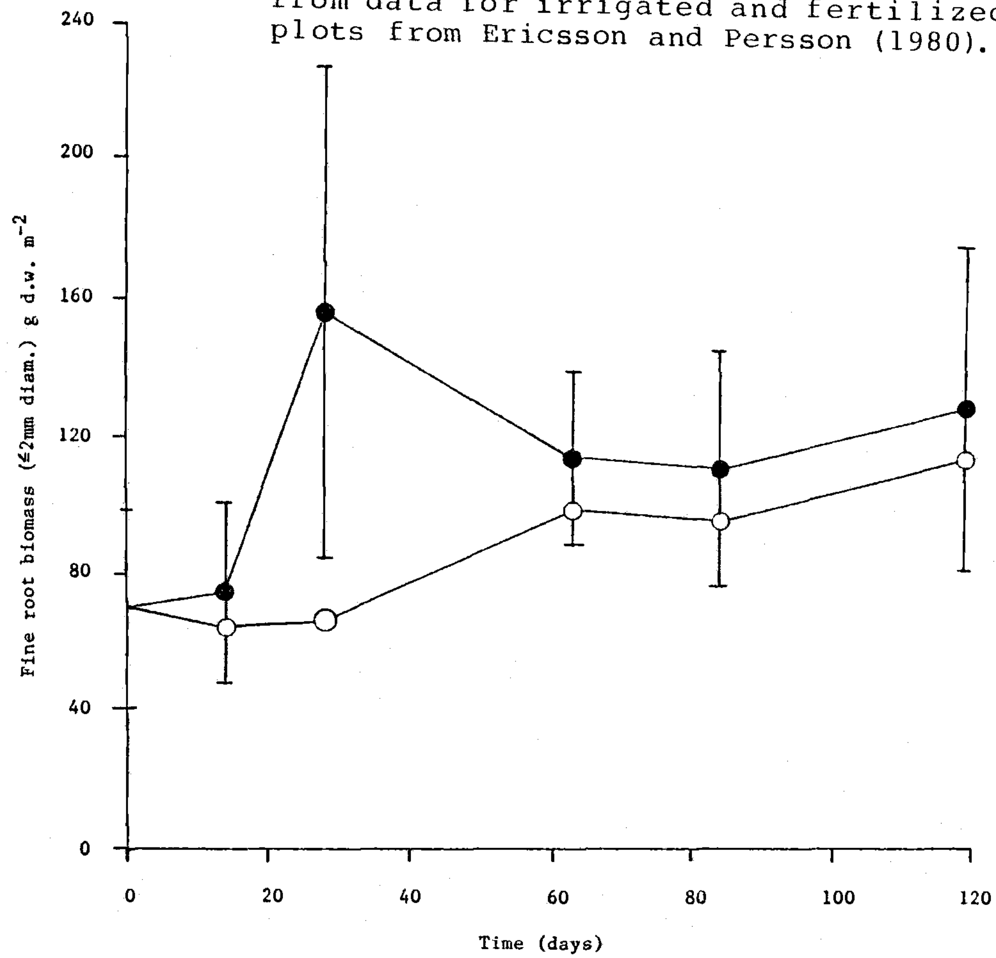


Figure II.10. Predicted and measured fine root biomass from data for irrigated and fertilized plots from Ericsson and Persson (1980).



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CHAPTER III.

PERCHLORIC ACID EXTRACTION OF STARCH FROM DOUGLAS-FIR TISSUE

by

John D. Marshall

ABSTRACT

A simplified method of extracting starch from tissue using 35% perchloric acid is presented. The perchloric acid extracted as much as 249 (SE 17.5) mg starch g $d.w.^{-1}$ and as little as 1.3 (SE 1.3) mg starch g $d.w.^{-1}$. The extract contained relatively small amounts (43 (SE 2.0) mg g $d.w.^{-1}$) of carbohydrates other than starch. The amounts of these "background carbohydrates" remained unchanged across the full range of starch contents, indicating they do not serve as reserve materials. A method of checking for completeness of starch extraction is described.

INTRODUCTION

Because starch is an important storage compound, its extraction and analysis have received much attention (McCready 1970). Starch analysis procedures generally consist of three steps: (1) extraction of soluble sugars, (2) hydrolysis of starch, and (3) measurement of sugar released in the hydrolysis. The starch may be hydrolyzed either by a mineral acid or by starch-degrading enzymes (McCready 1970).

Enzyme hydrolysis is subject to error due to interference from phenolic compounds (Ebell 1969) and protection of starch granules by lipids (McCready 1970). Haissig and Dickson (1979) suggest that these interferences can be circumvented by a methanol:chloroform:water extraction of the tissue prior to enzymatic hydrolysis, however this assumption has not been checked.

Acid hydrolyses are subject to error due to their lack of specificity--they usually extract non-starch polysaccharides along with the starch, which leads to overestimates of starch contents (McCready 1970). This error can be reduced by using less concentrated acid (Tetley 1974, Hansen and Moller 1975). Further, starch can be separated from most other polysaccharides by co-precipitation with iodine (McCready 1970).

One recently developed starch extraction method,

using a lengthy percolation with 35% perchloric acid (Hansen and Moller 1975), has yielded a remarkably wide range in starch contents in roots (8-33% d.w.), root bark (9-49%) and needles (4-29%) of Scots pine (Ericsson 1980, Ericsson and Persson 1980, Ericsson et al. 1980), especially if one compares them to results obtained using enzymatic hydrolyses or rapid perchlorate extractions, which rarely reached values above 10% dry weight (Kreuger and Trappe 1967, Ritchie 1982, Haissig and Dickson 1979). Because the carbohydrate content of the perchlorate extract in these studies never reaches zero, however, it is probable that the perchlorate extract includes carbohydrates other than starch. Nonetheless, the wide range in the extractable carbohydrate contents indicates that the plant uses and replaces these carbohydrate reserves, at least to a baseline of approximately 4%. For my particular interest in assessing the influence of reserves on root longevity, I wished to obtain complete extraction of reserve carbohydrates from roots. I therefore used the perchlorate extraction method. The purposes of this experiment were to determine the amount of non-starch carbohydrate extracted with 35% perchloric acid from Douglas-fir tissue and to determine the extent of its use as a reserve material.

METHODS AND MATERIALS

Plant tissue came from an experiment in which starch

depletion was followed in Douglas-fir (Pseudotsuga menziesii) seedlings completely shielded from light (see Chapter II). Three replicate samples of shoots, secondarily thickened roots, and roots without secondary thickening (fine roots) were used in these analyses. Tissues from the beginning and end of the experiment were contrasted.

Tissue was dried in a forced-draft oven at $70^{o}C$ for 24 hours and ground in a Wiley mill to pass a 40 mesh sieve. Samples of approximately 100 mg were extracted with 80% ethanol until the extract was colorless (AOAC Methods 1980). Samples were washed with 80% ethanol into a 5 cm section of Tygon tubing packed at the bottom with glass wool. The top was then packed with glass wool and a disposable plastic tip from a 100 μl Eppendorf pipettor was filled with glass wool and inserted into the tubing. The tubing was then connected to a percolation system similar to that described by Hansen and Moller (1975), which dripped 35% perchloric acid at a rate of 1-3 ml/hr from a Marriotte flask through a stopcock and into the sample. The perchloric acid extract dripped into a 500 ml volumetric flask containing 400 ml of continuously stirred water; this was necessary to dilute the perchlorate in order to avoid hydrolyzing the starch so completely that the starch would no longer co-precipitate with iodine. After eighteen hours, the diluted extracts

were brought to volume with 35% $HClO_4$, so that the starch was dissolved in a 7% $HClO_4$ solution. Tetley (1974) has shown that starch hydrolysis is mimimal at this concentration. Hexose concentration of this solution was determined by the anthrone reaction (Yemm and Willis 1954) using glucose standards in 7% $HClO_4$ (Hansen and Moller 1975). Starch was precipitated out of the extracting solution using the iodine-precipitation method of McCready (1970). The precipitate was dissolved in 0.5 ml 35% $HClO_4$, two ml of water was added and mixed, and hexose concentration of this solution was determined by the anthrone reaction.

To verify that the iodine precipitate was indeed starch, duplicate samples of the washed iodine precipitate were incubated with amyloglucosidase (Sigma Chemical Co. No. A-7255) , a starch-degrading enzyme. The samples were re-dissolved in two ml of distilled water in stoppered tubes placed in a boiling water bath. This solution was cooled and 0.5 ml each of purified amyloglucosidase solution and 1 M sodium acetate buffer (pH 4.2) were added and mixed. Tubes were stoppered and incubated at 50 ^{O}C for six hours (Smith 1969, Haissig and Dickson 1979). At the end of the incubation, the iodine precipitation procedure was repeated and the hexose concentration of the precipitate was again determined by the anthrone reaction. Starch contents were compared

using simple t-tests (Steel and Torrie 1980).

RESULTS

Over the course of the experiment, perchlorate-extractable carbohydrate fell sharply and Iodine-precipitable carbohydrate decreased almost to zero (Fig. III.1). On the other hand, there were no significant changes in concentrations of carbohydrate not precipitable by iodine. Incubation with amyloglucosidase hydrolysed $93.1 \pm 1.3\%$ of the iodine precipitate from the shoots and $97.1 \pm 0.6\%$ of the iodine precipitate from the roots. The vast majority of the iodine precipitate was, therefore, starch. Recoveries of starch standards, measured in quadruplicate, were $101.5 \pm 2.2\%$ for the perchloric acid extraction step, $94.8 \pm 0.1\%$ for all steps through the iodine precipitation, and $3.5 \pm 1.2\%$ through the second iodine precipitation, after enzymatic hydrolysis of the starch.

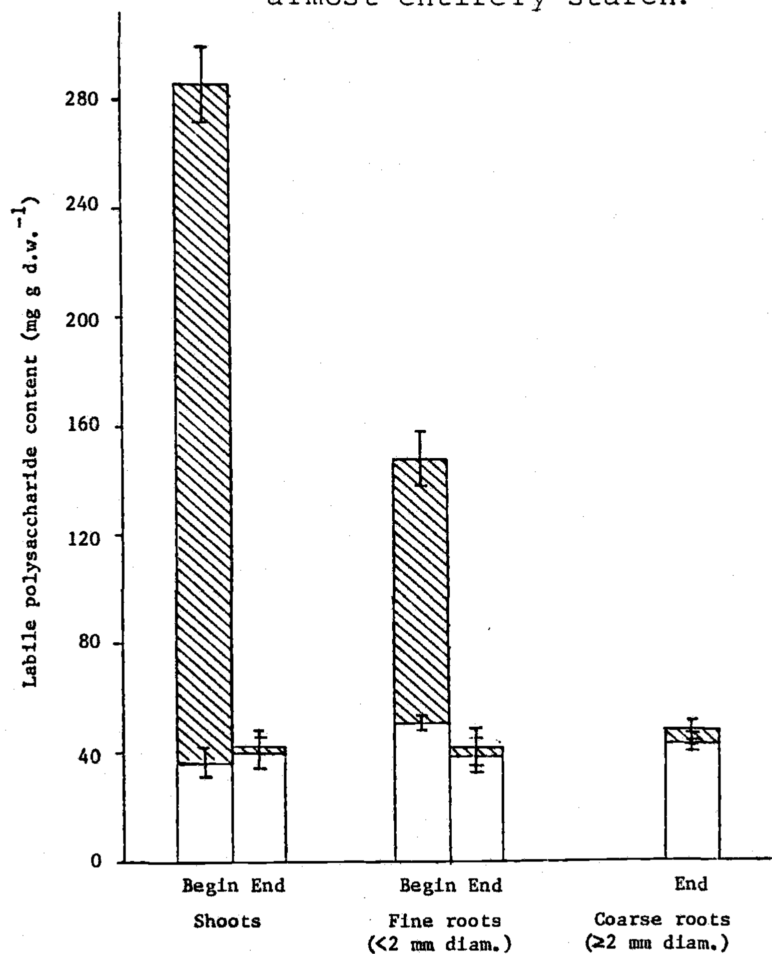
DISCUSSION

The starch extraction procedure described yields results comparable to those obtained by Hansen and Moller (1975). Values decreased to as low as 40 mg g $d.w.^{-1}$, similar to the lowest figures reported by Ericsson et al. (1980). Root starch contents at the beginning of the experiment were about half the maxima measured by Ericsson and Persson (1980).

Although some carbohydrates other than starch are extracted from Douglas-fir tissue by perchloric acid, their concentrations remain unchanged despite large changes in starch content. The precise amount of "background carbohydrate" can be estimated by covering a plant until its reserves are exhausted and the tissue dies. If the concentration of perchlorate-extractable carbohydrate is corrected for non-starch background, the remaining carbohydrate is essentially all starch. Moreover, rates of starch depletion are equivalent to losses of carbohydrates in maintenance respiration (see Chapter II). Thus, starch was by far the dominant reserve material in these Douglas-fir seedlings.

A check for completeness of starch extraction would be useful for interpreting results obtained by the various starch extraction procedures. The procedure used here: complete extraction with $HClO_4$, iodine precipitation, and enzymatic hydrolysis of the precipitate, can serve as a standard against which other techniques are compared.

Figure III.1. Labile polysaccharides extracted by 35% perchloric acid from Douglas-fir tissue before and after prolonged dark-treatment. Shaded areas represent iodine-precipitable carbohydrate, which is almost entirely starch.



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APPENDIX

EXTRACTION AND MEASUREMENT OF SUGAR AND STARCH

by

John D. Marshall

PREPARATION OF SAMPLES FOR ANALYSIS

Tissue was dried at 70°C for 24 hours in a forced-draft oven and ground in a Wiley mill to pass a 40 mesh sieve. Samples of approximately 100 mg were then weighed to the nearest 0.1 mg onto a piece of Whatman #1 filter paper 9.0 cm in diameter. The filter paper was folded to form a flattened tube about 1 cm wide at the wider end with the plant tissue on the inside. The narrower end of the tube was inserted into the wider end, sealing the plant tissue inside.

Because chlorophyll interferes with colorimetric sugar determinations, it must be removed from the sample prior to sugar extraction (AOAC Methods 1980). Furthermore, if starch is to be extracted, it is necessary to remove lipids and other non-polar compounds that may protect starch from hydrolysis (McCready 1970). The sample, folded in a filter paper tube, was placed in a 25 ml test tube with about 10 ml of 100% acetone and left overnight. The first acetone extract was poured off and the extraction was repeated until the extract was colorless. Acetone extracts were discarded.

ETHANOL EXTRACTION

The acetone-extracted sample, still folded in filter

paper, was placed in a 100 ml test tube and covered with 80% ethanol (see page 97). The sample was then placed in a water bath maintained at a temperature at which the ethanol gently boiled. The tube was covered by a small plastic funnel blocked with a boiling chip. It was necessary to cover the tube to minimize evaporation of ethanol from the extracting solution. Evaporation of ethanol would change the ethanol concentration and therefore the solvent characteristics of the extracting solution. After one to two hours, the extract was decanted into a 100 ml beaker, covered, and saved. The extraction was repeated until the extract was colorless, usually two to three times. Subsequent extractions of a given sample were combined.

REMOVAL OF INTERFERING SUBSTANCES FROM ETHANOL EXTRACTS

Although most of the chlorophyll and other colored substances were removed by the acetone extraction, the ethanol extract often showed marked coloration. I therefore added about 100 mg of insoluble polyvinylpyrollidone (PVP) to each extract, stirred, and allowed the PVP to adsorb non-polar components of the solution, with occasional stirring, for at least 30 minutes (Sanderson and Perera 1966). The solution was then filtered through Whatman #1 filter paper into a 100 ml volumetric flask, rinsing the beaker twice and the sample in the filter once with distilled water.

When filtration was complete, 100 μl of saturated neutral lead acetate $(Pb(OAc)_2$ solution was added and mixed into the ethanol solutions (Joslyn 1970). If the solution did not become noticeably cloudy within a few minutes, an additional 100 μl of lead acetate solution was added. After at least thirty minutes, 500 μl saturated sodium oxalate solution was added for each 100 μl $Pb(Oac)_2$ solution, precipitating any lead still in solution. The samples were brought to volume and allowed to stand overnight. The following morning, the uppermost 10 ml in the volumetric flask was poured through a dry Whatman #1 filter into a test tube, discarding the first few drops (Loomis 1937). The hexose concentration of the filtrate was then determined using the anthrone reaction (Yemm and Willis 1954). It was necessary to allow the lead precipitate to settle overnight because some of the lead precipitate would otherwise pass through the filter paper, yielding a turbid sample. The lead precipitation left a residue on the volumetric flasks that required acid-washing to remove.

PERCHLORIC ACID EXTRACTION OF STARCH

The ethanol-extracted sample was washed from the filter with three rinses of five ml each of 35% perchloric acid $(HClO_4)$ into a 125 ml Erlenmeyer flask. I used 35% perchloric acid because this is the highest

concentration at which cellulose is not hydrolyzed (Hansen and Moller 1975). Two more rinses of 5 ml each were used to rinse the sample from the sides of the flask. The sample was then covered and shaken overnight on an orbital shaker. The following morning, samples were filtered through Whatman #1 filters, rinsing the erlenmeyers twice and the sample in the filter once with distilled water. They were brought to volume in a 100 ml volumetric flask. Aliquots of these samples were used in the anthrone determination. It is necessary to add 25 ml 35% perchloric acid to the standards used in this determination because perchloric acid affects color development in the anthrone reaction (Hansen and Moller 1975).

COLORIMETRIC DETERMINATION OF CARBOHYDRATES WITH ANTHRONE

An aliquot of from 0.25 to 1.0 ml of sample solution was placed into a test tube in a cold water bath (Yemm and Willis 1954). I added 5.0 ml anthrone reagent and mixed on a vortex mixer until the sample was clear. The sample was then returned to a cold water bath. If the mixture was cloudy and did not clear upon heating, I added less sample solution or more anthrone reagent. The mixture was heated in a boiling water bath for exactly twelve minutes, then cooled in tap water for a few minutes. The perchlorate-anthrone mixture should be heated under a fume hood. Absorbance was read at 625 nm

using undiluted anthrone reagent as a blank. Because the sugar-anthrone reaction is not stoichiometric, a set of glucose standards was run with each set of samples and treated in exactly the same way as the samples. Standards were chosen to match the expected range of values, but never exceeded 100 mg/l glucose if 1.0 ml aliquots were to be used. With smaller aliquots, proportionally higher standards could be used. It is necessary to avoid exposing the samples to bright light after the anthrone reaction is complete because light decomposes the color formed in the reaction. If samples cannot be read immediately, they should be covered and stored in the refrigerator along with the standards with which they are to be read. Starch contents must be multiplied by 0.9 to account for hydration in the conversion from starch to sugar. Starch contents and sugar contents are then divided by original sample weights. Starch contents obtained from the calibration curves can be expressed on a dry weight basis by the following calculation:

Starch content (mg g d.w.$^{-1}$)=

[sugar content of sample solution(mg/l)] X [0.11] X [0.9mg starch/1.0mg glucose] X [1/sample weight(g)]

IODINE PRECIPITATION OF STARCH FROM PERCHLORIC EXTRACT

Samples were oven-dried, rolled in filter paper, and extracted in 80% ethanol as described above. The ethanol extract was discarded.

Perchloric acid extracts starch from tissue by hydrolyzing starch granules into fragments that are more easily dissolved. These starch fragments can be co-precipitated with iodine. Perchloric acid continues to hydrolyze the fragments, however, until they are no longer iodine precipitable (Tetley 1974). It is therefore necessary to restrict the duration of starch exposure to high concentrations of perchloric acid. A system similar to that of Hansen and Moller (1975) was assembled to drip perchloric acid through a sample and immediately dilute it, thereby dissolving the starch without hydrolyzing it so completely that it would no longer complex with iodine.

Samples were washed with 80% ethanol into a 5 cm section of Tygon tubing packed at the bottom with glass wool. The top was then packed with glass wool. A disposable plastic pipet tip from a 100 μl automatic pipettor was filled with glass wool and fitted into the end of the section of tubing in order to standardize drip rates.

Samples were extracted 18 hours at drip rates of 1-3 ml/hr of 35% perchloric acid. At the end of the

extraction, samples were brought to volume with 35% perchloric acid so that the starch was dissolved in 7% perchloric acid, a concentration at which further hydrolysis of starch fragments is minimal (Tetley 1974). A 5 ml aliquot of this solution was placed in a 15 ml centrifuge tube with 5 ml 20% NaCl and 2 ml iodine solution as described by McCready (1970) (see page 97). After mixing, this material was allowed to stand overnight in a refrigerator.

The following morning the precipitate was centrifuged at high speed for 10 minutes. The supernatant was decanted using a pasteur pipette attached to an aspirator. The pellet was carefully washed and decanted three times with 2 ml EtOH-NaCl solution. A 2 ml aliquot of EtOH-NaOH solution was added and mixed on a vortex mixer until the blue color vanished, indicating dissociation of the starch-iodine complex. The sample was again centrifuged at high speed for ten minutes, decanted, and washed twice more with EtOH-NaCl to remove iodine and NaOH. Salt crystals frequently precipitated along with the starch. An aliquot of 0.5 ml 35% perchloric acid was added and mixed to re-dissolve the starch. Two ml of water was added and mixed. An aliquot of this sample was used for the anthrone colorimetric sugar determination along with standards containing 7% perchloric acid.

ENZYMATIC HYDROLYSIS OF IODINE PRECIPITATE

Samples were prepared, extracted, and precipitated exactly as above. After the final washing of the iodine-free precipitate, however, samples were redissolved in stoppered tubes with 2 ml of water in a boiling water bath until dissolution was complete (usually less than 10 minutes). Aliquots of 0.5 ml each of amyloglucosidase enzyme solution and 1.0 M Na acetate buffer (pH 4.2) were then added and mixed, tubes were again stoppered and allowed to stand in a 50°C water bath for 6 hours (Smith 1969, Haissig and Dickson 1979).

At the end of the enzyme hydrolysis, 3 ml of 20% NaCl and 1.2 ml of iodine solution were added to the samples, mixed and allowed to stand overnight in a refrigerator. The precipitate was then washed exactly as described above and carbohydrate content of the unhydrolyzed residue was determined.

REAGENTS

ANTHRONE REAGENT: Add 200 ml distilled water to 500 ml concentrated H_2SO_4 slowly, with stirring, in an ice or cold water bath, to make 655 ml 72% H_2SO_4. To this solution add 175 mg anthrone (Matheson, Coleman, and Bell) powder/100 ml, or 1.146 g to 655 ml. These concentrations need not be exact. Allow to mix for at least two hours or, if possible, overnight. Store in refrigerator (Yemm and Willis 1954).

80% ETHANOL: Dilute 840 ml 95% ethanol to 1000 ml.

35% $HClO_4$: Dilute 500 ml 70% $HClO_4$ to 1000 ml, with stirring, in an ice or cold water bath (Hansen and Moller 1975).

20% NaCl: Add 20 g NaCl to 80 ml water and mix. Bring to volume (100 ml) and continue to mix until all NaCl dissolves (McCready 1970).

IODINE (I_2-KI) SOLUTION: Add 7.5 g I_2 and 7.5 g KI to small amount distilled water and grind in mortar. Bring to 250 ml volume. Filter to remove undissolved I_2 (McCready 1970).

EtOH-NaCl SOLUTION: To 350 ml 95% ethanol, add 80 ml H_2O and 50 ml 20% NaCl solution to total 500 ml solution (McCready 1970).

EtOH-NaOH SOLUTION (0.25 N): To 350 ml 95% EtOH, add 100 ml H_2O and 25 ml 5N NaOH for 500 ml total (McCready 1970).

ACETATE BUFFER (1.0 M): Dissolve 54.67 g sodium acetate in 500 ml distilled water and add acetic acid until pH 4.2 is reached (Smith 1969).

ENZYME SOLUTION: Dissolve 250 mg amyloglucosidase (Sigma Chemical Co. No. A-7255) in about 50 ml distilled water. Filter through Whatman #1 filter paper, rinsing several times. Rinse with acetone. Allow acetone to evaporate. Wash residue, with water, into 100 ml volumetric flask and bring to volume. Store in refrigerator for no longer than two or three days.

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