Enzyme Activity as an Index of Growth Superiority of *Pinus clausa* var. *clausa* on Two Soils

By

RUSSELL MacBAIN BURNS

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TABLE OF CONTENTS

	PAGE
INTRODUCTION	1
OBJECTIVES	3
LITERATURE REVIEW	6
Mineral Nutrition of Sand Pine	7
Photoassimilation of Carbon Dioxide	8
Concentration	8
Light Intensity	9
Exposure -- Length and Temperature	12
Enzymes	14
MATERIALS, EQUIPMENT, AND METHODS	17
Plant Material	17
Photoassimilation Chamber	23
Photoassimilation of $^{14}CO_2$	25
Separation of Ethanol-Soluble Components	27
Measurement of Radioactivity	28
Thin Layer Chromatography	29
Sugars	29
Organic Acids	31
Amino Acids	31
Radioautography	31
Measurement of Enzyme Activity	32

Fructoaldolase 33

Glyceraldehyde-3-Phosphate Dehydrogenase-NAD Dependent 33

Phosphoglycerate Kinase 33

Glucose-6-Phosphate Dehydrogenase 33

6-Phosphogluconate Dehydrogenase 35

Acetone Powders -- Preparation and Protein Extraction . 37

Preparation of Polyacrylamide Gels and Electrophoresis 38

Detection of Protein and Isoenzyme Bands 39

Proteins and Dehydrogenase Isoenzymes 39

Glucose-6-Phosphate Dehydrogenase and Malate Dehydrogenase 40

Sample Size and Statistical Analysis 41

RESULTS AND DISCUSSION 43

Photoassimilation of $^{14}CO_2$ 43

Clarification of Extracts and Quenching 51

Sugars 57

Organic Acids 65

Amino Acids 65

Effect of Nutrient Level on Seedling Morphology and ^{14}C Incorporation 67

Color and Weight 67

^{14}C Incorporation 70

Biochemical Pathways Involved 75

SUMMARY 94

APPENDIX 97

LITERATURE CITED 107

LIST OF TABLES

TABLE		PAGE
1	Source and concentration of elements used in the complete nutrient solution	20
2.	Chemical and physical properties of soil used to raise half-sibling seedlings.	21
3	Color and Rf values from chromatograms of known sugars used to identify unknowns	30
4	Distribution of photoassimilated ^{14}C in green sand pine seedling tissue	56
5	Seedling weight and photoassimilated ^{14}C distribution in response to nutrients	71
6	Influence of N and P fertilizer on the distribution of weight and radioactivity	72
7	Comparisons of the soil x tree interaction showing the probability of a chance occurrence and the soil on which the highest values were obtained	80
8	Distribution of half-sibling seedlings possessing 3 to 7 cotyledons	87
9	Protein Rf measurements taken from half-sibling seedlings grown on Lakeland coarse sand	98
10	Protein Rf measurements taken from half-sibling seedlings grown on Paola sand	101
11	Protein Rf measurements taken from parent trees growing at their original locations	104

LIST OF FIGURES

FIGURE		PAGE
1	Flow chart of procedures followed in part I	4
2	Flow chart of procedures followed in part II	5
3	Diagram of the photoassimilation chamber	24
4	Reactions for measuring fructoaldolase, glyceraldehyde-3-phosphate dehydrogenase, and 3-phosphoglycerate kinase	34
5	Reactions for measuring glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase	36
6	Distribution of radioactivity in seedlings allowed to photoassimilate $^{14}CO_2$	44
7	Distribution of radioactivity in seedling tissues exposed to $^{14}CO_2$ in the light and dark	46
8	Quenching curves developed to compensate for loss of counting efficiency	50
9	The ^{14}C incorporated into green tissue of *Pinus clausa* in response to different amounts and kinds of acid used to release $^{14}CO_2$ from $KH^{14}CO_3$	52
10	Comparison of clarifying agents	54
11	Chromatogram showing bands of standard and unknown sugars	58
12	Radioautogram of a sugar TLC plate (6 weeks exposure)	59
13	Radioautogram of the same TLC plate used in Figure 12 (2 weeks exposure)	60
14	Moribund seedling showing constriction near groundline	63

LIST OF FIGURES -- CONTINUED

FIGURE		PAGE
15	Organic acid TLC plate	66
16	Amino acid TLC plate for fertilizer treatment N_2P_1	68
17	Amino acid TLC plate for fertilizer treatment N_2P_2	69
18	Seed germination from superior and non-superior sand pine trees at 5 locations designated A to E	86

Abstract of Dissertation Presented to the
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ENZYME ACTIVITY AS AN INDEX OF GROWTH SUPERIORITY
OF *PINUS CLAUSA* VAR. *CLAUSA* ON TWO SOILS

By

Russell MacBain Burns

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Chairman: Dr. William L. Pritchett
Co-Chairman: Dr. Robert G. Stanley
Major Department: Soil Science

This study attempted to 1) identify the metabolic pathways and enzymes involved in the photosynthetic fixation of carbon and to determine 2) if metabolic intermediates were altered by changes in the supply of nitrogen and phosphorus; 3) if activity level of specific enzymes differed in tissue of superior and nonsuperior trees and their half-sibling seedling progeny; 4) if the activity was altered by the soils in which seedlings grew.

Distribution of photoassimilated $^{14}CO_2$ was measured in ethanol-soluble fractions of green tissue from 3-month-old Ocala sand pines grown from seed in sand culture and complete nutrient solutions containing two levels each of nitrogen and phosphorus. Portions of the metabolic pathways involved in carbon fixation were determined. The activity and electrophoretic migration rate of malate dehydrogenase isoenzymes, glucose-6-phosphate dehydrogenase isoenzymes, and proteins were compared in green tissue from saw log-size superior

and neighboring, nonsuperior sand pine trees and their half-sibling progeny grown in pots on Lakeland coarse sand and Paola sand.

Sugars contained 75%, organic acids 19%, and amino acids 6% of the radioactive carbon in the ethanol-soluble fraction from green tissue. Radioactivity was highest in fructose, glucose, and galactose. Nitrogen directly affected chlorophyll formation, seedling growth, and photosynthetic incorporation of ^{14}C. Phosphorus appeared to be the principal rate-limiting element in the incorporation of carbon in sugars and in some organic acid precursors of amino acids. Results indicated that enzymes of glycolysis, the Calvin cycle, and the tricarboxylic acid cycle were principally involved in fixation of carbon in sand pine seedlings allowed to photoassimilate $^{14}CO_2$ for 10 minutes in a controlled environment.

Measurements of activity and migration of isoenzymes did not provide an index of growth superiority in parent trees or in half-sibling seedling progeny. Superior parent trees, but not their progeny, lacked one or more protein bands found in nonsuperior trees indicating that a genetic marker exists. However, no relationship was found between the location of malate dehydrogenase and glucose-6-phophate dehydrogenase isoenzymes and protein bands.

Activity and Rf values of some malate dehydrogenase isoenzyme and protein bands were altered by the soil in which seedlings grew as well as by genetic factors. Both the isoenzyme and protein bands and factors influencing them were identifiable.

INTRODUCTION

Superior trees are fast growing and have a desirable morphology. They are sought and propogated principally to shorten the rotation age of plantations, i.e., the time needed for trees to reach merchantable size. Selection of superior trees is based primarily on a comparison between the candidate and neighboring contemporaries of the same species. True superiority is adjudged by the ability of grafted stock and progeny to exhibit the same superior characteristics as the selected candidate. The approach is sound but very time consuming.

A more rapid way to screen candidate superior trees may be by comparing levels of enzymes that catalyze growth processes. Growth, the primary index of superiority, is cumulative, genetically controlled (Squillace, 1965), and greater in superior trees than among others of the general population. Assimilation, the basic growth process, depends upon the speed of certain biochemical reactions. Enzymes control the rate of these reactions and, thereby, the rate at which a tree grows.

Many enzymes are involved in tree growth. This study measures activity of just a few. The problems are to select for assay those enzymes that control a biochemical reaction pathway involved in the synthesis of anabolins, which most differ between superior and nonsuperior trees, to determine if their activity is influenced by

the soil in which the pine trees grow, and to determine what effect fertilization with phosphorus (P) and nitrogen (N) has on photosynthetic fixation of carbon.

Nutrient levels influence rate of growth. Sandy soils in which sand pines grow are deficient principally in P and N (Brendemuehl, 1967). Sand pines respond to fertilization with these elements.

Ocala sand pine, *Pinus clausa* var. *clausa* Ward, was studied. It is native to droughty, infertile sandhill soils in Florida and has become increasingly important in reforestation of these difficult sites. Superior tree selections were readily available for seed collection and tissue sampling.

OBJECTIVES

The study was divided into two parts, each of which had two objectives. In part I, experiments were designed to 1) identify the metabolic pathways and the enzymes involved in the photosynthetic fixation of carbon, and to determine 2) how significantly these pathways might be altered by changes in the supply of plant nutrients. Figure 1 depicts a flow chart of procedures used to accomplish these objectives. In part II, objectives were to determine 3) if the activity level of specific enzymes differs in tissue of superior and nonsuperior trees and their half-sibling progeny, and 4) if the activity was altered by the soils in which seedlings grew. Figure 2 illustrates the procedures followed in the second part of the study.

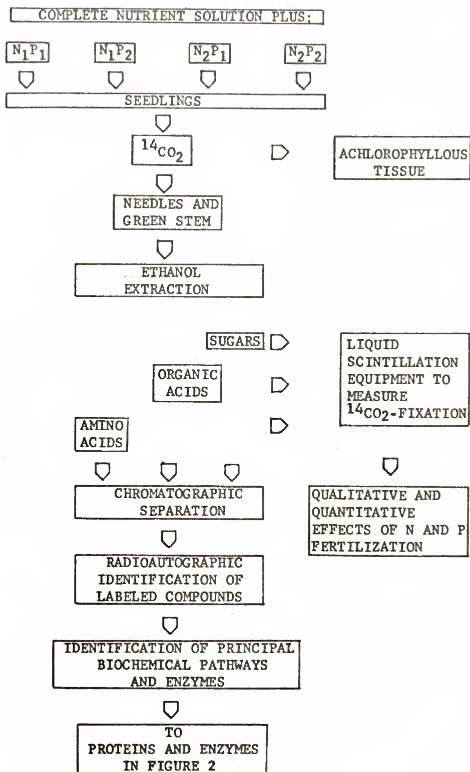

Figure 1.--Flow chart of procedures followed in part I

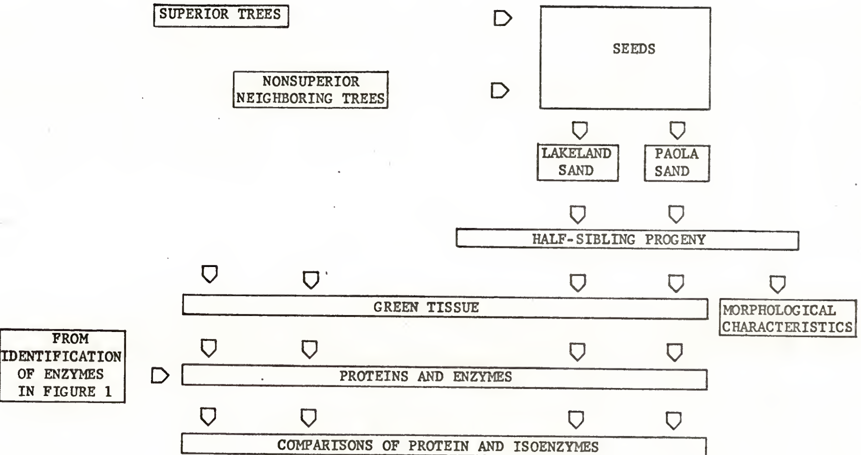

Figure 2.--Flow chart of procedures followed in part II

LITERATURE REVIEW

Literature pertinent to the development of an enzyme index for superior trees under the proposed approach encompasses a wide variety of subjects ranging from nutritional requirements for sand pine seedling culture to methodology for enzyme assay. To date no one has published results of an approach with an enzyme assay. The experiments in this study involve a number of variables, the effects of which had to be considered in sequence. Factors other than those to be tested (constants) had to be maintained within acceptable limits, or at desirable levels, in order to determine accurately the effect of the variable under consideration. Pertinent literature is reviewed in those sections dealing with specific aspects of this research, so that it will be in context.

Mineral Nutrition of Sand Pine

Similar nutrient solutions were employed in sand and water culture (Hewitt, 1952; Small and Leonard, 1969) and included a wide range of concentrations of macroelements (Hacskaylo, 1962; Hoagland and Arnon, 1950). This suggested that each species required a particular nutrient regime or that pines exhibited optimum growth under a diverse range of nutrient conditions. This was especially true of N and P.

Published (1967) and unpublished work by Brendemuehl indicated that *P. clausa* growth was enhanced by N but only after the P deficiency inherent in infertile, acid sand had been corrected. Brendemuehl (personal communication) recommended using N and P in a ratio of 1 to 2 in acid-washed sand, with N supplied at the rate of 75 ppm.

The form of N used also affects growth and protein synthesis in conifers. Durzan and Steward (1967) grew white spruce [*Picea glauca* (Moench.) Voss] and jack pine (*Pinus banksiana* Lamb.) seedlings for 478 days in sand irrigated with nutrient solutions containing either ammoniacal nitrogen (NH_4-N) or nitrate nitrogen (NO_3-N). The fresh weight of white spruce seedlings was greater with NO_3-N, but more free, non-protein bound, amino acids were found in seedlings fed NH_4-N, especially in stems and leaves. Jack pine seedlings supplied with NH_4-N were heavier and contained more total free amino acids than those grown with NO_3-N. Because free amino acid reserves were lowest with NO_3-N, Durzan and Steward (1967)

concluded that more NO_3- than NH_4-N was synthesized into proteins by conifers. Work with Southern pines (Barnes and Naylor, 1959; Barnes, 1962; and Pharis, Barnes, and Naylor, 1964) tended to substantiate this conclusion.

Photoassimilation of Carbon Dioxide

Concentration

The average concentration of CO_2 in the atmosphere is about 300 ppm at sea level. Of this amount only about two-thirds is available for photosynthesis (Moss, 1962). He found that the ability of plants to utilize CO_2 varied with species. Corn (*Zea mays* L.), for example, has a CO_2-compensation point of less than 10 ppm; Norway maple (*Acer platanoides* L.), 145 ppm. Furthermore, this equilibrium between CO_2 production and utilization varies with light intensity and temperature. Moss reported that in a closed system the average plant reduces CO_2 concentration to a level of only 50 to 100 ppm.

At best, only an approximation can be made of CO_2 uptake because photosynthesis and photorespiration occur simultaneously. At most light intensities much of the CO_2 respired is utilized before it can diffuse to the atmosphere. Zelitch and Day (1968), working with parent and mutant progeny of tobacco (*Nicotiana tabacum* L.) at several concentrations of CO_2 (including CO_2-free air), presented evidence suggesting that the increased rate of CO_2 uptake in hybrid plants (higher photosynthetic efficiency) may be attributed to genetic interference with the photorespiratory process, i.e., net photosynthesis was high because photorespiration was lower in mutant siblings.

Growth of plants, and more specifically growth of pine seedlings, will occur and, in fact, will be enhanced at CO_2 concentrations above 300 ppm. Zelawski and Kinelska (1967) working with Scotch pine (*Pinus sylvestris* L.) at relatively low light intensities (maximum approximately 1,000 ft-c), found that the rate of photosynthesis was almost directly proportional to the concentration of CO_2 within the range of 200 to 400 ppm. At concentrations above 450 ppm the rate of photosynthesis declined and approached the point where the response to increased CO_2 was negligible irrespective of light intensity. Similar findings were reported by Hughes and Cockshull (1969) with China aster (*Callistephus chinensis*) at low light intensity. Dry matter production of plants grown in 600 ppm CO_2 was slightly higher than in those grown at 900 ppm, and considerably higher than in plants grown at 325 ppm. The natural diffusion gradient for CO_2 between leaf and atmosphere is altered at high concentrations and may cause recycling of respired CO_2 at higher than normal rates. Extremely high CO_2 concentrations, 2,000 to 4,000 ppm, caused stomata of some plants to close (Pallas, 1965). He found that dicots were more tolerant of high CO_2 concentrations than monocots.

Light Intensity

Uptake of CO_2 is affected by light intensity. Zelawski and Kinelska (1967) presented a graph showing that the shift in CO_2 compensation point with varying light intensity had a parabolic configuration. At low intensities small changes induced drastic shifts in the CO_2 compensation point. The magnitude of the shifts diminished in almost exponential fashion as light intensity approached 47.5 and 100 % illumination and, although intermediate intensities were

not tested, assumedly at intervening intensities. Unfortunately, light saturation was not attained in their experiments.

Light saturation was reached in a study of eastern hemlock (*Tsuga canadensis* L.) in Wisconsin (Adams and Loucks, 1971). Hemlock is tolerant of dense shade and develops well as an understory plant. Illumination of foliage at midday varied between 25 and 500 ft-c in the forest. Under controlled conditions the rate of net photosynthesis increased sharply up to 1,000 ft-c and then began to decrease. At 3,000 and 3,500 ft-c net photosynthesis was approximately the same indicating that the light compensation point had been reached. The data suggested that, for this shade-tolerant conifer, changes in intensity above 1,000 ft-c would not greatly alter CO_2 uptake. Cooper (1957) reported that young sand pines are tolerant of shade. In this respect the two conifers are alike.

Keller and Koch (1962) examined the influence of mineral nutrition upon CO_2 exchange in poplar (*Populus euramericana marilandica*) and found that light saturation occurred at 2,000 ft-c in N-deficient leaves. Light saturation was not reached at even 4,000 ft-c in "well-fed" leaves. However, at low intensities of up to 500 ft-c net assimilation was the same in N-deficient and "well fed" leaves. With regard to the dependence of net photosynthesis upon CO_2 concentration and light intensity, their findings at least partially substantiated those of Zelawski and Kinelska. At 4,000 ft-c the CO_2 uptake of poplar leaves was strongly influenced by foliar N content. N-deficient leaves assimilated only 60% as much CO_2 as normal leaves, were proportionately smaller in size, and contained only 55% as much chlorophyll per unit area. There was a close correlation between the chlorophyll and N content of poplar leaves.

Light intensity influenced NO_3 uptake and the subsequent induction of NO_3 reductase in cereals (Chen and Ries, 1969). Rye seedlings took up NO_3 slowly in dark and rapidly in light. After a 12-hr exposure, seedlings subjected to about 300 ft-c contained as much NO_3-N as those illuminated with 800 to 1,500 ft-c, suggesting that dependence of NO_3 reductase activity on light was satisfied at the lowest intensity. Presumably NO_3 reductase was produced in the dark. Light and prior uptake of NO_3 were essential for the induction of the enzyme. Once these conditions were met enzyme production continued to increase for the next 24 hours, even in the dark. Within the range of 300 to 1,500 ft-c induction of NO_3 reductase was proportional to light intensity.

Sand pine seedlings appeared to be umbraphilic with respect to light tolerance and juvenile growth (R.M. Burns, unpublished data). Tests conducted in an experimental nursery support the observation; seedlings raised in partial shade were taller, larger, and more verdant than those grown in full sunlight. Results suggested that chlorophyll-catalyzed photooxidation induced by high light intensities, and increased respiration in response to high summer temperatures, as normally encountered on exposed sands, contributed to slower growth in direct sunlight.

Meidner (1970), working with sun and shade leaves from a variety of herbaceous and woody plants, noted that the light compensation point was most closely related to leaf thickness. Thinner shade leaves had the lowest light compensation point. Kramer and Kozlowski (1960) made a similar observation with light saturation of sun and shade leaves of European beech. Differences in saturation

levels exist because higher intensities are needed to affect chlorophyll molecules deeply imbedded in thick leaves. Pine needles are thicker than hardwood leaves and much less efficient photosynthetically. Bonner and Galston (1952) reported that light saturation of pine foliage did not occur even at intensities approaching full sunlight, 10,000 to 12,000 ft-c.

Exposure -- Length and Temperature

Uptake of CO_2 depends upon the volume involved, the amount of chlorophyll present, the rate of diffusion inward, and the rate of photosynthesis. The latter is temperature, light, and CO_2 dependent. Small and Leonard (1969) exposed 6-week-old legumes in plastic bags to a volume of $^{14}CO_2$ gas with radioactivity of 5 microcuries (μc). An induction period of 15 min in direct sunlight was used. No mention was made of how length of exposure for complete utilization of $^{14}CO_2$ was determined but, in view of the previously cited work, disposal of residual, labelled gas obviously was necessary.

Paired leaves on intact hybrid and parent *Mimulus* (Monkey flower) plants were used by Decker (1959) to measure the effect of CO_2 concentration from 100 to 500 ppm on photosynthesis at temperatures of 20, 30, and 40 C and 2,000 ft-c. He found that apparent photosynthesis increased as CO_2 concentration increased and temperature decreased. Apparent respiration increased with temperature but decreased with CO_2 concentration. The CO_2 compensation point increased almost linearly with temperature. The dependence on temperature was ascribed to a temperature coefficient larger for respiration than for photosynthesis, the rate of respiration being more than 3 times higher in light than in darkness. For *Mimulus*, at least, to approach

the CO_2 compensation point with the shortest possible exposure time at a fixed CO_2 level necessitates use of a low temperature.

Dr. W. Zelawski (personal communication) suggested evolution of $^{14}CO_2$ into a closed system in small increments, then circulating the gas through the illuminated chamber for 2 hr. This procedure was used for preliminary work because it provided sufficient time for translocation of some ^{14}C-labelled products to the roots (Lister et al., 1968; Small and Leonard, 1969) and insured biochemical fixation of a large volume of CO_2 containing sufficient quantities of ^{14}C, for detection.

Devlin (1968) noted that an optimum rate of photosynthesis occurred at 30 C during short-term exposure and at 22 C during long-term exposure of *Chlorella*. *Nitzschia closterium* reached an optimum rate at 26 C and *N. palea* at 32 C at high light intensities. The photosynthetic rate of several trees and seedlings was highest at 20-30 C (Kramer and Kozlowski, 1960). Vose and Spencer (1969) and Zelawski and Kinelska (1967) reported that temperature in closed photoassimilation chambers usually is maintained within a range of 20 to 27 C.

The effect of temperature on photosynthesis and respiration was measured using sand pine (*P. clausa* var. *immuginata*) seedlings from west Florida (Pharis and Woods, 1960). Apparent photosynthesis (mg. CO_2 taken up per hr) was highest at 23 C and actual photosynthesis (apparent photosynthesis + apparent respiration, as mg. CO_2 exchanged per hr) was equally as high at 23 and 28 C. This was explained by the fact that apparent respiration increased with temperature throughout the range tested (18 to 48 C) whereas apparent photosynthesis peaked at about 23 C.

Enzymes

Enzymes are proteins that catalyze anabolic or catabolic reactions along biochemical pathways. The net result is growth and reproduction. The regulatory mechanisms for protein synthesis and enzyme activation are not fully understood. One interesting and widely accepted theory, developed through intensive experimentation with microorganisms, was advanced by Jacob and Monod (1961). Although not yet verified in higher plants or animals, it has been used to explain how specific enzymes are induced and why rate changes occur during development and maturation of higher plants (Borchert, 1967; Firenzuoli, et al., 1968; McClintock, 1961), also as a basis for interpreting the genetic implications of evolution in maize (Efron, 1970) and mutant enzymes (Schwartz, 1962), photorespiration in tobacco (Zelitch and Day, 1968), and induction of NO_3 reductase in rye (Chen and Ries, 1969).

In its simplest form the mechanism involves an operon, composed of a structural gene and an operator gene, and a regulator gene. Activity of the structural gene is controlled by the operator gene. The structural gene dictates the pattern for synthesis of a specific enzyme, and the operator gene determines the rate and timing of synthesis. The operon may be activated by presence of exogenous substrate and external conditions to induce *de novo* synthesis of specific enzyme(s) involved in the sequential metabolism of the substrate.

The regulatory gene determines the quantity of enzyme(s) produced. It produces a specific repressor which, when activated by metabolites, acts upon the operator gene to block the mechanism of the operon. Repression may be influenced by a feed-back control arising from the accumulation of specific catabolic products in the cytoplasm.

Level of enzyme activity varies during plant growth (Borchert, 1967), during the advent and end of specific physiological processes (Chen, Towill, and Loewenberg, 1970), and with certain changes in environment (Chen and Ries, 1969). Exposure of seed to conditions conducive to germination induces changes in the activity level of enzymes involved in conversion of stored food to energy and substrate necessary for assimilation (Firenzuoli et al., 1968). Activity may reach a peak in a short time and then decline as the substrate is depleted or as repressive metabolites are produced as a feed-back control.

External conditions that influence normal growth processes also cause change. Sometimes induction of enzyme activity is under the influence of more than one external factor. Machlis and Briggs (1965) report that day length as well as temperature may control breaking of winter dormancy of trees. Premature growth flushes during unseasonably warm winter weather may be prevented by a photo control. Chen and Ries (1969) found that light as well as substrate was needed to induce formation of NO_3 reductase. Light also induced changes in enzyme activity in etiolated bean seedlings (Filner and Klein, 1968).

The use of enzyme activity measurements to predict the potential for rapid growth in individual plants is not entirely new. Hybrid vigor (heterosis) in maize is detectable 2 to 6 days after germination by comparing the level of isocitric dehydrogenase activity of hybridized seedlings with that of progeny of inbred parent plants (Roos and Sarkissian, 1968). Although hybridization is known in pine the study material was not hybridized.

The rapid growth characteristic of superior sand pine results from a fortunate combination of germ plasm which, through its sequential control of enzyme synthesis, activity, and repression, governs the rate of its physiological processes. The hypothesis under investigation is that the activity of biological catalysts in rapidly growing, superior trees differs from that in slower growing, nonsuperior pines.

Because of the great number of enzymes involved in plant growth and development, some system or method usually is employed to determine the one(s) involved in particular processes. Roos and Sarkissian benefited from the works of Gowan (1952), Hageman, Leng, and Dudley (1966), and others involved in the heterotic behavior and breeding of corn. In nutritional studies, examination can be made of enzymes containing the element under investigation. Van Lear and Smith (1970), for example, examined isoenzymes of polyphenoloxidase, peroxidase, and ascorbic acid oxidase in tissue of pine seedlings grown without copper or without iron because these enzymes contain copper or iron.

MATERIALS, EQUIPMENT, AND METHODS

Plant Material

Seeds and foliage were collected from 30 sand pine trees (*P. clausa* var. *clausa*) on the Ocala National Forest in Marion and Putnam Counties, Florida. Five of the trees, numbered 77, 82, 120, 193, and 199, were superior selections made jointly by personnel of the Florida Forest Service, U. S. Forest Service, St. Regis Paper Company, and the Forest Physiology and Genetics Laboratory of the University of Florida. Within 100 feet of each superior tree 5 nonsuperior trees of similar age were selected as representatives of the general population growing under conditions similar to the superior tree.

Cones of the 1967 seed crop were collected from the 30 trees. They were opened in a force-draft oven at 60-63 C and the extracted seeds were stored at temperatures below 5 C. Needle samples from parent trees were collected and used during the summer of 1970. Needles were placed in plastic bags, quick-frozen in liquid N_2, transported to the laboratory on dry ice, and stored at -20 C until used.

Seeds composited from nonsuperior pines were used throughout the first series of experiments. On April 21, 1968, they were surface sterilized with sodium hypochlorite and planted in 8 polyethylene-lined pots, each filled with approximately 27 pounds of dried, acid-washed sand. Pots were cylindrical, measured 21 cm in diameter and height, and were made with a 25-mm diameter hole through the side at

the bottom. A one-hole stopper masked with glass wool was fitted in the hole to provide for irrigation and drainage without loss of soil. Fifty seeds were planted in each pot. Distilled water and subirrigation were used to germinate the seeds. They were allowed to germinate for 30 days and were then thinned to 25 per pot. Those that germinated later were discarded.

Seedlings were raised under greenhouse conditions using complete nutrient solutions patterned after those of Hacskaylo (1962). Solutions contained either 7.5 ppm (N_1) or 75.0 ppm (N_2) of N and either 15.0 ppm (P_1) or 150.0 ppm (P_2) of P (Table 1). Seedlings in 2 pots were supplied with one of the 4 solutions (N_1P_1, N_1P_2, N_2P_1, or N_2P_2) twice weekly throughout the first series of experiments.

Seedlings and soil from one pot of each fertilizer treatment were transferred to tubules on June 19, 1968. Tubules were 15 cm long, 2.5 cm in diameter, and were constructed from rigid polyvinyl chloride (PVC) water pipe. They were fitted with a one-hole neoprene stopper masked with glass wool to permit irrigation and drainage yet prevent loss of sand. Subirrigation and immediate drainage were used to feed seedlings and promote soil aeration in pots and tubules. To prevent accumulation of nutrient salts, seedling containers were flushed with deionized water at biweekly intervals.

Seeds from superior and nonsuperior trees were used in the second series of experiments. On December 1, 1969, 50 seeds from each tree were planted in each of two polyethylene-lined greenhouse pots previously described. One pot contained a Lakeland coarse sand from Calhoun County and the other a Paola sand from Marion County,

Florida. These soils were used because within the limited, natural range of sand pine they, and their respective hyperthermic and thermic counterparts, Astatula and Lakewood sands, comprise a major portion of the sandhill sites upon which the species grows. Each contained 7.5 cm of topsoil (A_1 horizon) and about 12.5 cm of soil from an underlying horizon (C for Lakeland and A_2 for Paola).

Procedures outlined by Jackson (1958) and by the American Society of Agronomy and American Society for Testing and Materials (1965) were used to analyze soils. Soil pH was measured using a 1 to 5 soil-water suspension and a glass electrode. Cation exchange capacity was determined using normal neutral ammonium acetate. Available nutrients were extracted from the soil with ammonium acetate buffered at pH 4.8. The concentration of individual elements in solution was measured using the equipment or procedures that follow: Ca and Mg with a Beckman Model DU flame spectrophotometer; K with a Beckman Model B flame spectrophotometer; P using the chlorostannous-reduced molydophosphoric blue color method in a sulfuric acid system with a Spectronic 20 colorimeter; and Al with a Perkin-Elmer model 303 atomic absorption spectrophotometer. Organic matter was determined using the Walkley-Black chromic acid oxidation procedure. The modified Kjeldahl method was used to determine total N. Particle size distribution was determined using the hydrometer method, and sand fractions were separated by dry sieving. Soil moisture at 15 and 1/3 atmospheres was measured using pressure plate apparatus. Physical and chemical analyses of these soils are summarized in Table 2.

Table 1.--Source and concentration of elements used in the complete nutrient solution

Nutrient element	Source	Nutrient Solution*			
		N_1P_1	N_1P_2	N_2P_1	N_2P_2
		----------ppm of ion-------------			
N	NH_4OH and HNO_3	7.5	7.5	75.0	75.0
P	H_3PO_4	15.0	150.0	15.0	150.0
K	KOH	200.0	200.0	200.0	200.0
Ca	$CaCl_2 \cdot 2H_2O$	100.0	100.0	100.0	100.0
Mg	$MgSO_4 \cdot 7H_2O$	50.0	50.0	50.0	50.0
S	$MgSO_4 \cdot 7H_2O$	65.9	65.9	65.9	65.9
B	H_3BO_3	0.4	0.4	0.4	0.4
Mn	$MnCl_2 \cdot 4H_2O$	0.04	0.04	0.04	0.04
Zn	$ZnCl_2$	0.05	0.05	0.05	0.05
Cu	$CuCl_2 \cdot 2H_2O$	0.02	0.02	0.02	0.02
Mo	$H_2MoO_4 \cdot H_2O$	0.03	0.03	0.03	0.03
Fe	Fe - EDTA	5.00	5.00	5.00	5.00

*Solutions were adjusted to pH 5.85 with HCl or NaOH

Table 2.--Chemical and physical properties of soil used to raise half-sibling seedlings

Chemical Analyses

SOIL	HORIZON	pH	CEC[2] me./100g	Ca	Available Nutrients[1] Mg	P	K	Al	OM[3]	N[4]
				---------	---------	ppm	---------	---------	--percent--	
Paola	A_1	4.7	3.30	67	Trace	0.7	11	1.5	1.82	0.022
	A_2	5.5	---	22	Trace	2.2	4	8.5	----	-----
Lakeland	A_1	5.6	2.18	45	10	0.5	15	47.5	1.14	0.046
	C	5.6	---	15	2	0.2	4	53.5	----	-----

[1] Ammonium acetate (pH 4.8) extraction. Spectrophotometric determinations except for P, which was colorimetric.

[2] Ammonium saturation method.

[3] Walkley-Black method.

[4] Kjeldahl method.

Table 2.-- Continued

Physical Analyses

SOIL	HORIZON	Particle size distribution[5] Sand fraction[6] VCS	CS	S	FS	VFS	Total	Silt	Clay	Moisture Content[7] 15 Atmospheres	1/3 Atmospheres
		percent									
Paola	A_1	Trace	2.7	57.7	34.5	0.8	95.7	2.5	1.8	1.71	3.41
	A_2	Trace	2.6	55.4	38.2	1.0	97.2	1.0	1.8	0.64	2.25
Lakeland	A_1	4.4	45.2	37.8	4.5	0.5	92.4	4.3	3.3	2.28	4.16
	C	5.2	46.0	35.6	5.3	0.8	92.9	2.8	4.3	1.34	2.36

[5] Hydrometer method.

[6] Dry Sieving.

[7] Pressure membrane apparatus.

Seeds were germinated and seedlings grown using distilled water and subirrigation. Approximately 45 days after planting, seedlings were thinned to 25 per pot. On August 25, 1970, needles and green stems were harvested, quick frozen in liquid N_2, then stored at -20 C until needed.

Photoassimilation Chamber

A chamber for photosynthetic fixation of CO_2 in pine seedlings was designed and built specifically for this project. It was constructed entirely of plexiglass and consisted of a cubic chamber, with dimensions of 30.5 cm, surrounded on 5 sides by a 10-cm water jacket, Figure 3. Access to the chamber was provided through a gas-tight bottom board.

The environment of the chamber was controlled. Constant light intensity of 2,000 ft-c was provided by two overhead, narrow-spot Sylvania cool-lux lamps (PAR 300/2NSP). Temperature was maintained at 25 $\pm$ 1 C with ice-cooled water piped through the water jacket. Air and introduced CO_2 were circulated through a closed system at 9 to 10 ft^3 per hr by a sealed pump. Even distribution of gases within the chamber was assured by a magnetically driven fan. Gases entered the chamber through the base of the fan and left via 4 exhaust ports in the bottom board. Tests with smoke showed that circulation within the chamber was good and that no leakage occurred.

Two CO_2 traps and an air drier supplemented the closed system. The drier (sulfuric acid solution of density 1.40) maintained a relative humidity of 37% in the air bubbled through it. CO_2 traps containing a 20% solution of KOH were used to

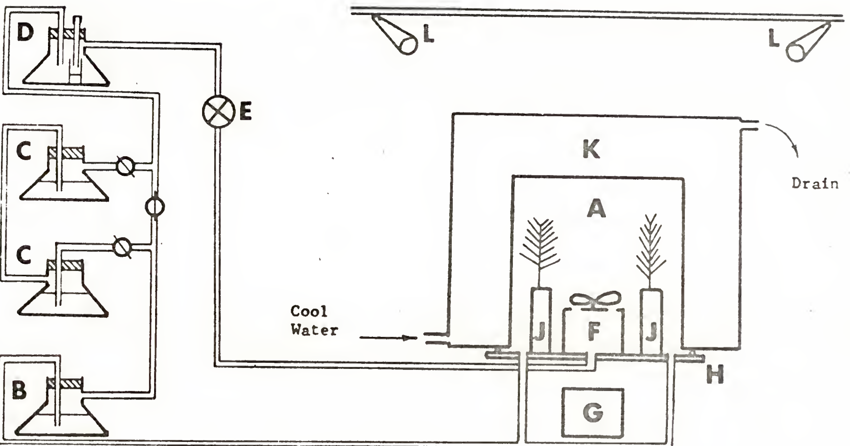

Figure 3.--Diagram of the photoassimilation chamber (A) and components of the closed system: dehumidifier (B), 2 CO_2 traps (C), CO_2 generator (D), Pump (E), magnetic-driven fan (F), magnetic stirrer (G), removable bottom board (H), intubuled seedlings (J), heat screen (K), and adjustable lights (L)

cleanse the system of CO_2 and $^{14}CO_2$. Traps were bypassed when $^{14}CO_2$ was added to the system.

CO_2 and $^{14}CO_2$ gas was generated by injection of 10 ml concentrated H_2SO_4 into a 5 ml aliquot of $KHCO_3$ and $KH^{14}CO_3$.

$Ba^{14}CO_3$ with a specific activity of 39.2 x 10^3 μc/mM was used as the source of $^{14}CO_2$. To provide the 20 μc of activity and 500 ppm of CO_2 needed for each replicated run in the photoassimilation chamber required 0.10065 mg of $Ba^{14}CO_3$ and 124.6313 mg of $BaCO_3$. Because of difficulty in weighing such minute quantities of $Ba^{14}CO_3$, an alternate procedure was devised to insure accurate replication. CO_2 and $^{14}CO_2$ were evolved from sufficient $BaCO_3$ and $Ba^{14}CO_3$ for 20 replications (2.0130 mg $Ba^{14}CO_3$ and 2.493 g $BaCO_3$) in a closed system using 2 equivalents of lactic acid. Gases were trapped in a solution containing an excess of KOH (1.5 g in 100 ml of boiled, glass distilled water). A 5.0 ml aliquot registered 4.5 x 10^7 counts per min (cpm) on liquid scintillation equipment (101% of the activity calculated to be in a sample containing 20 μc of activity).

Photoassimilation of $^{14}CO_2$

Seedlings were sealed in their tubules with parafilm and liquid latex to prevent uptake of $^{14}CO_2$ by roots (Stemmet, DeBruyn, and Zeeman, 1962; Fadeel, 1963) and microorganisms in the soil. Seedlings representing each fertilizer regime were treated simultaneously. They were preconditioned to the temperature, humidity, and light intensity of the chamber for 20 min while atmospheric CO_2 was trapped. Preconditioning in light enables

plants "...to generate any reducing agents active in photosynthesis" or "...to deplete any reducing agents" in darkness (Stutz and Burris, 1951). After preconditioning, 500 ppm of CO_2 containing 20 μc activity was released into the circulating air of the system from $KH^{14}CO_3$ and $KHCO_3$, using excess acid. At the conclusion of the photoassimilation period residual $^{14}CO_2$ was trapped.

Tubules were removed from the chamber and plunged into ice water. Seedlings were washed free of sand, blotted dry, and placed in individually tared and labelled plastic bags. Bags were sealed after evacuating the air and then plunged into liquid N_2. Frozen seedlings were stored at -20 C until seedlings were partitioned and weighed.

Low temperatures were maintained during weighing. Bags containing seedlings were blotted dry of condensation and weighed on a Mettler balance. Seedlings were removed and divided into: 1) epicotyl (needles and chlorophyllous stem), 2) nonchlorophyllous stem (hypocotyl), and 3) roots; then (1) and (2) were weighed; weight of 3 was obtained by subtraction. Seedling parts were returned to the bag and stored at -20 C before making ethanol extracts.

Ethanol-soluble extracts were made of weighed seedling parts. Needles and green stem, nongreen stem, and roots were individually ground with a cold mortar and pestle using liquid N_2, washed into Ehrlenmeyer flasks with liquid N_2 and 95% ethanol, and boiled for 10 min. Cooled extract was vacuum filtered into scintillation flasks. Residues were discarded.

Separation of Ethanol-Soluble Components

Extracts were evaporated just to dryness in a pan of warm sand (80-90 C), cooled, and the residue taken up in 5 ml of 15% ethanol. Four ml were fractionated into sugars, amino acids, and organic acids using ion-exchange resins. The remaining 1 ml was retained to sample radioactivity of the extract.

Procedures for fractionation of ethanol extracts on ion exchange columns were those of Shiroya et al. (1962, 1966) modified by Riech (1970). The extract was pipetted, a drop at a time, directly onto two seriate resin columns each 5 cm long x 1 cm diameter. The first contained 50-100 mesh Dowex 1-x 8 resin converted from the Cl^- to $HCOO^-$ form with formic acid, and the second contained 200-400 mesh Dowex 50W-x 8 in the H^+ form. The extract was washed through both columns with 150 ml of glass-distilled, deionized water added at the rate of 30 to 40 drops per min. The elution contained sugars. Amino acids were elutriated from the 50W-x 8 resin column with 70 ml of 2N NH_4OH, and organic acids from the 1-x 8 column with 70 ml of 5N formic acid. Using this procedure in control experiments, Riech reported 98% recovery of glucose-^{14}C in the sugar fraction, 91% recovery of Leucine-^{14}C in the amino acid fraction, and 98% recovery of orotic acid-^{14}C in the organic acid fraction.

Eluted fractions were evaporated to dryness (80-90 C) and taken up in 6 ml of 10% ethanol. Half (3 ml) of each fraction was reserved for further separation by thin layer chromatography; the remainder, and the 1 ml sample of ethanol extract, were used to obtain measurements of radioactivity.

Riech (1970) suspected that heating extracts on a hot plate to 80 or 90 C might cause loss of some of the more volatile amino and organic acids and modified his technique for concentrating extracts. The modified technique was not used because plant tissue was boiled in 95% ethanol to prepare the extract. Volatile acids do not survive the preparatory steps (Ting and Dugger, 1965). Vacuum evaporation and lyophilization were used to concentrate small volumes, but both procedures were excessively time-consuming and neither proffered special advantages, so the practice was abandoned.

Organic acids did not redissolve well in the diethyl ether used to remove impurities. Water or ethanol were substituted and found to give better resolution. Because it evaporated more rapidly during spotting of plates, 80% ethanol was used.

Measurement of Radioactivity

Samples of ethanol extract, sugars, amino acids, and organic acids were evaporated to dryness (80-90 C). The residue, dissolved in 0.5 ml absolute ethanol, was taken up in 10 ml of scintillation fluid (BBOT)[1], and the radioactivity counted for 5 min. Counts of radioactivity were measured using Model 3380 Packard

[1]BBOT is 2,5-bis [2-(5-tert-Butylbenoxazolyl)] thiophene and the scintillation fluid or "cocktail" consisted of 4 grams of BBOT dissolved in 1 liter certified grade toluene.

Tri-Carb liquid scintillators, units were normalized, and internal standards used.

Thin Layer Chromatography

Sugars, organic acids, and amino acids were separated into component parts by thin layer chromatography (TLC). Commercially available plates were used. For sugars and organic acids separations were made in one direction on glass plates coated with silica gel. Two-dimensional separation on cellulose-coated acetate plates was used for amino acids.

Sugars

Plates were scored vertically so as to contain spots and convert them to bands (Stahl, 1969). Each was spotted with unknown sugar solution and with solutions of known sugars. Tests showed that the clearest bands and best definition were obtained with 40 to 50 µl of unknown sugar solution per spot.

Sugars were separated by processing the plates twice with a solvent composed of n-propanol, ethyl acetate, and water (6:3:1). Plates were completely dried in a fume hood each time the solvent front reached the upper edge. Bands were detected and colors developed by gradually heating plates sprayed with a mixture of anisidine hydrochloride, aniline diphenylamine, and phosphoric acid (5:5:1) from 27 to 36 C in a force-draft oven over a period of from 5 to 10 min (Lewis and Smith, 1969 as modified by Riech, 1970). Sugars were identified using a combination of colors and Rf[2] values from samples of known sugars, Table 3.

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[2] $Rf = \frac{\text{Distance compound moved}}{\text{Distance solvent moved}}$

Table 3.--Color and Rf-values from chromatograms of known sugars used to identify unknowns

Sugars	Molecular Weight	No. of Carbon Atoms	Color	Range of Rf
Raffinose	594.5	18	Non-Descriptive	.04-.06
Sucrose	342.3	12	Brownish-yellow	.12-.15
Galactose	180.2	6	Brown	.12-.15
Glucose	180.2	6	Brown	.15-.18
Fructose	180.2	6	Yellow	.16-.19
Mannose	180.2	6	Brownish-yellow	.17-.21
Arabinose	150.1	5	Brownish-blue	.18-.21
Ribose	150.1	5	Non-Descriptive	.21-.25
Xylose	150.1	5	Non-Descriptive	.24-.27

Organic Acids

Preliminary tests showed that the quantity of organic acids in green tissue was low and that separation of the unknowns was difficult. Spots made from 40 to 80 µl of solution required 4 separate processing treatments with water-saturated ethyl ether-formic acid (7:1) to obtain discernible separation into distinct bands (Ting and Dugger, 1965). The bands stained yellow against a blue background when air-dried plates were sprayed with a tincture of bromcresol green (0.04 g bromcresol green in 100 ml 95% ethanol, then 0.1N NaOH was added drop by drop until a blue coloration appeared) (Krebs, Heusser, and Wimmer, 1969).

Amino Acids

Soltanabadi's (1966) procedure was used for the two-dimensional separation of amino acids on cellulose-coated acetate plates. Spots were separated in the first dimension using a solvent composed of 2-propanol, formic acid, and water (40:2:10), and, after drying, were separated in the second dimension with tertbutanol, methyl ethyl ketone, 3% NH_4OH, and water (50:30:10:10). Spots were developed on thoroughly air-dried plates with ninhydrin spray (0.5 g ninhydrin in 100 ml acetone) and heat (65 C for 30 min).

Radioautography

Counts of radioactivity using liquid scintillation equipment provided a quantitative measure of photoassimilated ^{14}C in ethanol soluble fractions of plant parts, i.e., sugars, organic acids, and amino acids. Radioautography was used to obtain a comparative measure of radioactivity in individual compounds

separated and identified by TLC. Films were exposed to the TLC plates for 2 weeks or 6 weeks; exposure for 6 weeks recorded the relative concentration of ^{14}C in all compounds. At 2 weeks only the most radioactive compounds exposed the film. Although methods are available for more quantitative measurements of photoassimilated ^{14}C, radioautography served the purpose of this study, i.e., to identify those compounds incorporating the most ^{14}C.

Measurement of Enzyme Activity

Needles and green stems were homogenized in a Sorvall Omni-mixer with a 2-fold weight of extracting solution (10 mM 2-mercaptoethanol, 2 mM EDTA, 100 mM phosphate buffer, pH 7.2) for 1 min at -15 C. The brei was extracted for 60 min at 0 C with magnetic stirring. The insoluble fraction was sedimented by centrifugation at 37,000 x g for 10 min at 4 C, then the supernatant was centrifuged at 100,000 x g for 30 min at 0 C. Clear supernatant was used for determination of enzyme activity.

The activity of three enzymes of glycolysis: fructose diphosphate aldolase (EC 4.1.2.13), glyceraldehyde-3-phosphate dehydrogenase-NAD dependent (EC 1.2.1.12), and 3-phosphoglycerate kinase (2.7.2.3), and two enzymes of the pentose phosphate shunt: glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were measured using optical tests based on the extinction coefficient of pyridine nucleotide coenzymes at 340 mμ and a Beckman model DB spectrophotometer with a Sargent recorder. Procedures were those employed by Firenzuoli et al. (1967) but were modified for use on the Beckman spectrophotometer. Extinction was measured at a wavelength of 340 mμ instead of 366 mμ.

Fructoaldolase

Measurement of fructoaldolase activity involved a coupled reaction (Figure 4) with two accessory enzymes (triose isomerase and glycerol-3-phosphate dehydrogenase). The reaction mixture contained: 50 mM triethanolamine hydrochloride (TRA-HCl) buffer, 5 mM EDTA, 0.15 mM nicotinamide adenine dinucleotide-reduced form (NADH), 4 mM fructose-1, 6-diphosphate, 0.72 IU per ml glycerol-3-phosphate dehydrogenase, 0.72 IU per ml triosephosphate isomerase, and green tissue extract. The product of the reaction was glycerophosphate.

Glyceraldehyde-3-Phosphate Dehydrogenase-NAD Dependent

One accessory enzyme (phosphoglycerate kinase) was used in the coupled reaction, depicted in Figure 4. The reaction mixture included: 50 mM TRA-HCl, 5 mM EDTA, 0.15 mM NADH, 1.5 mM adenosine triphosphate (ATP), 3.3 mM $MgSO_4$, 10 mM cysteine-hydrochloride, 7 mM 3-phosphoglycerate, 1.8 IU per ml phosphoglycerate kinase, and extract. The product of the reaction was glyceraldehyde-3-phosphate.

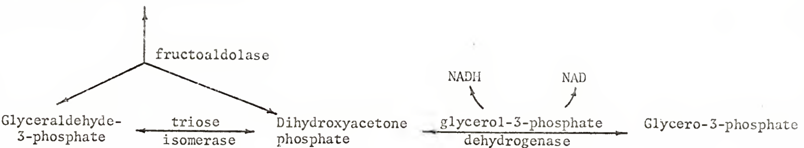
Phosphoglycerate Kinase

This is the same reaction as that used for glyceraldehyde-3-phosphate dehydrogenase reversed. The reaction mixture was identical except that 0.9 IU per ml glyceraldehyde-3-phosphate dehydrogenase substituted for 3-phosphoglycerate kinase. The product was 3-phosphoglycerate.

Glucose-6-Phosphate Dehydrogenase

The reaction mixture contained 50 mM TRA-HCl (pH 7.6), 5 mM EDTA, 0.5 mM NADP, 1.8 mM glucose-6-phosphate, and extract. The product was gluconolactone-6-phosphate (Conn and Stumpf, 1964). In aqueous solution containing the obligatory metal ion

FRUCTOALDOLASE



GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND 3-PHOSPHOGLYCERATE KINASE

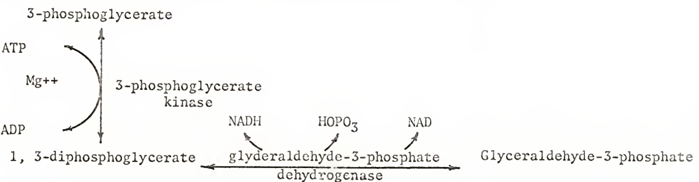


Figure 4. Reactions for measuring fructoaldolase, glyceraldehyde-3-phosphate dehydrogenase, and 3-phosphoglycerate kinase

cofactor Mg++ the reaction may proceed to form 6-phosphogluconate (Figure 5), the substrate for phosphogluconate dehydrongenase.

6-Phosphogluconate Dehydrogenase

The reaction mixture contained 50 mM TRA-HCl (pH 7.6), 5 mM EDTA, 0.5 mM NADP, 6.6 mM $MgSO_4$, 1 mM 6-phosphogluconate, and extract. The product was ribulose-5-phosphate.

Using the spectrophotometric assays, only very low and short-lived enzyme activity was detected by these procedures. On several occasions the reaction reversed after starting. Use of internal standards and commercially prepared enzymes showed that procedures were valid. Polyphenols, resins, terpenes (Firenzuoli, Vanni, and Mastronuzzi, 1969; Anderson, Lowe, and Vaughn, 1969), quinones, and tannins formed after cells are ruptured by extraction procedures (Anderson and Rowan, 1967) and other endogenous substances in tissue extracts interfere with biochemical reactions and inhibit enzyme activity. Attempts to prevent oxidation of phenols and quinones with reducing agents (Stokes, Anderson, and Rowan, 1968; Wildes et al., 1969), to remove or nullify them with polyvinylpyrollidone (Jones, Hulme, and Wooltorton, 1965; Walker and Hulme, 1965; Loomis and Battaile, 1966), and to extract sufficient proteins for analysis from acetone powders failed, as did efforts to concentrate enzyme extracts.

An alternate approach proved more successful and was used in this study. Acetone powders were made from green tissue, and the enzymes and proteins were extracted from the powders. The quantity extracted was too small for spectrophotometric use but was ample for electrophoretic detection and colorimetric measurements

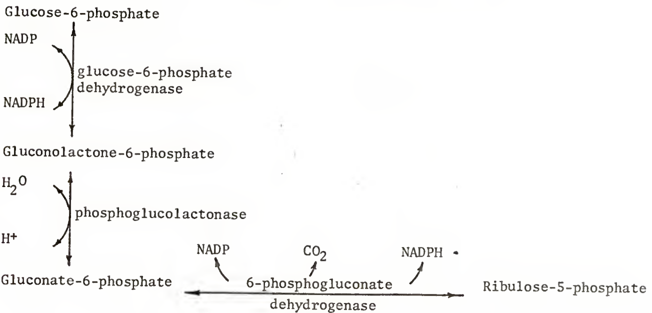


Figure 5. Reactions for measuring glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase

of enzyme activity. The technique is not as accurate as the optical test, but it has been used successfully (DeJong, Jansen, and Olson, 1967; Efron and Schwartz, 1968). Considering the comparative use made of these data, it provided an acceptable alternative.

Acetone Powders--Preparation and Protein Extraction

To prevent denaturation of enzymes all operations were carried out at temperatures below 5 C with cold reagents, tissue, and glassware (Hare, 1970).

Twenty grams of frozen tissue and approximately 125 ml of 80% acetone were "grated" then "liquified" in an Oster Blender. The brei was poured through a No. 60 soil sieve. Fibers were discarded and the suspension sedimented at 37,000 x g for 10 min. The supernatant was discarded and the pellet was mixed into fresh 80% acetone. The suspension was sedimented, as before, and the pellet blended into 100% acetone using a glass homogenizer and teflon pestle. After vacuum filtration through a plastic filter-funnel, the residue was washed with 100% acetone until white. The residue, air-dried by vacuum filtration, was sifted through No. 35 and No. 100 soil sieves. These fines were stored at -20 C.

Proteins were extracted from acetone powders with a solution containing: 2.5 M urea, 22.5 mM potassium metabisulphite, 56.8 mM ascorbic acid, 6.5 mM dithiothreitol (Clelands reagent), "Tween 20", tris buffer [tris (hydroxymethyl) amino methane], and water to give a product containing 0.004% "Tween 20" at pH 8.5 (Hare, 1970). Protein extracts were made by mixing acetone powder and extracting solution 200:3, extraction for 1 hr, and centrifugation at 37,000 x g for 10 min. The supernatant, extracted with a syringe, contained dissolved proteins. The pellet was discarded.

Preparation of Polyacrylamide Gels and Electrophoresis

Preliminary tests were made comparing polyacrylamide gel formulations and layering techniques proposed by Bakay and Nyhan (1969), Davis (1964), and Hare (1970). Use of a large-pore sample gel as an anticonvection medium proved inconvenient and unnecessary; the high concentration of urea in Hare's extracting solution served as well when layering was done by syringe under the upper buffer of the electrophoresis chamber. Separation of glucose-6-phosphate dehydrogenase isoenzymes was enhanced with a large-pore, spacer gel, but the clearest definition of malate dehydrogenase isoenzymes was obtained without one, i.e., by layering directly on the small-pore, running gel. Davis' large- and small-pore gels gave the best resolution of proteins.

Buchler Polyanalist disc electrophoresis apparatus was used. Buffers were those suggested by Hare: tris-glycine (pH 8.9) containing bromphenol blue dye in the upper chamber and tris-HCl (pH 8.1) in the lower chamber. Twelve tubes were processed simultaneously using 50 ma of current.

Gels were precharged, i.e., processed without a layer of protein extract, to remove contaminants. Protein extract was layered on gels under the buffer using a syringe calibrated in μl. The urea in the extract prevented dispersion into the upper buffer. Gels developed for glucose-6-phosphate dehydrogenase isoenzymes and for protein bands were layered with 40 μl of protein extract on the spacer gel; 20 μl of extract was layered directly onto the small pore, running gel used for development of malate dehydrogenase isoenzymes and for protein bands. Electrophoresis was complete when the colored front reached the lower end of the gel.

Detection of Protein and Isoenzyme Bands

Gels were removed from the electrophoresis tubes with a fine jet of water then placed in 100 x 12 mm test tubes.

Proteins and Dehydrogenase Isoenzymes

Gels were covered with 10% trichloroacetic acid then agitated in a reciprocal shaker for 30 min. Three drops of 0.2% coomassie blue were mixed with the acid, and the immersed gels were stored for 48 hr in the dark. Stained gels were rinsed and stored in 8% acetic acid in the dark at 4 C until scanned with a Gilford Model 2000 gel scanner (densitometer).

Existing techniques for preparing substrates to color isoenzymes of a specific enzyme in polyacrylamide gels were used (Johnson, Brannaman, and Zscheile, 1966; Macko, Honold, and Stahmann, 1967; Roggen, 1967).

The staining procedure involves action of the enzyme on its substrate in a coupled reaction that results in the reduction of nitroblue tetrazolium (NBT) to a colored product (Goldberg, 1963). Bands stain blue. The intensity of the stain is related to the pH of the solution, temperature, length of incubation, and the activity of the enzyme (Gabriel and Wang, 1969).

Dietz and Lubrano (1967) recommended 90-min incubation for optimum development of lactate dehydrogenase isoenzymes and cautioned against using less than 45 or more than 120 min for quantitative work. The upper limit was set because substrate in the vicinity of the most active isoenzymes is exhausted while that near less active isoenzymes allows staining to continue. This was true with malate dehydrogenase

for which a 45-min incubation period was used. However, comparisons of staining intensity with glucose-6-phosphate dehydrogenase substrate showed that the most active isoenzymes continued to darken even after 6 hr, indicating a low level of enzyme activity and an adequate supply of substrate. Background staining, attributed to protein sulfhydryl groups (Dietz and Lubrano, 1967), occurred with long periods of incubation, so development of isoenzymes of glucose-6-phosphate dehydrogenase was reduced to 3 hr or less.

The basic incubation media was prepared using two solutions to which specific substrate and pyridine nucleotide coenzymes were added. Both were made with 0.05 M tris-HCl buffer pH 7.5. Solution I contained 30 mg NBT (dissolved in 95% ethanol) and 100 mg $MgSO_4 \cdot 7H_2O$ dissolved in 50 ml buffer. Solution II was composed of 150 mg EDTA dissolved in 40 ml hot buffer to which 3 mg phenazine methosulfate (PMS) was added when cool. The solution was brought to 50 ml volume with buffer.

Both NBT and PMS solutions are light-sensitive, and required storage in brown bottles and use in subdued light.

The incubation mixtures for developing glucose-6-phosphate dehydrogenase and malate dehydrogenase bands were made up in quantities to develop 21 gels.

Glucose-6-Phosphate Dehydrogenase and Malate Dehydrogenase

Gels were inundated in an incubation mixture containing 10 mg NADP, 84 mg glucose-6-phosphate, 14 ml solution I, and 14 ml solution II, then incubated at 37 C in the dark.

The incubation mixture consisted of 188 mg malic acid, 14 ml solution I, and 14 ml solution II (adjusted to pH 7.5 with NaOH) to which 20 mg NAD was added. Gels were covered with the incubation

mixture and incubated at 37 C in the dark. At the end of the incubation period 8% acetic acid was substituted for the incubation mixtures. Gels were stored at 4 C in the dark until scanned on the densitometer.

Reaction mixtures for staining isoenzymes that involve use of one or more accessory enzymes are difficult to formulate. Experiments aimed at developing or modifying existing procedures (Bergmeyer, 1965) to stain fructose-1 6-diphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase isoenzyme bands were not entirely successful. Bands were obtained but results were not consistently reproducible.

Individual bands were identified by their position relative to the dye front (Rf value). Activity of identified isoenzymes measured by staining intensity was quantified using heights of densitometer scanning peaks. Gabriel and Wang (1969) report that quantification of isoenzyme activity using reduced tetrazolium is accurate for comparative purposes. Comparisons using the number, Rf, and activity of dehydrogenase isoenzymes were made between the superior tree and each of its 5 nonsuperior, neighboring trees, and between the half-sibling progeny of the superior tree and that of each of its neighbors growing on two soils. Similar comparisons were made using the number, Rf, and staining intensity of protein bands.

Sample Size and Statistical Analysis

Use of two electrophoresis chambers permitted processing 24 gels simultaneously and replication of samples from a single location

and soil at least 3 times. Measurements were obtained from scanned gels representing the average of each tree x soil x protein combination.

A randomized complete block design was used for the experiment involving measurements of isoenzymes and proteins. The number of bands, Rf values, and quantified staining intensity data from densitometer scannings were subjected to analysis of variance.

Location of the 5 superior trees and their associated nonsuperior contemporaries, from which seeds and tissues were collected, served as blocks. Comparisons were made among soils on which parent trees grew (original), and those on which seedlings were grown (Lakeland sand and Paola sand), and among trees from each location. Orthogonal partitioning of sums of squares was used to make the contrasts specified under soils and trees. Dunnett's procedure (Steel and Torrie, 1960) was used to compare the superior tree with each neighbor. Selected comparisons also were made (at a single degree of freedom) by partitioning the tree x soil interaction sums of squares.

RESULTS AND DISCUSSION

Photoassimilation of $^{14}CO_2$

To obviate the need for processing all seedling parts, time-course studies were used to estimate an optimum period for photo-assimilation of $^{14}CO_2$. The purpose was to limit time for translocation of labelled compounds into the nonchlorophyllous portions of the seedling, yet permit incorporation of easily measurable quantities in green tissue. Radioactivity of the ethanol-soluble fraction of needles and green stem, stem, and roots was measured for seedlings allowed to photoassimilate $^{14}CO_2$ for 15, 30, 60 and 120 min.

Results summarized in Figure 6 show that, with minor exceptions, there was a progressive increase in radioactivity in stem and root tissue with the length of the photoassimilation period, and the proportion of activity in green tissue decreased as the length of photoassimilation increased. It appears that even with photoassimilation as short as 15 min, ample time is provided for some translocation of labelled compounds to the roots. The proportionately high concentration of radioactivity in the stem, compared to root tissue for all lengths of exposure suggests that the stem of young sand pine seedlings serves more as storage tissue than primarily as an avenue for translocation. This does not discount possibilities that some CO_2-fixation may occur in non-green tissue or that a high rate of metabolism in the roots may catabolize and respire some labelled compounds. Extrapolation of the data suggested a 10 min photoassimilation period as near optimum.

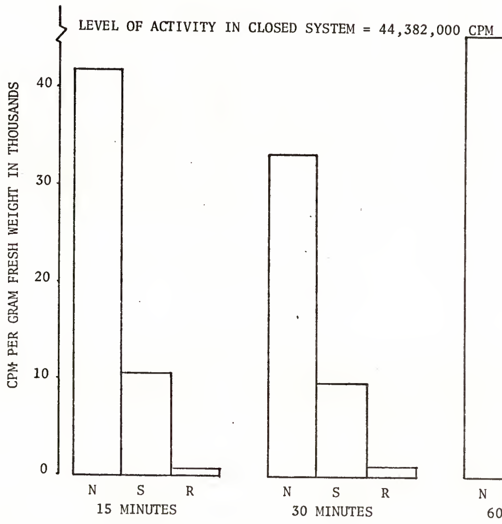


Figure 6. Distribution of radioactivity in seedlings allowed to photoassimilate $^{14}CO_2$

Seedlings were separated for analysis into

Results of the time-course experiment raised two questions: 1) would assimilation of $^{14}CO_2$ and CO_2 continue during the post-illumination period while the chamber was being cleansed of radioactive gas and seedlings were being processed for freezing? 2) Why was such a small proportion of the 44 million cpm released into the closed system utilized even after a 2 hr photoassimilation period? Experiments were conducted to answer these questions.

The first was designed to answer question 1 and to test the conclusion that a 10 min photoassimilation period was near optimum for fixing measureable amounts of labelled carbon in green tissue without allowing appreciable amounts to translocate to the stem and roots, i.e., to limit radioactivity primarily to green tissue.

Working at night to reduce the likelihood of contamination by incident daylight, comparison was made of the radioactivity in ethanol extracts of green tissue, stem, and roots between seedlings subjected to a) preconditioning and exposure to $^{14}CO_2$ for 10 min in darkness or b) preconditioning and photoassimilation for 10 min using 2,000 ft-c light. Post-induction periods in both treatments were in darkness, and seedlings were processed for preliminary freezing in subdued light (just enough to ascertain that all sand was washed from roots).

Results presented in Figure 7 support the previous contention that photoassimilation for less than 15 min confines metabolites with ^{14}C to green tissue without permitting appreciable amounts to translocate to the stem and roots. Reducing the photoassimilation period from 15 to 10 min increased the proportion of radioactivity in green tissue by about 3% compared to activity in stem and roots. It also reduced radioactivity by 70% in

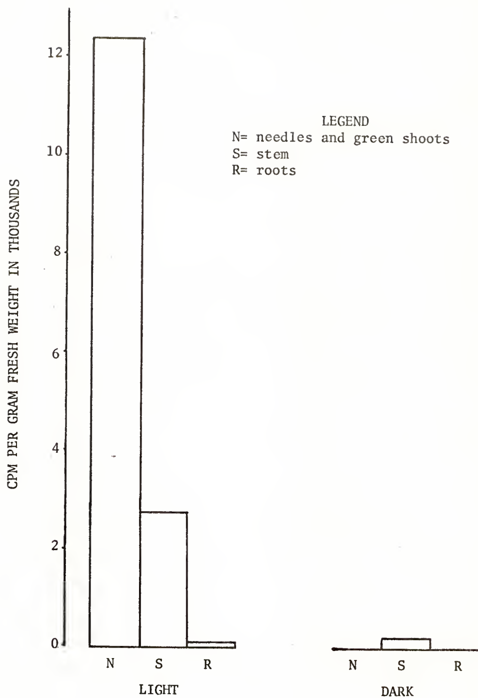


Figure 7. Distribution of radioactivity in seedling tissues exposed to $^{14}CO_2$ in the light and dark

green tissue, 75% in stems, and 87% in roots. Shorter photoassimilation periods might have increased the proportion of radioactivity in green tissue and greatly limited translocation to the stem and roots, but extrapolation of 15 and 10 min data suggests that radioactivity in green tissue also might be lowered to a level of questionable utility. For purposes of this study a 10 min photoassimilation period appeared near optimum.

In the dark radioactivity was recorded in the stem, but none in the roots or green tissue of all 4 seedling replicates. Results, while not analyzed statistically, emphasize the need to extinguish light at the conclusion of the photoassimilation period and to process seedlings in subdued light.

The large discrepancy between the radioactivity of labelled carbon in the system and the amount photoassimilated has at least 5 possible explanations:

1. Uptake of CO_2 was limited by some unexplained physiological condition(s).
2. Preferential uptake of CO_2 over $^{14}CO_2$.
3. An excess of CO_2 was used.
4. Activity was lost during preparation of ethanol extracts or was masked by chlorophyll and other extraneous material.
5. Not all the radioactive carbon was released from $KH^{14}CO_3$ by the lactic acid.

The system was gas tight. Photo- and dark-respiration do not account for the large discrepancy. Possibilities 1 and 2 are beyond the scope of this investigation, and 3 was intentional. Experiments were undertaken to test 4 and 5.

Aliquots of green tissue extract prepared from 5 sand pine seedlings were pipetted into scintillation vials to form an arithmetic series from 0.5 through 5.0 ml in 0.5 ml increments. A blank of 95% ethanol was used. To each vial 10 μl ^{14}C-sucrose was added. All were evaporated to dryness, then the residue was taken up in 0.5 ml absolute alcohol and diluted with 10 ml BBOT scintillation cocktail. One additional vial containing 2.5 ml green tissue extract was similarly prepared and processed except that the extract, including ^{14}C-sucrose, was clarified with charcoal before evaporating the liquid. Counting efficiency and cpm were recorded on identical liquid scintillators at two cooperating laboratories (Agronomy and Pesticides).

Chlorophyll in the scintillation fluid lowered both counting efficiency and cpm. High concentrations of chlorophyll, such as those found in tissue from high nitrogen fertilizer treatments, were most seriously affected. Effectiveness of the scintillators was lowered in direct proportion to the amount of chlorophyll present. Clarification with charcoal improved counting efficiency by 800% and increased cpm by 250% in the vial containing 2.5 ml of chlorophyll-containing extract. Some loss of radioactivity occurred during clarification but, considering gains experienced, it was considered tolerable. At best, however, the counting efficiency was only 0.70 in scintillation fluid containing no green tissue extract, indicating that the cocktail or absolute ethanol lowered counting efficiency. These possibilities were tested.

Results of the tests showed that the cocktail and the absolute ethanol used with scintillation equipment in the Forest Physiology and Genetics Laboratory (F.P.G.L.) caused quenching in scintillator

counters at cooperating laboratories. The cooperators equipment had been standardized against cocktail other than BBOT. To compensate for quenching caused by chlorophyll and ethanol and for differences in cocktail, curves were drawn to equate all measurements of radioactivity at 100% counting efficiency regardless of cooperator equipment used, Figure 8. BBOT alone was responsible for a reduction in counting efficiency of more than 20%. Absolute ethanol caused a further reduction of about 11%. Increasing the proportion of BBOT to absolute ethanol improved efficiency by about 2% but reduced cpm by more than 14%. Linear regressions of the form Log_e AES Ratio = a+b Log_e cpm were fitted to a plot of the data. Test of the regression by analysis of variance showed that the probability of obtaining an F value larger than that obtained for regression was less than 0.0001 for cooperator equipment. No meaningful loss of accuracy occurred from fitting straight line functions to the data. The slope of each curve appears to be unique for each scintillator.

Seedlings appear to utilize only a minute amount of the $^{14}CO_2$ released into the photoassimilation chamber. Possibly not all of the 20 μc of activity (45 million dpm) was evolved from $KH^{14}CO_3$ by lactic acid even though two equivalents of acid were used to one of the bicarbonate.

To test the effectiveness of the acid, a comparison was made of radioactive carbon photoassimilated by seedlings when 10 ml of concentrated lactic, hydrochloric, or sulfuric acid were used. Stoichiometrically, this provides twice the amount of lactic and hydrochloric and 4 times the amount of sulfuric acid needed to evolve 500 ppm of CO_2 containing 20 μc of activity from the potassium

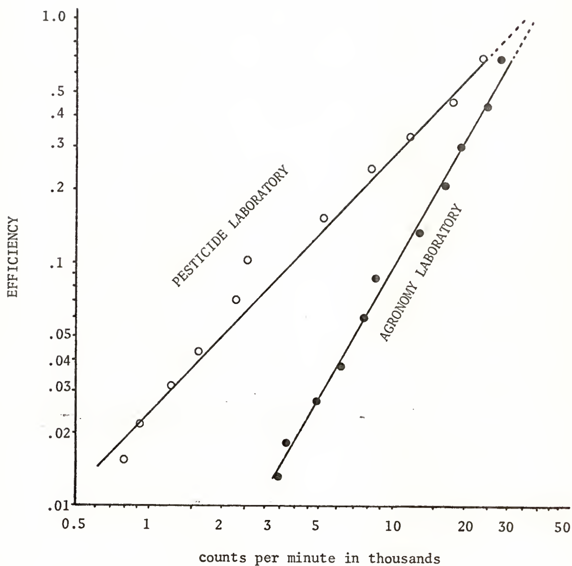


Figure 8. Quenching curves developed to compensate for loss of counting efficiency

bicarbonate. To verify results of a previous experiment, which indicated that postillumination processing of seedlings should be done in darkness, one additional treatment was included. Two equivalents of lactic acid were used to evolve CO_2, but lights were left on for an additional 5 min following the 10 min photoassimilation period (while CO_2 in the chamber was trapped). Then, seedlings were processed in fluorescent rather than subdued light. Adjustments were made for quenching, and cpm were expressed per gram of fresh tissue weight.

Inorganic acids more effectively evolved CO_2 from $KHCO_3$ than lactic acid, Figure 9. On an equivalent basis HCl was as effective as H_2SO_4, but in this experiment, only two equivalents of HCl were used as compared to four of H_2SO_4.

Photoassimilation of ^{14}C continued during 5 additional min of light while the chamber was cleansed of $^{14}CO_2$. The exact amount could not be determined because seedlings subjected to the additional photoassimilation period also were processed in fluorescent rather than subdued light. Some loss of ^{14}C may have occurred to the atmosphere due to photorespiration.

Clarification of Extracts and Quenching

Extraneous material in ethanol extracts caused quenching, i.e., loss of counting efficiency and a reduction in recorded cpm, in the liquid scintillator. Dark green extracts from seedlings supplied high rates of N proved particularly troublesome. Attempts to remove the chlorophyll by filtration or centrifugation failed. Powdered, activated charcoal was used routinely in previous experiments as a clarifying agent; and even though some loss of

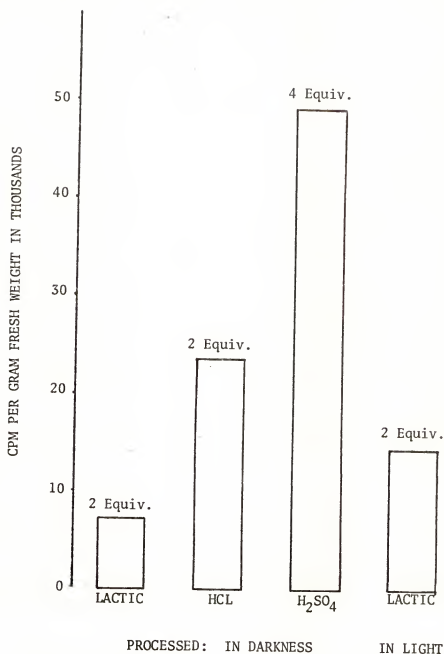


Figure 9. The ^{14}C incorporated into green tissue of *Pinus Clausa* in response to different amounts and kinds of acid used to release $^{14}CO_2$ from $KH^{14}CO_3$

radioactivity occurred, gains in counting efficiency more than compensated for losses. Powdered, activated alumina was compared with powdered, activated charcoal to provide a more efficient clarifying agent.

Ethanol extracts from 2 sets of seedlings (each represented by the 4 fertilizer treatments) were prepared from seedlings that had photoassimilated $^{14}CO_2$. Color of the extracts ranged from light to dark green (no color charts for foliage were available).

N_2P_1 N_2P_2 N_1P_2 N_1P_1

Dark green -------- Light green

One set of extracts was clarified with activated alumina (80-200 mesh size chromatographic grade) using 3 additions of approximately 0.5 g each plus 3 min spinning at maximum speed on a clinical centrifuge after each addition. Comparison with untreated extract showed no color change although the alumina had attained a light yellowish-green color.

The other set of extracts was clarified with powdered activated charcoal using 3 additions of approximately 0.3 g each, as above. All clarified extracts were light yellowish-green and only a slight difference in color remained between extracts of N_2P_x and N_1P_x fertilized seedlings. Because alumina failed to clarify the extracts, they were treated with charcoal. Meaningful differences in counting efficiency or cpm between charcoal clarified and alumina plus charcoal clarified extracts were attributed to alumina.

Figure 10 shows that when both counting efficiency and retention of radioactivity are considered, clarification with charcoal was as good or superior to clarification with both alumina and charcoal. Alumina by itself was not an efficient clarifying agent.

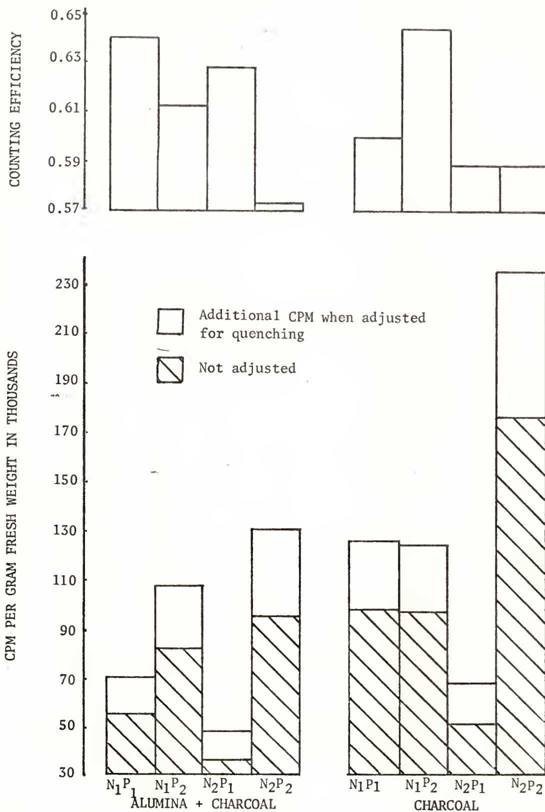


Figure 10. Comparison of clarifying agents

Objectives of another series of experiments were: to identify metabolic pathways most responsible for fixation of atmospheric carbon in green sand pine seedling tissue; to determine the affect of each of the 4 levels of fertilizer on the seedlings, on radioactivity in the ethanol-soluble fraction of green tissue, and on the relative amounts of labelled sugars, organic acids, and amino acids produced.

Ethanol extracts made from green tissue of fertilized, 3-month-old sand pine seedlings, after $^{14}CO_2$ was photoassimilated for 10 min at 2,000 ft-c and 25 $\pm$ 1 C, were fractionated on ion exchange columns. Radioactive counts were made of sugar, organic acid, and amino acid fractions. Sugars and acids were separated into constituent compounds chromatographically, for identification, and radioautograms were made of TLC plates to permit comparison of radioactivity among constituents.

Table 4 shows the actual and proportional distribution of ^{14}C in the ethanol soluble fractions. The insoluble fraction, composed primarily of cellulose, hemicellulose, and lignin, was not assayed. Shiroya et al. (1962, 1966) working with white and red pine seedlings and Balatinecz, Forward, and Bidwell (1966) working with 8-month-old jack pine found no more than 10% of photoassimilated ^{14}C in the ethanol insoluble fraction. Extrapolation of their data suggests that virtually all the ^{14}C photoassimilated in 10 min was contained in ethanol-soluble compounds. In 6-month-old sand pines Riech (1970) reported very little translocation of labelled, ethanol-soluble compound from needles supplied by photoassimilated $^{14}CO_2$, even after 8 hours.

Table 4.--Distribution of photoassimilated 14C in green sand pine seedling tissue

Ethanol Soluble Fractions

Sugar	Organic Acid	Amino Acid	Total
Cpm x 10^3/g fresh tissue			
55.7	13.8	4.8	74.3
Percent of fraction			
75.0	18.6	6.4	100.0

Of the ethanol-soluble fraction, sugars contained 75%, organic acid 19%, and amino acids 6% of the labelled carbon in green tissue. About 40% of the activity was lost during fractionation of the ethanol extract on ion exchange columns. Although this loss is approximately 2 1/2 times as great as that reported by Riech using the identical procedure, the proportionate distribution of ^{14}C he reported in 6-month-old sand pines was almost identical i.e., 76% sugars, 17% organic acids, and 7% amino acids.

Sugars

Glucose, fructose, and galactose were the 3 most common sugars identified for seedlings raised at all levels of fertilization. Rf values for glucose and fructose overlapped (Table 3) but were differentiated by color. When sprayed with developer and heated, glucose stained brown, and fructose yellow. Rf values for glucose and fructose differed enough from galactose to avoid confusion, but Rf's for galactose and sucrose were similar. Here, too, color was used to differentiate between them: galactose was brown, and sucrose was brownish-yellow. One other band developed primarily in the extract from seedlings raised at low levels of nitrogen. Its Rf identified it as a pentose, but it bracketed the ranges of ribose, arabinose, and mannose. The brownish-blue color indicated it was arabinose.

The chromatogram illustrated in Figure 11 shows other bands at relatively high Rf values, but they were either faint or not present at all levels of fertilization. They were not specifically identified; yet, since Rf values in sugars are inversely proportional to molecular weight and number of hydroxyl groups (Lewis and Smith, 1969) or the number of carbons, this suggests they were probably tetroses or

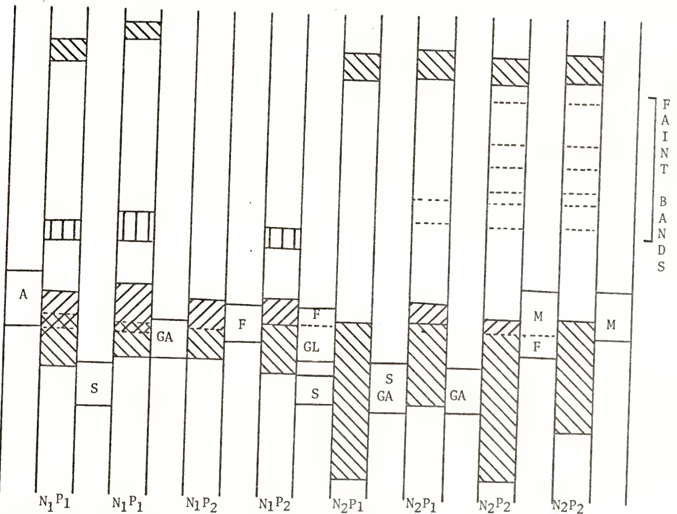


Figure 11. Chromatogram showing bands of standard and unknown sugars

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Hatching serves only to emphasize location of unknown sugar bands and indicate where overlapping occurred.

Known sugar standards: A=Arabinose; S=Sucrose; GA=Galactose; F=Fructose: G=Glucose: M=Mannose

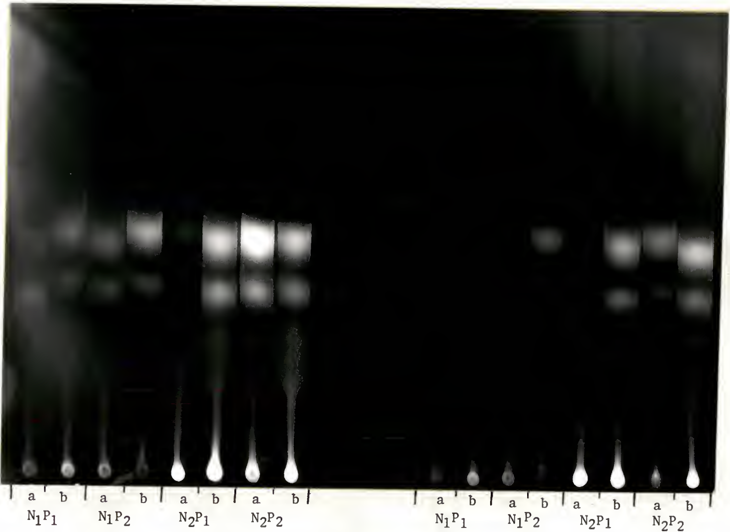

Figure 12. Radioautogram of a sugar TLC plate (6 weeks exposure)

Extract from the two seedling replicates is designated a and b

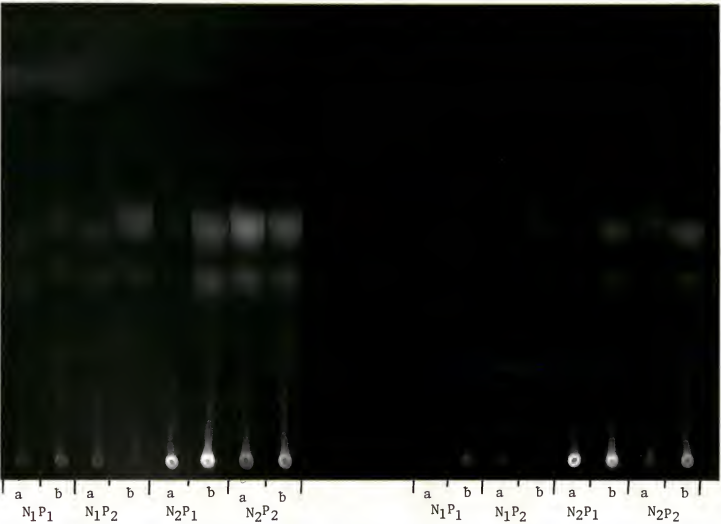

Figure 13. Radioautogram of the same TLC plate used in Figure 12 (2 weeks exposure)

Extract from the two seedling replicates is designated a and b

trioses. The bands were for the most part in extracts from seedlings raised at high levels of N and P.

Radioautograms of TLC plates show that most of the ^{14}C was contained in glucose and fructose, less in galactose, virtually none in high Rf, low molecular weight sugars, and none in pentoses (Figures 12 and 13). No difference in radioactivity between glucose and fructose was apparent here even though the green tissue included active apical, leaf, and stem meristem.

Rangnekar and Forward (1969) reported differential fixation of ^{14}C in fructose and glucose following 6 days of $^{14}CO_2$ assimilation. From 2.7 to 8.4 times as much radioactivity was found in fructose as in glucose in the stem, root, and bud of red pine seedlings. The pattern was reversed in needles: 1.4 times as much ^{14}C was recorded in glucose. The high proportions in active meristematic tissue was attributed to metabolism of the glucose moiety of translocated sucrose and the presumed sequestration of fructose within a cell compartment or in the individual cell. This explanation is in agreement with the theory for higher plants. Sucrose acts as a protective derivative and source of glucose, the primary metabolite of all living organisms (Arnold, 1968).

Some labelled carbon remained at and near the origin. The concentration was particularly high for seedlings raised at the high N level. Their low mobility with respect to sucrose and raffinose suggested that they were oligosaccharides, possibly verbascose, composed of sucrose plus 3 galactose units. Presence of oligosaccharides containing sucrose might serve to explain absence of sucrose on the chromatograms. A body of evidence exists

to show that sucrose is a primary translocate in higher plants (Clauss, Mortimer, and Gorham, 1964; Gordon and Larson, 1968; Shiroya et al., 1962; Willenbrink, 1966; Hofstra and Nelson, 1969), including conifers. Hida, Sacko, and Harada (1962), however, found that the sucrose content in pine needles appeared lower than in foliage of other conifers. In some plants raffinose, (Pridham, Walter, and Worth, 1969) stachyose, and verbascose (Webb and Burley, 1964; Trip, Nelson, and Krotkov, 1968) were found among translocates (sucrose plus 1, 2, and 3 galactose units, respectively). It seems apparent that oligosaccharides were present. They may serve as translocates in young sand pine seedlings.

The extract from one seedling fertilized at the high level of N and low level of P remained at the origin of the TLC plate. Extract from all other seedlings raised under this fertilizer regime migrated upward during separation. The seedling is believed to have been moribund. Seedlings raised at low P levels (P_1) suffered comparatively high mortality in both pots and tubules. Mortality among the faster growing N_2P_1 seedlings was higher than among slower growing N_1P_1 seedlings. Dead and dying seedlings were characterized by a constriction of the stem at groundline reminiscent of damping off disease (Figure 14). The constriction and area immediately adjacent to it was black. It appeared charred. Dr. R. Schmidt, Forest Pathologist at F.P.G.L., identified a species of *Verticillium* in one of several tissue sample cultures. Alexopoulos (1952) recognizes *Verticillium* as a cause of wilt disease in many plants and as a fungus associated with damping-off.

Figure 14. Moribund seedling showing constriction near groundline

Another possible cause of mortality among seedlings raised at the low level of P might have been chemical burn. HCl was used to adjust the acidity of nutrient solutions to pH 5.85 $\pm$ 0.05. Prior to adjustment solutions containing the low level of P were pH 9.8 and pH 10.5, whereas those containing the high level were pH 3.5 and pH 5.8. Accumulation of Cl^- during the 2 weeks between flushings of plant containers with deionized water could have resulted in a Cl build-up and burn.

Regardless of the cause of death, seedlings supplied the N_2P_1 nutrient solution had a comparatively high rate of mortality. The possibility exists that one healthy-looking but moribund seedling was used in these experiments. If this were a fact, assimilation processes may have all but ceased thereby causing a low level of ^{14}C fixation in sugars. More likely, however, is the possibility that the stem was girdled causing accumulation of translocation products in the stem and green tissue above the girdle. If these products were high molecular weight oligosaccharides, such as stachyose or verbascose, their movement from the origin would be slight. The seedlings' moribund condition would permit progressively less assimilation of ^{14}C in comparatively low carbon sugars such as fructose, glucose, and galactose and reduced respiratory loss of labelled compounds. Subsequent coupling with previously formed sucrose might result in an accumulation of translocated ^{14}C-labelled high carbon sugars, such as stachyose and verbascose, at the origin of the TLC plate. Examination of the radioautogram shows this to have been the case.

Organic Acids

Incorporation of ^{14}C in the organic acid fraction amounted to only about one-fourth that in sugars, (Table 4). Separation by TLC required several treatments with solvent to obtain separation for identification of some components, Figure 15. Exposure of plates to x-ray film for two weeks yielded no clearly discernible bands of radioactivity; 6 weeks exposure was required to obtain faint bands. The intensity of the bands attests to the low activity obtained on liquid scintillation equipment.

Several bands appeared on the radioautograms that did not correspond to those obtained visually on TLC plates. These were labelled "hot" on the chromatogram. "Hot" bands were found only in syrupy extract. The Rf of one "hot" band coincided with that of malic acid standards, another with glutamic acid. Glutamic acid is a dicarboxylic amino acid formed from the organic acid α-ketoglutarate and NH_3. An intermediate is α-iminoglutarate which requires NADH + H^+ and glutamic dehydrogenase to catalyze the second reaction (Devlin, 1968). It is conceivable that α-iminoglutarate or the acidic glutamic acid was absorbed on the 50w x 8, H^+-form resin to become a contaminant contributing to the syrupy consistency of the extract.

Malic acid was the only organic acid identified containing detectable amounts of ^{14}C.

Amino Acids

This fraction of ethanol-soluble, green tissue extract contained the least radioactivity, about one-twelfth of that in sugars. Activity was so low that spots could barely be detected

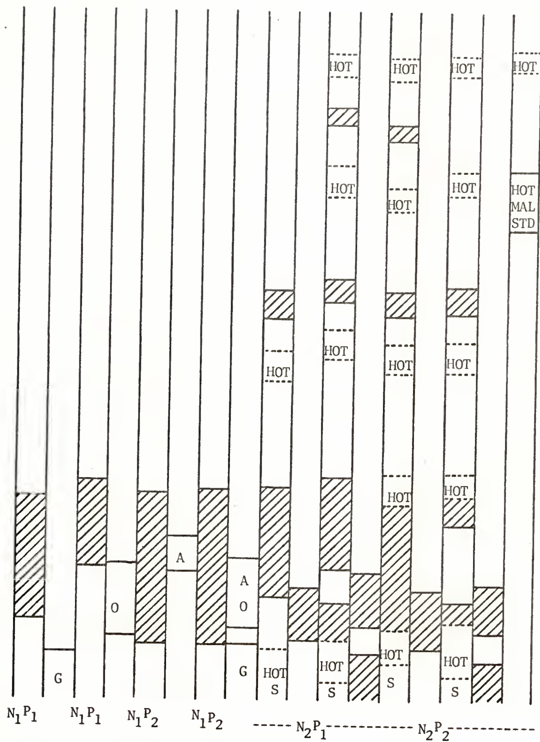

Figure 15. Organic acid TLC plate

Hatched bands are unknowns

Organic acid standards are labelled: glutamic acid (G), oxalic acid (O), ascorbic acid (A), and malic acid (M)

Radioactive bands (HOT) appeared in syrupy (S) extract

on x-ray film after 6 weeks of exposure. Prints could not be made.

Only extract from trees fed the high level of nitrogen contained radioactive spots, Figures 16 and 17. They were identified by using Soltanabadi's (1966) amino acid map as: glutamic acid, aspartic acid, and, because of the close proximity of spots, either lysine or arginine, most probably the latter.

Labelled malic acid, glutamic acid, and aspartic acid suggest activity of the tricarboxylic acid cycle, amination of α-ketoglutarate to glutamic acid, and possibly transamination to form aspartate leading to arginine in the urea cycle (Wilson, King, and Burris, 1954). Because of the extremely low incorporation of ^{14}C in amino acids, no further work was done with amino acids and the enzymes involved in their synthesis.

Effect of Nutrient Level on Seedling Morphology and ^{14}C Incorporation

Distribution of weight and ^{14}C in seedlings supplied 7.5 ppm (N_1) or 75.0 ppm (N_2) N, and 15.0 ppm (P_1) or 150.0 ppm (P_2) P, is summarized in Table 5. The proportional distribution of weight and radioactivity as influenced by N and P nutritional levels is shown in Table 6.

Color and Weight

Foliage was darker green in N_2 than N_1 treatments and darkest in the N_2P_1 treatment. P, by itself, had no noticeable influence on color. (See results of experiment testing clarifying agents on page 53.)

N more than P influenced seedling weight and size. The entire seedling and each of its component parts was larger and heavier

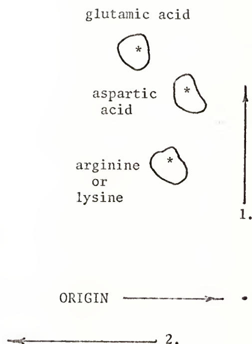

Figure 16. Amino acid TLC plate for fertilizer treatment N_2P_1

Radioactive spots are labelled *

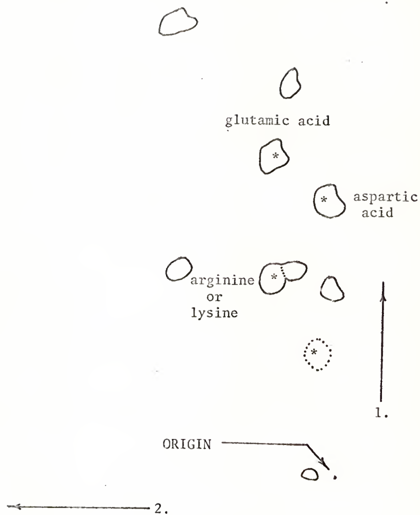

Figure 17. Amino acid TLC plate for fertilizer treatment N_2P_2

Radioactive spots are labelled *

One * spot (dotted outline) did not appear on the TLC plate

in N_2 than N_1 treatments. The effect of P was not as pronounced and appeared to have been centered in green tissue and roots. P_2 treatments contained the heaviest seedlings. The N_2P_2 fertilizer regime produced the largest and heaviest seedlings.

Ratios between green and nongreen tissue weight were virtually unaffected by P but strongly influenced by N. The ratio was lowest for N_1 seedlings which suggests that, like the more common top-root ratio, a favorable balance existed between green and nongreen tissue. This was expected because N_2 treatments produced seedlings with the most green tissue.

Fertilizer combinations most conducive to green tissue production contained the high level of N. N_2P_2 produced seedlings with heavy foliage, stem, and roots. N_2P_1 produced proportionately less root and stem tissue and, therefore, seedlings with the highest green-nongreen tissue ratio. Seedlings grown in N_1P_1 were smallest but the weight distribution between green and nongreen tissue was most equally balanced.

^{14}C Incorporation

Foliage of N_2-fed seedlings was heavier and darker green than those fed N_1. It contained more chlorophyll and photo-assimilated more $^{14}CO_2$, consequently, green tissue extracts from N_2-supplied seedlings were more radioactive. The affect of N and P on ^{14}C incorporation becomes apparent only when radioactivity is adjusted for differences in tissue weight, i.e., when expressed on a weight of green tissue basis.

Both P and N influenced the level of radioactivity in ethanol extract and most of its component fractions. Highest

Table 5.--Seedling weight and photoassimilated ^{14}C distribution in response to nutrients

	NUTRIENT REGIME			
ITEM	N_1P_1	N_1P_2	N_2P_1	N_2P_2
Weight in grams				
Entire seedling	0.375	0.425	0.929	1.196
Needle + green stem	.146	.168	.542	0.675
Stem	.024	.024	.042	.048
Roots	.205	.233	.345	.473
Ratio				
Green/nongreen wt.	.594	.675	1.367	1.264
Adj. cpm/g green tissue x 10^3				
Ethanol extract	55.2	69.0	41.1	74.7
Sugars	38.9	58.0	54.9	71.2
Organic acids	10.9	22.0	10.0	12.5
Amino acids	5.5	3.0	5.9	4.6

Nutrient treatments were: 7.5 ppm (N_1) or 75.0 ppm (N_2) of N and 15.0 ppm (P_1) or 150.0 ppm (P_2) of P

Table 6.--Influence of N and P fertilizer on the distribution of weight and radioactivity

ITEM	NUTRIENT REGIME			
	NITROGEN LEVELS		PHOSPHORUS LEVELS	
	N_1 (7.5 ppm)	N_2 (75 ppm)	P_1 (15 ppm)	P_2 (150 ppm)
	--------Percent of seedlings---------------			
Heaviest seedling	5.6	94.4	44.4	55.6
Heaviest green tissue	0.0	100.0	50.0	50.0
Heaviest stem	11.1	88.9	50.0	50.0
Heaviest root	5.6	94.4	44.4	55.6
Lowest green-nongreen tissue wt. ratio	100.0	0.0	50.0	50.0
Highest adj. cpm per gram green tissue				
Ethanol extract	55.6	44.4	44.4	55.6
Sugars	37.5	62.5	12.5	87.5
Organic acids	50.0	50.0	50.0	50.0
Amino acids	12.5	87.5	62.5	37.5

^{14}C incorporation in the extract was recorded in P_2 and N_1 treatments. Treatment combinations with the highest radioactivity were N_2P_2 and N_1P_2.

Sugars contained 75% of the photoassimilate ^{14}C and, in this fraction, the level of activity was influenced almost exclusively by P. Seedlings supplied N_2 were more radioactive than those supplied N_1, but, on the average, P contributed more to ^{14}C incorporation than N. Seedlings grown in N_2P_2 contained the most ^{14}C.

Interpretation of the affects of N and P on fixation of ^{14}C in organic and amino acids is confounded by presence of, what appears to be, glutamic acid in both acid fractions. To facilitate interpretation, the presumed contaminant in the organic acid fraction was considered to be α-iminoglutarate, the oxidized precursor of glutamic acid. This was possibly true as no α-iminoglutarate was used as a standard for identification. Organic acids from seedlings supplied P_2 and N_1 contained the most ^{14}C. Here, as in ethanol extract and sugars, incorporation of ^{14}C appears to be influenced more by P than N. Highest radioactivity was recorded in the N_1P_2 treatment combination.

In amino acids, as in the other fractions, ^{14}C fixation was influenced more by the level of P than N. However, the difference is much less pronounced in amino acids. The apparent increase in importance of N on carbon fixation is not interpreted as indicating an extraordinary relationship between ^{14}C incorporation and N but rather an expression of the requirement for N in amino acid synthesis via amination of ketoacids

utilizing carbon derivatives. Radioactivity was highest in P_1 and N_2 treatments and in the N_2P_1 fertilizer regime.

P appears to be a rate-limiting element in photosynthetic incorporation of carbon in sand pine grown on acid-washed sand. It had its most pronounced affects on the fixation of carbon in sugars, the principal assimilate and translocate in sand pine, and presumably, in some organic acid precursors of amino acid synthesis.

A N-P ratio of 1:2 produced the heaviest seedlings, a low green-nongreen tissue ratio, and the highest level of photoassimilated ^{14}C in ethanol extract and sugars. Except for the green-nongreen tissue ratio, the most beneficial combination of N and P included the high level of N (75.0 ppm) and the high level of P (150.0 ppm), i.e., the N_2P_2 nutrient regime recommended by R. H. Brendemuehl (personal communication). The green-nongreen tissue ratio was most nearly balanced using the same ratio of N to P. The amount of radioactivity in the amino acid fraction of green tissue extract was slightly higher for the N_2P_1 than the N_1P_1 regime. In organic acids, N_2P_2 was second best to N_1P_2.

These data suggest that, of the treatments used, the ratio of one part N to 2 parts P is best for sand pine seedlings on infertile sands. Furthermore, they suggest that procedures involving assimilation of labelled compounds can be used for rapid determination of optimum nutrient regimes involving more than just two elements.

Biochemical Pathways Involved

The highest proportion of photoassimilated ^{14}C was contained in the sugar fraction of the ethanol extract from green tissue. Fructose, glucose, and to a lesser extent galactose, contained virtually all the labelled carbon in sugars. Organic acids contained some ^{14}C principally as malic acid. Virtually no activity was contained in the amino acid fraction, but the little there was, was identified as components leading to, and involved in, the urea cycle.

Sugars constitute the major photosynthates leading to formation of organic and amino acids and eventually to protein synthesis. These data suggested that the glycolytic pathway, Calvin cycle, and the tricarboxylic acid cycle were mechanisms for interconversion of these compounds. Furthermore, they suggested that activity of key enzymes along these biochemical pathways might provide an index of superior sand pine tree growth.

Objectives of the final phase of the study were to determine: 1) whether differences exist in the isoenzymes of glucose-6-phosphate dehydrogenase and malate dehydrogenase and in proteins of superior and nonsuperior sand pine trees and their half-sibling, seedling progeny, and 2) if the soils on which seedlings were grown altered their morphology or the migration rate and activity of isoenzymes and protein bands.

Seeds collected from parent superior and nonsuperior sand pines were planted in Lakeland and Paola sands. Germination, number of cotyledons, and foliar color were recorded.

The protein extract from green tissue of parent plants and half-siblings was separated by gel electrophoresis then stained

to develop isoenzymes of malate dehydrogenase, glucose-6-phosphate dehydrogenase and protein bands. Stained gels were scanned and measurements were made of the number and Rf value of bands and the intensity of densitometer tracings.

Measurement of glucose-6-phosphate dehydrogenase isoenzymes was limited to the 3 bands near the center of the gels. This limitation was caused by degredation products (Bakay and Nyhan, 1969) and "nothing" dehydrogenase that produced false bands; and, protein sulfhydryl groups (Dietz and Lubrano, 1967) and lightly stained, unresolved proteins (Hall et al., 1969) that caused a foggy, colored background at both extremes of the gels. Bakay and Nyhan interpreted the 3 bands as subresolved glucose-6-phosphate dehydrogenase isoenzyme and identified them, by rate of migration, as fast, medium, and slow components. They were treated as 3 distinct isoenzymes.

Quantified data were subjected to analysis of variance; selected orthogonal and single-degree-of-freedom comparisons were made of soil and tree treatment main effects and the soil x tree interaction.

Isoenzymes and proteins bands are separate entities. Any one can act as a marker indicating genetic variation between superior and nonsuperior trees. The Rf and activity of three glucose-6-phosphate dehydrogenase and six malate dehydrogenase isoenzymes and the Rf and staining intensity of 17 protein bands were examined. To obviate repeated qualification to differentiate between isoenzyme activity and protein band staining intensity, the term "activity" is used hereafter with both isoenzymes and protein bands.

To be meaningful, consistent differences have to exist in tissue of superior and nonsuperior trees growing on the same soil. Two individual comparisons differed statistically, but examination of the data showed that neither was consistent for all nonsuperior trees. For example, average Rf's for glucose-6-phosphate dehydrogenase isoenzymes 1, 2, and 3, were significantly higher in superior trees than in nonsuperior trees labelled D, but not in nonsuperior trees A, B, C, and E. Statistically, no meaningful differences were found among parent trees or among their progeny for these isoenzymes. None of the isoenzymes studied here provided an index of superiority.

The only indication of a possible genetic marker was discovered in the raw data (Appendix Tables 9, 10, and 11). No protein band existed between Rf 0.29 and 0.39 in superior parent trees; other parent trees contained at least one band within this range. The marker was absent among siblings.

The Rf and activity of some isoenzymes and protein bands are affected by the soil in which trees grow as well as by genetic factors. In some instances, both the band and the controlling influence can be identified. Two orthogonal comparisons were made among soils irrespective of superiority of tissue source. The first compared results obtained from Lakeland (L) with that from Paola (P) sand. Half-sibling seedling tissue was used. Since tissues were similar, the comparison was between soils developed under thermic (L) and hyperthermic (P) conditions. The natural range of Ocala sand pine is limited almost exclusively to hyperthermic soils.

In this comparison the significant response obtained can be attributed to soil.

The second comparison among soils measured the response from half-sibling seedlings grown on thermic and hyperthermic sand (L+P) with that from parent trees growing *in situ* (O, for original soils) on hyperthermic sands. The significant response obtained can be attributed to genetic factors, half-sibling vs parent, or to age, seedling vs grown tree, as well as to the soil. Differences in age were discounted, however, because only green tissue from the current year's growth was used and because the tissue served an identical function involving the same enzymes and proteins in both trees and seedlings.

The Rf of malate dehydrogenase isoenzymes 2, 3, and 4 and of protein bands 12 and 16 were significantly higher for seedlings grown in L and P than for parents (O). Rf's in the L vs P comparison did not differ significantly suggesting that differences in the rate of migration of isoenzymes 2, 3, and 4 and of protein bands 12 and 16 was influenced by a combination of soil and genetic factors.

Activity of malate dehydrogenase isoenzymes 2 and 3 was significantly higher in seedlings grown in L than P, but activity in the L+P vs O comparison did not differ significantly. This suggests that their activity was influenced by the soil more than by genetic factors or else the parent-half sibling comparison would also have been significant. Activity of protein bands 7, 12, 14, 16, and 17 was significantly higher in L than P. Two of these bands, 12 and 16, plus three additional bands, 2, 9, and

15 were significantly higher in the L+P vs O comparison. This implies that activity of some protein bands, e.g., 12 and 16, was influenced by soil and genetic factors, some by soil, e.g., 7, 14, and 17, and still others by genetic factors, e.g., 2, 9, and 15.

Factors believed to have influenced the Rf or activity of specific isoenzyme and protein bands are entered under "Remarks" in Table 7 for future discussion.

The implication that the soil on which trees grew strongly influenced the Rf and activity of isoenzymes and proteins was substantiated by comparisons made of the soil x tree interaction. Interpretation of results summarized in Table 7 is facilitated by considering separately comparisons made under columns headed: Lakeland vs Paola, Paola vs Original, and L vs P+O (Lakeland vs Paola plus Original). Isoenzymes and protein bands are listed numerically in the column to the left of the comparisons according to migration rate; 1 was fastest. Consistency among superior and among nonsuperior trees was essential to identify genetic markers and to provide an index of superiority. It was also essential to substantiate differences attributable to soils, genetic factors, and soil plus genetic factors suggested by the comparisons made among soils.

The original groupings for comparisons in the soil x tree interaction were made to determine if anticipated differences in isoenzymes and proteins between superior and nonsuperior tissue could be detected in siblings grown on soils other than those on which the parents grew. Here they serve to test the validity of inferences suggested in the soils comparison.

Table 7.--Comparisons of the soil x tree interaction showing the probability of a chance occurrence and the soil on which the highest values were obtained

		LAKELAND (L) versus PAOLA (P)							PAOLA vs ORIGINAL (O)		L vs P+O	
		TREE(S)										
Item	Band No.	A	B	C	D	E	Super	All but Super	All	Super	Super	Remarks
								MALATE DEHYDROGENASE				
Rf	2								**(P)			Soil & Genetics
	3							*(L)	**(P)			Soil & Genetics
	4								**(P)	*(L)		Soil & Genetics
	5							**(L)				
	6	*[1]					*[1]	**[1]				
Activ.	1				*(L)				*(O)			
	2	**(L)	**(L)	**(L)			**(L)	**(L)			*(L)	Soil
	3	*(L)	**(L)	*(L)		**(L)	*(L)	**(L)			*(L)	Soil
	4	*(L)	**(L)	**(L)		**(L)	**(L)	**(L)			*(L)	
	5		*(L)			**(L)	**(L)	**(L)	**(O)	**(O)		
	6					*[1]	**[1]	**[1]	**[1]		*[1]	
No. of Isoenzymes							*(L)	**(L)				

[1]Significance is due to lack of this isoenzymes in A, E, and superior trees growing on Paola sand.

* = 5% and ** = 1% probability

Table 7.--Continued

		LAKELAND (L) versus PAOLA (P)							PAOLA vs ORIGINAL (O)		L vs P+O	
		TREE (S)										
Item	Band No.	A	B	C	D	E	Super	All but Super	All	Super	Super	Remarks
							GLUCOSE-6-PHOSPHATE DEHYDROGENASE					
RF	1								*(O)			
	2						*(L)	*(L)				
							PROTEIN					
RF	1							*(L)				
	2							**(P)				
	3					*(L)		**(L)	**(O)			
	4		*(L)					*(L)		*(P)		
	5			*(L)					**(O)	*(O)		
	6								*(O)			
	7								**(O)			
	8								**(P)			
	9			*(L)								
	12							**(P)	**(P)	**(P)		Soil & Genetics
	13									*(P)		
	14									**(P)		
	15						*(L)	**(L)	*(P)		*(L)	
	16								**(P)	**(P)		Soil & Genetics

Table 7.--Continued

Item	Band No.	A	B	C	D	E	Super	All But Super	All	Super	Super	Remarks
		LAKELAND (L) versus PAOLA (P)							PAOLA vs ORIGINAL (O)		L vs P+O	
		TREE (S)										
								PROTEIN				
Activ.	2								**(P)	*(P)	*(L)	Genetics
	4		**(L)					*(L)	*(P)		*(L)	
	5			*(L)				*(L)				
	6		*(L)				*(L)		**(L)			
	7						**(L)	**(L)			*(L)	Soil
	8		**(L)	*(L)				**(L)	**(O)			
	9			*(L)	*(L)			**(L)				Genetics
	10						*(L)	*(L)				
	11		*(L)			**(L)		**(L)				
	12		**(L)	**(L)	*(L)	**(L)	**(L)	**(L)	**(P)		**(L)	Soil & Genetics
	13							**(L)			*(L)	
	14		**(L)	*(L)	*(L)	**(L)	**(L)	**(L)			**(L)	Soil
	15	*(L)	**(L)	**(L)		**(L)	**(L)	**(L)	**(P)		**(L)	Genetics
	16					**(L)	**(L)	**(L)			**(L)	Soil & Genetics
	17	*(L)	*(L)	**(L)		*(L)	*(L)	**(L)			*(L)	Soil
No. of Bands								*(L)			*(L)	

Paola vs Original comparisons were made on sandy soils from the same general area. For this reason no consistent and significant response should be expected in the Rf or activity of isoenzymes or protein bands influenced primarily by soil. A response attributable to genetic factors might be anticipated in appropriately labelled bands because the comparison is primarily between parent vs half-sibling progeny.

Table 7 shows that the labels are correct. In siblings, Rf of isoenzymes 2, 3, and 4, and of protein bands 12 and 16, and the activity of protein bands 2, 4, 8, 12, and 15, and, in parents, activity of protein band 6 were highest by a statistically significant degree. Protein bands 4, 6, and 8 were not labelled, but all the rest bore a genetic or a genetic and soil designation. Conversely, no significance was recorded for the activity of malate dehydrogenase isoenzyme 2 and 3 or protein bands 7, 14, and 17, influenced by soil, or of band 16, labelled soil and genetics. The only exception was in protein band 9 which bore a genetic label but exhibited no significant or consistent response.

A comparison of Lakeland vs Paola plus Original was made to examine differences in Rf and activity of isoenzymes and proteins attributable to "native vs non-native" soils in superior trees. Combining the data from Paola and Original soils also introduced the element of genetic makeup, half-sibling vs parent, into the comparison; thus it is not surprising that mixed results were obtained. The Rf and activity of many labelled and unlabelled isoenzymes and proteins exhibited a significant response. In

every instance the Rf and activity were highest on Lakeland sand.

Comparisons of Lakeland vs Paola examine the effect of a native and non-native soil on the Rf and activity of isoenzymes and protein bands in progeny from superior and nonsuperior trees. Individual comparisons were made for 1) superior trees, 2) nonsuperior trees collectively, and 3) nonsuperior trees bearing a common, identifying letter. Comparison 3 provides a statistical breakdown of comparison 2 but is meaningless by itself.

The Rf and activity of all significantly affected isoenzymes and most significantly affected protein bands in the 3 comparisons were higher on Lakeland than Paola sand for seedling from superior and nonsuperior parents alike. With respect to soil, genetic factors, and soil plus genetic factors, the data defy interpretation. Had the effect of soil, genetic factors, and the combined effect of soil and genetics on isoenzymes and protein bands been anticipated, provision could have been made to clarify results by use of backcrossed or control-pollinated stock or possibly rooted cuttings from parents.

It is tempting and sometimes convenient to identify protein bands with isoenzymes that react similarly to a given stimulus by direct comparison of gels. Both are protein. Gabriel and Wang (1969) successfully accomplished staining for both protein and enzyme on paired gels using triphenyltetrazolium. With nitroblue tetrazolium the practice does not appear to be reliable. Malate dehydrogenase isoenzymes 2 and 3 reacted similarly to protein bands 12 and 16 in the L vs P and L+P vs O soils comparisons,

yet the Rf's of the isoenzymes averaged 48.7 and 37.4 while those of the protein bands averaged 40.3 and 24.8. Possible application of this method was further tested by developing protein bands in gels previously stained for isoenzymes and then comparing these with gels stained either for isoenzymes or protein bands. Although all the gels had been processed simultaneously in the same electrophoresis chamber, the position of the bands did not coincide. Development of isoenzymes and proteins in paired halves of gels split lengthwise was not tried.

A clue to deciphering the consistently high response to Lakeland soil in Lakeland vs Paola plus Original and Lakeland vs Paola comparisons was sought among measurements made of seedlings prior to harvest. Seed germination was consistently higher on Paola than Lakeland sand (Figure 18). The difference might be attributed to a response to origin of the soils; it seems more probable that it reflects the influence of soil texture on moisture available for imbibition by seeds planted at the surface. Texture of Lakeland sand was coarse while that of Paola sand was medium.

No genetic variance was expected because seeds were from the same trees, and samples for planting were selected at random. A comparison of cotyledon numbers (Table 8) showed no meaningful differences in the proportional distribution between soils or between superior and nonsuperior progeny. There was no apparent difference in size of cotyledons.

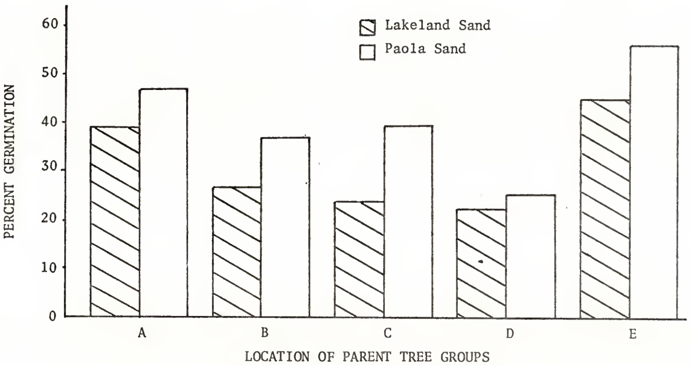

Figure 18. Seed germination from superior and nonsuperior sand pine trees at 5 locations designated A to E

Table 8.--Distribution of half-sibling seedlings possessing 3 to 7 cotyledons

PARENT TREE LOCATION	AVERAGE OF NONSUPERIOR TREES					SUPERIOR TREE				
	3	4	5	6	7	3	4	5	6	7
	Percent									
	LAKELAND SAND									
77	0	26	66	8	0	0	4	58	38	0
82	0	38	52	10	0	0	48	44	8	0
120	0	28	56	15	1	11	44	45	0	0
193	1	28	54	15	1	0	41	45	14	0
199	2	43	53	2	0	0	30	53	17	0
	PAOLA SAND									
77	0	28	61	10	1	0	7	64	29	0
82	2	30	53	15	0	0	33	59	8	0
120	1	34	55	10	0	0	45	48	7	0
193	1	23	55	21	0	0	32	61	7	0
199	2	42	49	7	0	0	13	60	27	0

Color of the foliage was fairly uniform on each soil, but it differed markedly between soils. Color differences increased with age indicating that possibly soil aeration and moisture in Lakeland coarse vs Paola medium sand, but most probably the availability of nutrients was primarily responsible. A comparison of colors was made shortly before harvest against standards in Munsell Color Charts for Plant Tissue. It showed that the foliage of seedlings grown on Lakeland sand was dark greenish-yellow, approximately 7.5 GY 4/6 to 7.5 GY 5/6, while foliage on Paola sand had a lighter hue, 2.5 GY 8/10 to 5 GY 7/8. No other differences in the foliage were observed. Some fascicled needles developed on all seedlings, and the needles were of about the same size.

These data raise several questions: 1) What characteristics of the soil differ sufficiently to alter color of seedling foliage? 2) Are these differences in any way related to the consistently high Rf and activity of isoenzymes and proteins observed in seedlings raised on Lakeland soil?

Photosynthetic rate can be as much as 3 times higher in dark green than light green leaves (Kramer and Kozlowski, 1960). A corollary that serves to explain the consistently higher activity of enzymes and proteins on Lakeland than Paola sand is that the darker leaves produced more substrate for protein synthesis because they contained more chlorophyll. Since seedlings of genetically similar origin were raised under identical conditions except for the soils, differences in chlorophyll content are ascribed to available nutrients in the two soils. The possibility exists that chlorophyll formation also could have been affected

by a deficiency or an excess of water in the soils, but, considering the slight difference in texture between the sands, and the care with which seedlings were raised, the probability that either factor played a meaningful role seems extremely remote.

Table 2 showed that Lakeland sand contained twice as much total N, considerably more Mg, and more K than Paola sand. N and Mg are constituents of chlorophyll; a deficiency of either causes chlorosis. The amount of N in soil is a major determinant of leaf protein (Wynd and Noggle, 1945). In the plant, it is an element essential for amino acid and protein synthesis. Conceivably, the divergence in N between Lakeland and Paola sand could have modified the amino acid and enzyme components of sand pine seedlings growing on them in a manner similar to that reported in slash pine seedling tissue following addition of N (Stanley and Smith, 1970), i.e., by altering isoenzyme patterns. In this respect differences in available Mg could also have had an effect. The Mg ion is also a cofactor in many biochemical reactions including conversion of glucose-6-phosphate to β-lactone catalyzed by glucose-6-phosphate dehydrogenase (Conn and Stumpf, 1964), and influences the reactivity of at least one glucose-6-phosphate dehydrogenase isoenzyme in some animals (Hori and Matsui, 1967; Hori, Tsutomu, and Matsui, 1967). Aside from the difference in foliar color, no apparent symptoms of N or Mg deficiency were observed.

The K requirement of most plants is high. Bollard (1955) reported that dried leaves of healthy plants contain about 15,000

ppm. Brendemuehl (unpublished data) found that dried needles of Choctawhatchee sand pine seedlings grown in a Lakeland sand with 14 ppm available K contained 6,560 ppm K. So, although K accumulates in needles of sand pine seedlings against a concentration gradient, the relatively low level of available K in the Lakeland and Paola sands of this study suggests that the sand pine seedlings on both soils grew at suboptimum, but not necessarily deficient, levels of K. Deficiency symptoms such as those characterized for foliage of white pine (Hacskaylo, 1962), loblolly pine and Virginia pine (Sucoff, 1961) seedlings were not apparent.

Lakeland sand contained slightly less available Ca, 1/4 as much available P, and 10 times the Al of Paola sand. Sucoff reported no deficiency symptoms in loblolly pines supplied with 0.8 ppm Ca nor in Virginia pines raised with as little as 0.2 ppm Ca. It seems unlikely, therefore, that the supply of Ca was limiting in either Paola (44 ppm) or Lakeland (30 ppm) sand. No deficiency symptoms were evident.

As in most acid sandhill soils, P availability was low. This was especially true in the Lakeland sand, pH 5.6, where the presence of a comparatively high concentration of Al, 50.5 ppm, suggests that P was fixed as the insoluble hydroxy-phosphate of Al. Although the threshold concentration for P deficiency in Ocala sand pine seedlings is unknown for sandy soils, the data suggest that it may lie between concentrations found on Lakeland and Paola sand. Seedlings on Lakeland sand, 0.35 ppm P, evidenced the dark green color characteristic of a P deficiency (Bonner and Galston, 1952) while those on Paola sand, 1.45 ppm P, did not.

The relatively dark green color of seedlings grown on Lakeland vs Paola soil may be attributed to a P deficiency and to a greater availability of N and Mg. Because seedlings were destroyed during preparation of acetone powders for protein extraction, foliar analyses could not be made to corroborate suppositions based upon foliar color and the soils' available nutrient content. If plants were deficient in P, apparent conflict exists.

P is essential in plant metabolism. Its high-energy bonds provide a mechanism for storage and energy transfer (Arnon, 1953). How, then, can plants growing in soil containing only 0.35 ppm of available P and signalling a P deficiency symptom exhibit high protein and enzyme activity levels? Lacking corroborative plant tissue analyses, the answer can only be conjectural. Two possible explanations follow.

The rate of P absorption reaches its maximum early in the growth cycle; at a time when the amount absorbed per unit of growth is higher than at later stages of growth (Dean and Fried, 1953). In this study young seedlings were used. They could have absorbed enough P during their first month or so of existence to sustain a normal metabolic rate for about a month. In the interim, natural recycling of P and translocation from relatively old to meristematic tissue could sustain sufficient sources of high energy compounds to provide the increased protein synthesis ascribed to comparatively high levels of available N and Mg found in Lakeland sand.

Plants react differently to a P deficiency. Eaton (1949, 1950, and 1952) raised sunflowers, soybeans, and black mustard plants in sand-solution culture with and without P. Analyses of stem tissue extracts showed an accumulation of carbohydrates in all plants but a significant increase in water-soluble N, nitrate, ammonia, amino, and amide, only occurred in soybean. Accumulation of carbohydrates and nitrogen is symptomatic of P deficiency and was explained by interference with a) protein synthesis at the nitrate-reduction stage or b) protein synthesis at the amide stage.

Sunflowers were grown for 64 days and, according to Eaton, would have eventually matured under P-deficiency conditions, presumably because they were able to utilize P of complex organic compounds. The sand pines in this study seemed to react similarly to sunflowers. Under field conditions both varieties of sand pine grow to maturity on P-deficient soils. If protein synthesis was not blocked by a P deficiency in the period of growth studied, then differences in levels of N and Mg could account for the consistently higher protein and enzyme activity levels found in seedlings on Lakeland than on Paola sand.

A solution to the apparent dilemma offers opportunity for further investigation into the differenctial uptake of available nutrients under stress conditions imposed by deficiency of one or more nutrients, as related to the affect of soil and plant nutrients on protein production and enzyme activity. The continued quest for a genetic marker and index of superior growth using isoenzymes affords opportunities for future investigations.

The physiological response of superior tree selection to soils and soil nutreint levels other than those found in the parent habitat also offers opportunities for future research.

SUMMARY

This was a study of sand pine growth on sandhill soils. It included measurements of the affect of nutrient levels on fixation of photoassimilated ^{14}C and comparisons of isoenzyme and protein migration rates and activity between superior and nonsuperior trees and between their half-sibling, seedling progeny grown on Lakeland and Paola sands.

1. Photoassimilation of $^{14}CO_2$ in a closed system and at steady-state conditions is directly related to length of exposure.
2. Translocation of ^{14}C-labelled photosynthate from green seedling tissue was negligible for photoassimilation periods of 10 min or less. Thereafter, the proportion of labelled compounds remaining in green tissue decreased in relation to nongreen tissue.
3. Young Ocala sand pine seedlings did not incorporate $^{14}CO_2$ in the dark.
4. Chlorophyll in ethanol extracts decreased the efficiency and the accuracy of liquid scintillation measurements. Both were improved by use of quenching curves and clarification of the extract with powdered activated charcoal. Quenching curves were unique for each scintillation counter.
5. The ethanol-soluble fraction of green tissue contained most of the ^{14}C photoassimilated within 10 min. Sugars

contained 75%, organic acids 19%, and amino acids 6% of the ^{14}C in this fraction.

6. N treatments seemed to affect chlorophyll formation, seedling growth, and the photosynthetic incorporation of ^{14}C.
7. P appeared to be a rate-limiting element in the photosynthetic incorporation of C in sugars and in some organic acid precursors of amino acids.
8. A N-P ratio of 1:2 in complete nutrient solution produced seedlings with a high ratio of nongreen to green tissue and resulted in a high level of ^{14}C incorporation in sugars.
9. The activity and Rf values of malate dehydrogenase and glucose-6-phosphate dehydrogenase isoenzymes did not provide an index for rapid growth in superior Ocala sand pine tree selections or in their seedling progeny.
10. Absence of protein bands between Rf 0.29 and 0.39 in superior trees was the only indication of a genetic difference between superior and nonsuperior parent trees. This genetic marker was not found among half-sibling, seedling progeny.
11. The activity and Rf of some malate dehydrogenase isoenzyme and protein bands were affected by the soil on which seedlings were grown as well as by genetic factors. Both the bands and the factors influencing them were identified.

12. Procedures developed in this study may be applicable to research seeking to detect genetic aberrations in trees exhibiting a superior growth rate and to the development of optimum levels of tree nutrition.

APPENDIX

Table 9.--Protein Rf measurements taken from half-sibling seedlings grown on Lakeland coarse sand

Band	TREE					
	A	B	C	D	E	Super.
	Rf x 100					
1	97	95	97	95	95	96
	97	97	98	98	97	97
	96	93	92	91	92	92
	97	98	97	00	98	98
	99	99	93	96	98	98
2	00	00	00	00	00	91
	92	94	93	94	93	93
	90	88	87	86	87	87
	94	94	94	00	93	94
	93	93	00	00	92	92
3	88	88	88	87	86	00
	86	87	86	87	86	86
	83	82	81	79	81	81
	87	88	87	00	88	87
	83	83	83	87	83	82
4	82	81	82	81	81	81
	82	85	84	84	83	83
	80	79	77	76	78	78
	84	85	84	00	84	84
	78	79	79	82	78	78
5	00	00	00	00	00	00
	78	81	80	78	77	78
	00	00	74	73	74	75
	81	81	80	00	00	80
	73	73	72	76	72	72
6	00	00	00	00	00	00
	74	78	78	75	74	76
	74	73	00	00	00	72
	79	79	78	00	78	78
	70	70	70	73	69	69
7	00	00	00	00	00	00
	70	76	75	71	71	72
	71	71	69	69	69	70
	77	76	76	00	76	76
	67	67	00	68	64	64

Table 9.--Continued

Band	TREE A	B	C	D	E	Super.
	Rf x 100					
8	69	69	70	69	69	69
	00	72	72	70	66	00
	69	67	66	64	66	67
	73	73	73	00	72	72
	63	63	63	66	62	62
9	00	64	65	64	00	00
	66	68	67	67	64	66
	00	63	61	58	62	62
	68	69	68	00	00	68
	60	60	60	63	59	59
10	00	00	00	62	64	63
	60	62	61	61	61	62
	62	61	59	56	60	60
	66	66	66	00	66	66
	54	54	54	60	53	54
11	00	00	00	00	00	00
	56	58	57	57	57	00
	58	57	56	53	57	57
	63	63	61	00	61	62
	52	52	51	53	50	49
12	00	00	00	00	00	55
	52	54	53	53	52	55
	51	50	49	47	50	49
	56	55	55	00	55	55
	45	45	45	47	44	44
13	54	54	55	44	34	34
	46	48	49	49	49	51
	00	00	00	00	00	45
	51	49	50	00	51	51
	38	39	38	41	38	38
14	43	43	44	43	43	43
	00	44	47	47	47	49
	43	43	41	40	42	41
	49	48	47	00	48	47
	00	36	35	37	34	35

Table 9.--Continued

Band	TREE A	B	C	D	E	Super.
	-----------------Rf x 100					------------------
15	40	40	41	40	39	40
	38	41	40	41	41	42
	36	36	34	32	35	35
	41	41	40	00	40	40
	32	32	32	34	31	31
16	00	00	35	36	35	35
	32	33	33	34	34	36
	28	29	28	26	28	28
	34	33	32	00	33	34
	26	28	27	30	26	26
17	00	00	27	26	24	25
	26	27	26	28	27	31
	21	21	20	18	19	19
	24	25	23	00	24	24
	17	18	19	20	18	18

Table 10.--Protein Rf measurements taken from half-sibling seedlings grown on Paola sand

Band	TREE					
	A	B	C	D	E	Super.
	Rf x 100					
1	95	98	98	98	98	98
	97	97	97	98	98	97
	93	92	92	92	93	00
	97	97	99	00	97	97
	00	00	00	00	98	98
2	88	91	89	89	90	92
	93	92	92	97	93	93
	88	87	87	86	88	89
	93	95	93	00	91	92
	92	92	92	93	90	90
3	00	84	00	00	00	87
	86	86	82	82	00	87
	82	81	81	82	81	83
	86	87	86	00	85	86
	83	82	82	83	88	88
4	82	00	84	84	83	83
	83	82	75	86	84	83
	00	00	79	00	78	81
	83	82	82	00	82	83
	78	78	77	78	81	85
5	00	00	00	00	00	00
	76	77	00	83	77	00
	78	77	78	78	00	79
	00	00	00	00	00	00
	72	73	72	73	80	80
6	00	00	00	00	00	00
	74	75	71	77	72	76
	00	00	00	00	74	00
	77	78	78	00	77	78
	70	70	69	70	78	77

Table 10.--Continued

Band	TREE A	B	C	D	E	Super.
	-----Rf x 100-----					
7	00	00	00	00	00	00
	71	71	00	00	00	00
	74	72	00	75	71	00
	76	76	76	00	75	76
	00	65	64	65	73	73
8	71	72	71	71	72	71
	00	00	00	76	65	65
	72	70	71	71	00	72
	72	72	73	00	71	72
	64	63	62	64	61	61
9	00	65	00	00	00	00
	66	64	64	65	62	61
	67	66	66	67	67	62
	00	00	00	00	00	00
	60	60	59	61	69	68
10	64	00	63	63	64	64
	61	61	61	00	00	00
	61	59	60	00	61	60
	00	65	66	00	66	65
	54	55	54	55	62	57
11	00	00	00	00	00	00
	57	00	57	62	00	00
	58	55	56	58	58	58
	62	62	62	00	61	62
	51	51	50	52	58	00
12	58	59	60	60	58	58
	52	54	54	61	55	55
	53	51	52	54	53	54
	57	57	57	00	57	57
	46	48	45	47	54	53

Table 10.--Continued

Band	TREE					
	A	B	C	D	E	Super.
	-----------------Rf x 100--------------------					
13	55	56	55	55	55	55
	48	47	47	56	48	00
	00	48	49	50	51	53
	54	55	00	00	00	53
	00	45	00	00	52	50
14	44	52	49	49	44	45
	00	00	00	00	00	00
	00	00	42	45	44	51
	57	48	48	00	47	46
	39	35	37	40	46	45
15	00	45	44	00	00	00
	40	40	41	00	41	48
	32	33	34	41	36	00
	39	00	00	00	00	00
	31	31	30	33	36	36
16	33	34	33	44	00	34
	32	00	34	49	34	42
	29	25	28	37	29	38
	33	40	40	00	40	41
	26	27	26	29	00	00
17	24	00	27	00	26	23
	00	26	00	43	00	33
	00	00	00	00	00	00
	27	33	34	00	32	32
	18	19	22	21	28	00

Table 11.--Protein Rf measurements taken from parent trees growing at their original locations.

Band	TREE					
	A	B	C	D	E	Super.
	Rf x 100					
1	96	97	98	97	97	96
	00	98	98	98	97	97
	96	98	97	95	95	97
	96	98	98	98	98	98
	00	00	94	00	00	00
2	00	93	93	95	93	93
	00	92	95	94	91	92
	94	93	95	94	00	94
	90	91	91	91	90	91
	95	00	93	00	95	00
3	86	87	88	88	87	90
	90	88	89	89	86	87
	90	90	91	91	90	92
	86	88	88	87	87	88
	91	90	90	89	90	00
4	81	83	83	85	82	84
	86	83	84	84	82	82
	00	85	86	85	85	85
	00	00	00	00	00	00
	81	83	81	80	82	00
5	75	74	75	83	75	76
	79	80	75	76	73	76
	80	81	80	81	81	83
	81	83	82	82	82	83
	76	76	00	76	76	00
6	68	68	69	77	68	71
	71	69	70	71	68	66
	76	77	77	77	79	77
	00	00	00	00	00	00
	71	68	69	70	70	00

Table 11.--Continued

Band	A	B	C	D	E	Super.
	TREE					
	----------------RF x 100----------------					
7	00	64	64	74	00	65
	68	64	66	68	66	66
	69	69	69	69	69	69
	73	77	74	74	76	74
	63	63	00	63	64	00
8	60	00	59	69	00	00
	66	58	63	66	63	64
	00	64	64	67	00	68
	68	69	69	68	69	69
	00	00	00	00	00	00
9	56	57	56	61	61	00
	61	55	58	61	59	57
	63	62	61	63	64	64
	00	67	67	66	67	68
	00	00	00	00	00	00
10	52	52	51	58	58	58
	52	51	52	53	50	52
	61	61	59	58	59	60
	62	64	64	63	66	65
	00	00	00	00	00	00
11	48	48	48	54	00	54
	49	48	48	50	47	00
	59	60	56	57	00	59
	57	58	58	59	57	58
	00	00	00	58	00	00
12	00	00	46	49	49	49
	43	41	42	44	41	42
	54	54	53	54	00	56
	00	00	00	57	00	00
	00	00	00	54	00	00

Table 11.--Continued

Band	TREE A	B	C	D	E	Super.
	-----RF x 100-----					
13	41	42	42	42	42	00
	39	37	38	40	37	39
	50	47	50	53	00	00
	47	48	48	47	48	49
	00	00	00	47	00	00
14	00	37	39	38	37	00
	36	34	35	36	34	00
	45	45	44	52	52	00
	44	46	46	44	45	46
	44	00	00	45	00	00
15	00	35	00	00	00	00
	00	29	31	00	00	00
	36	38	38	49	00	00
	38	37	43	38	38	41
	00	00	00	00	00	00
16	00	26	00	00	00	00
	28	27	28	29	26	00
	35	35	34	45	46	00
	29	29	37	22	30	29
	00	00	00	00	00	00
17	00	21	28	00	00	00
	21	20	21	23	20	22
	32	32	31	38	39	00
	22	23	00	00	23	23
	31	00	00	00	00	00

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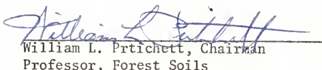
BIOGRAPHICAL SKETCH

Russell MacBain Burns was born August 25, 1926, at New York, New York. In June, 1944, he was graduated from Haaren High School. From 1944 until 1946 he served in the Infantry of the United States Army in Europe. Following his discharge from the Army, he attended the Associated Colleges of Upper New York and Michigan State University and, in 1950, received his degree of Bachelor of Science with a major in Forest Management. He worked for the Southern Forest Experiment Station of the U.S. Forest Service throughout the South, and while stationed at Oxford, Mississippi, attended the University of Mississippi. In 1959, he received the degree of Master of Science with a major in Biology and a minor in Mycology. In 1966 he was selected by the Forest Service for advanced training under the Government Employees Training Act at the University of Florida. The Ph.D. degree was received in August 1971, with a major in Soils and a minor in Forest Physiology.

Russell MacBain Burns is married to the former Mildred Ann Nastasia and is the father of three children, Stephen, John, and Russell. Memberships are held in Sigma Xi, The American Society of Plant Physiologists, Soil Conservation Society of America, Society of American Foresters, and the Florida Academy of Science.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

August, 1971



William L. Prtichett, Chairman
Professor, Forest Soils

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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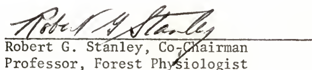

Robert G. Stanley, Co-Chairman
Professor, Forest Physiologist

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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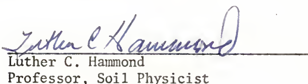

Luther C. Hammond
Professor, Soil Physicist

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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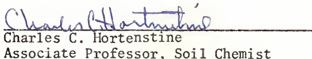

Charles C. Hortenstine
Associate Professor, Soil Chemist

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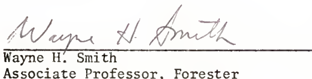

Wayne H. Smith
Associate Professor, Forester

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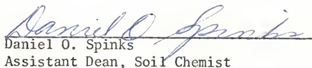

Daniel O. Spinks
Assistant Dean, Soil Chemist

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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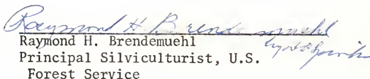
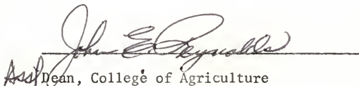


Raymond H. Brendemuehl
Principal Silviculturist, U.S. Forest Service

This dissertation was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1971



Asst Dean, College of Agriculture

Dean, Graduate School