AN ABSTRACT OF THE THESIS OF

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Abstract approved:
Signature redacted for privacy.
Joe B. Zaerr

The physiology of progressively drought-stressed ponderosa pine (Pinus ponderosa Dougl. ex Laws.) var. scopulorum and var. ponderosa was characterized in seedlings unwatered for about a month. Additional seedlings of var. scopulorum were shaded to 10% full light and compared to unshaded seedlings to determine the influence of light starvation on drought-stress tolerance. Pressure-volume and carbohydrate analyses indicated that needles of light starved seedlings had significantly lower symplastic osmotic concentrations, cell-wall capacitance, apoplastic water content, cellulose content, and higher cell volume-to mass ratios. The light-starved seedlings demonstrated reduced drought-stress tolerance by low recovery rates and loss of structural integrity at relative water contents below 60%.

A method was developed for the analysis of free amino acids extracted from the needles using

derivatization with dansyl chloride and high performance liquid chromatography. Concentrations of most amino acids increased in light-starved seedlings. Arginine and proline accumulated to highest concentrations in all drought-stressed seedlings; in unstressed, light-starved seedlings arginine and glutamine accumulated most, indicating the sequestering and storage of free ammonia is important under stress conditions.

Characterization of protein synthesis was by in vivo labeling with [^{3}H]leucine, one and two dimensional electrophoresis and fluorography. Drought stress induced or enhanced the synthesis of specific low M_r proteins. Western blots were used to identify putative 70-80 kDa heat shock proteins. Antibody against a 26 kDa osmotic stress-induced protein cross-reacted to antigen in salt-stressed, but not in drought-stressed seedlings.

Metabolic changes induced by severe drought stress, including synthesis of new proteins and increases in arginine and proline, were altered by light starvation which also reduced drought tolerance. Unshaded seedlings of both varieties demonstrated their drought tolerance in surviving water potentials as negative as -4.0 MPa and RWC below 65%. Physiological differences between the two varieties may have been due to differences in needle type, ie. the early development of secondary needles in var. scopulorum.

Physiology of Drought Stress in *Pinus Ponderosa* (Dougl. *ex* Laws.) and the Influence of Low Irradiance

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Nan C. Vance

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Professor of Forest Science in charge of major

Signature redacted for privacy.

Head of Department of Forest Science

Signature redacted for privacy.

Dean of Graduate School

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DEDICATION

This dissertation is dedicated to my mother
whose selfless devotion to her task
inspired me to persevere
with mine.

CONTRIBUTION OF AUTHORS

Dr. J. B. Zaerr supervised the research project in the capacity of major professor. He supervised and contributed his expertise to the initial study, the experiments and the writing of the manuscripts.

Dr. D. O. Copes supervised much of the laboratory work, contributed to the research strategy and provided the laboratory for the analysis of proteins. Dr. Copes was responsible for USDA Forest Service Grant No. PNW86-396, a major part of the funding of this project.

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INTRODUCTION

Background

Ponderosa pine (*Pinus ponderosa* Dougl. *ex* Laws.) is native to arid regions of the West where drought may last two months or more. Its establishment and survival depend on characteristics developed early in the seedling stage which enable it to tolerate, or acclimate to lack of available water and to high evaporative demand (Vance and Running 1985).

Ponderosa pine seedlings exhibit a remarkable ability to endure high evaporative demand and prolonged drought (Vance and Running 1985). They have survived drought as long as 100 days and water potentials as negative as -5.0 MPa (P. Heidmann, personal communication, 1986), yet the mechanisms that enable this species to tolerate extreme water deficit are not understood.

Drought tolerance is based on the ability of the plant to postpone, or tolerate tissue dehydration (Kramer

1980). Seedlings of different coniferous species (Heth and Kramer 1975) and of different populations within a species (Pharis and Ferrell 1966) can vary in drought tolerance; the cause, whether due to differences in ontogeny, genotype or physiology, remains obscure. In ponderosa pine, genetic traits associated with drought tolerance generally have been readily identifiable, morphological characteristics, such as extensive, deep rooting systems, which involve multi-gene control (Van Buijtenen 1976). There is little knowledge of the physiological traits associated with drought tolerance in coniferous trees other than the occurrence of stomatal control of moisture loss, osmotic adjustment and the tenuous involvement of abscisic acid (Blake and Ferrell 1977). Furthermore, none of these traits is associated with dehydration tolerance, but rather, dehydration postponement. It is not known if different traits are under coordinated control. For example, trees of some coniferous species actively postpone dehydration by early stomatal closure, yet appear to be less capable of tolerating dehydration when the limit of this delaying mechanism has been exceeded (Brix 1979); conversely, some drought adapted ecotypes seem to exhibit an earlier and more sensitive stomatal response to drought stress "signals" (F. Sorenson, 1986, personal communication).

Squillace and Silen (1962) examined the genetic variation in ponderosa pine in which seed for the study was collected from widely separated seed sources. The natural range of ponderosa pine includes regions encompassing the Cascades and North Plateau; the Intermountain area and Northern and Central Plains; and the Central and Southern Rockies. The study revealed that differences in growth and needle morphology were correlated with geographic and climatic variables. The authors suggested that climate favors natural selection for rapid growth in the northwestern portion of the range, but that slower growth in the southeastern part of the range may be linked to resistance to extreme climatic conditions. Read (1980) delineated the genetic variation in ponderosa pine using 13 traits as a basis for grouping the 80 seed origins covering the range of this species. The greatest genetic difference was determined to be between the two varieties, var. ponderosa and var. scopulorum, which divide the range into distinct regions. P. ponderosa var. ponderosa originates primarily in the Far West and Northwest (North Plateau) and P. ponderosa var. scopulorum, the eastern regions including the Rockies and Great Plains. Read's study supported several conclusions of the earlier study of Squillace and Silen (1962) which suggested greater frost hardiness in the variety scopulorum. However, genetic variation in traits

associated with drought resistance or tolerance was not demonstrated and remains speculative.

Drought Stress and Plant-Water Status

Dehydration tolerance is the least understood component of drought tolerance, but may be a critical factor in plant adaptivity, since early seedling survival may depend on this attribute. Martin et al. (1987) in indexing the dehydration tolerance of six woody tree species suggested that dehydration tolerance, while not related to rooting depth, a dehydration avoiding adaptation, was related to species distribution. This suggests that the mechanisms of dehydration tolerance should be identified and evaluated in the context of the total adaptability of the plant to drought.

Since the pressure chamber was adapted to measuring water potential (Ψ_x) of xylem sap in forest trees (Cleary and Zaerr 1980, Zaerr 1982), there has been wide acceptance of it as a useful measure of the plant's water status. Ψ_x is less useful when attempts are made to relate it to physiological processes (Newville and Ferrell 1980). Most studies of drought-induced water stress in conifers measure the performance of whole plants, indexed by Ψ_x (Morris and Zaerr 1978, Vance and Running 1985). While useful, this measure of performance

fails to account for the variation in drought tolerance or survival at any particular level of water potential (Pharis and Ferrell 1979, Vance and Running 1984). Although the thermodynamic status of water and physiological processes have been related, eg., reduction in turgor potential and abscisic acid accumulation (Blake and Ferrell 1977), no well-defined transducers to translate the thermodynamic state of water into physiological performance in plants has been demonstrated (Oertli 1976). Cell volume, or more commonly relative water content (RWC), is an important, and possibly a major determinant of metabolic activity and leaf survival (Sinclair and Ludlow 1985). Pressure volume analyses demonstrate that water potential and RWC are not linearly related, and that drought episodes may alter the relationship (Joly and Zaerr 1987). Flower and Ludlow (1986) suggested that leaf water potential has a limited role in determining plant survival under extreme water stress (in the context of this study, water stress is stress caused by drought). Jarvis and Jarvis (1963) found that among four water-stressed tree species, seedlings of each species died at a narrow range of RWC, whereas leaf water potential varied considerably. Within a species RWC was used to determine variation in drought resistance among seedlings of Douglas fir (Pseudotsuga menziesii (Mirb.) Franco) from different sources (Pharis

and Ferrell 1966). Variation has been demonstrated among other coniferous species and among populations within species in the cellular water content that is lethal (Heth and Kramer 1975, Van Buijtenen 1976, Brix 1978), but the variation has not been explained on a physiological basis.

Water Stress and Metabolism

Proline is one of a number of compatible solutes (amino acids and amino acid derivatives, polyamines, organic acids and sugars or sugar alcohols) which accumulate to high cellular levels in response to drought or osmotic stress (Jones et al. 1979, Yancey et al. 1982). Proline accumulation has received the greatest attention, because it accumulates to large concentrations in a wide variety of plants and has been shown to contribute to drought tolerance (Munns et al. 1979, Kapuya et al. 1985). Cyr (1983) demonstrated that in three water-stressed coniferous species, proline, alanine, glycine, hydroxyproline, and aspartic and glutamic acids accumulated as osmotic potential declined. Cyr suggested that in conifers the amino acid pool may also play a significant role in osmotic adjustment.

There is a substantial body of evidence supporting the hypothesis that the accumulation of proline may

protect plant cells from dehydration, and aid in recovery once water stress is relieved (reviewed by Aspinall and Paleg 1981); however, an adaptive role for its metabolism in drought stressed plants is disputed (Stewart and Hanson 1980. Stewart and Lee (1974) suggested that the high accumulation of proline functions as a source of solute for intra-cellular osmotic adjustment. Barnett and Naylor (1966) suggested that proline may function temporarily as a C-, N- and energy-storage compound. Studies demonstrating proline to be rapidly oxidized upon relief of stress support the energy-storage function of proline (Blum and Ebercon 1976, Jager and Meyer 1977). Stewart 1972 demonstrated that oxidation of proline was especially rapid when water stressed was relieved in plants having low carbohydrate status. While the accumulation of proline has been correlated with drought resistance in crop plant species (Singh et al.1972, 1973), the adaptive significance of proline accumulation was disputed on the grounds that proline had no apparent role in preventing the decrease in water potential (Hanson and Hitz 1982). The role of increased proline concentration in plant cells' ability to delay or tolerate dehydration awaits clarification.

Although proline has been the most studied amino acid, other amino acids may predominate under stress or other environmental conditions. Malhotra and Sarkar

(1979) reported that SO_2-treated jack pine seedlings accumulated alanine, valine, glycine, isoleucine, leucine, threonine, aspartic acid, tyrosine, lysine and arginine. Sulfur deficient trees were found to accumulate arginine which was correlated to a needle fungus infection (Turner and Lambert 1978). Kim et al. (1986) reported that high nitrogen fertilization of jack pine and black spruce seedlings caused the greatest accumulation of arginine and glutamic acid as well as proline. In coniferous gymnosperms arginine has been found to accumulate along with proline over the course of a season and appears to serve an important N-storage function for overwintering buds (Durzan 1967).

Light Starvation and Drought-Stress Tolerance

In addition to the accumulation of free amino acids, water stress has been shown to cause specific changes in the carbohydrate metabolism of tree seedlings. Water stress-associated decreases in starch and increase in sugars have been reported for black oak (Parker and Patton 1975), grand-fir (Magnussen 1981), and slash pine (McNabb 1985). However, Cyr (1983) found no decrease in starch from drought in white, and black spruce, and jack pine. The increase in sugar concentration resulting from water stress may serve to supply substrate for

maintenance respiration or carbon skeletons and reducing power for synthesized amino acids. Brix (1962) observed that when loblolly pines were increasingly water stressed, respiration first decreased, then dramatically increased.

Ackerson (1981) analyzed the relationship among carbohydrate status, photosynthesis, and adjustment to stress in cotton plants. He found that adapted plants (plants that maintained turgor at lower water potentials by osmotic adjustment) had more sugar than non-adapted plants; and when adapted plants were placed in 80 hours of darkness, they became de-adapted. Under lowered CO_2 and increased water deficit, an accumulation of sugar and corresponding hydrolysis of starch was observed in beet leaves (Fox and Geiger 1986). Stewart (1971) demonstrated that the effect of darkness in stressed bean leaf tissue was to increase the loss of starch over that of non-darkened tissue. The effect of darkness which reduces the carbon and energy income of the plant, is to reduce the accumulation of other compatible solutes as well. For example, the carbohydrate status of the plant affects the metabolism of proline. Water stressed plants starved by darkness did not accumulate proline to the same level as control plants (Stewart 1973). Stewart (1971) previously reported that when water-stressed plants accumulates proline, their carbohydrate status

affects the subsequent oxidation of proline when stress is relieved. The above studies suggest that adaptive metabolic responses to stress may depend upon the availability of reducing compounds, energy and carbon compounds which are influenced by the amount of light the plant receives (Kosuge and Kimpel 1985).

Gene Expression and Stress Metabolism

Since proline is synthesized at a higher than normal rate in a variety of water-stressed plants (Boggess et al. 1976), Boggess and Stewart (1980) suggest increased synthesis of enzymes in its metabolic pathway. Those conclusions have been supported by additional evidence (Voetberg and Stewart 1986). Dungey and Davies (1982) also indicated that there may be increased synthesis of enzymes associated with stress-induced proline metabolism. They suggested that increased protein synthesis may have been concerned with producing specific "stress proteins".

The relationship between the accumulation of stress proteins and stress metabolism or stress tolerance remains elusive. Few gene products associated with stress induction have been identified as enzymes. Sachs and Freeling (1980) identified a protein, which was induced by anaerobiosis, as the enzyme, alcohol

dehydrogenase (ADH). Hanson et al.(1984) also identified ADH gene products. The enzymes of metabolic pathways associated with water stress are numerous, generally constitutive and therefore, not so readily identified as ADH. However, Jacobsen et al. (1986) identified a water stress-induced increase in the synthesis of α-amylase. By hybridization with a cDNA clone derived from α-amylase RNA, they established that its increase was due to increased transcription of the α-amylase gene. The increased synthesis and activity of this enzyme suggest that regulation of gene expression may be a component in water stress-induced metabolic changes.

The effects of a variety of stresses on gene expression have been characterized in an array of crop plants (Theillet et al. 1982, Bewley et al. 1985, and reviewed by Sachs and Ho 1986). Recent investigations on the effects of various stresses on gene expression in higher plants have demonstrated qualitative changes in proteins. Water stress, among other perturbations, induces the production of "stress" proteins (Heikkila et al 1984). Several studies have demonstrated that specific heat shock proteins (hsps) may be induced by other stresses (Heikkila 1984, Nover 1984) or by heavy metals (Edelman et al. 1988, Lin and Key 1984). The increase in hsps has been reported to be correlated with acquisition of thermotolerance (Lin and Key 1984). At

present, the precise function of these and other stress-induced proteins have not been established (Bonham-Smith et al. 1988).

In higher plants, including woody tree species, protein synthesis has been shown to decline as water stress progresses, but can resume upon subsequent relief of stress (reviewed by Hsiao 1973, Brandle et al. 1977, Bewley 1981). In studies of proteins separated by two-dimensional electrophoresis, quantitative changes in the pattern of protein synthesis were demonstrated, i.e., some proteins appeared to be synthesized less and others more under water-stressed than under unstressed conditions (Bewley et al. 1983, Oliver and Bewley 1984).

An osmotic stress-induced 26 kDa protein, also reported to be induced by desiccation, has been strongly associated with tolerance of osmotic stress in tobacco cell cultures (Ericson and Alfinito 1984, Singh et al. 1987 and in roots and leaves of the Solanaceae family King et al. 1986). Although, Ericson and Alfinito (1984) report its accumulation to 12% of cellular protein, its role in osmotic adaptation has not been elucidated.

Polyethylene glycol (PEG) was used for water stress studies of protein synthesis patterns in maize (Bewley et al 1983) and barley (Dasgupta and Bewley 1984). Mannitol-induced water stress was applied to loblolly pine hypocotyls (Hulbert et al. 1988) and to slash pine

callus cultures (Valluri et al. 1988) for quantitating changes in protein synthesis. Unfortunately, information derived from such artificially manipulated stresses may be of little consequence to the plant's survival; responses to PEG or mannitol-imposed water stress may not represent normal tolerance mechanisms expressed by a plant that evolved in a climate characterized by episodes of drought.

Objectives

Lack of water has been a major selective force on forest trees and an important determinant of distribution and productivity. Yet, the physiological role in adaptivity of forest trees to dry environments is largely unstudied. I propose to analyze specific biochemical and metabolic responses to drought stress in the needles of ponderosa pine (Pinus ponderosa Dougl. ex Laws.), a western conifer of documented drought tolerance (Vance and Running 1985). By using ponderosa pine seedlings which dehydrate relatively slowly during drought, metabolic processes that are likely to be of adaptive significance may be more readily recognized. The study is designed so that several water-stress parameters can be associated with whole plant performance, cellular metabolism and gene expression that may be of survival value.

The primary objective: to study the physiological response to progressive drought stress in two varieties of ponderosa pine and the role of light starvation in those responses. Objectives supporting the main objective and the chapters corresponding to the objectives are presented below:

Objective 1. Characterize the water relations of ponderosa pine seedlings during progressive drought stress to determine the limits of drought-stress tolerance. Chapter 1 describes the water relations of ponderosa pine seedlings drought stressed until Ψ_x < 5.0 MPa. Pressure-volume and carbohydrate analyses were used to study drought stress and to assess characteristics of dehydration tolerance.

Objective 2. Determine if concentrations of free amino acids are influenced by the water and light status of the seedlings. Chapter 2 describes a method for separating and analyzing free amino acids extracted from needles of ponderosa pine. Chapter 3 compares free amino acid concentrations between unstressed and drought-stressed seedlings of the two varieties, and between unshaded and shaded seedlings.

Objective 3. Characterize drought-stress induced changes in protein synthesis and determine if the changes are influenced by the light status of the seedlings. Chapter 4 examines changes in patterns of protein synthesis by radiolabeling, electrophoretic and fluorographic techniques. Proteins induced by heat shock and osmotic stress are also compared using immunodetection procedures.

CHAPTER 1

INFLUENCE OF DROUGHT STRESS AND LOW IRRADIANCE ON PLANT WATER RELATIONS AND STRUCTURAL CONSTITUENTS IN NEEDLES OF PINUS PONDEROSA

Nan C. Vance, Joe B. Zaerr

Abstract

To understand the influence of light starvation on tolerance of drought stress in ponderosa pine (Pinus ponderosa Dougl. ex Laws.), unshaded and shaded seedlings of P. ponderosa var. scopulorum were subjected to drought. Seedlings of P. ponderosa var. ponderosa were also drought-stressed to compare varietal responses to drought. The irradiance received by shaded seedlings was 10% of full light. Water was withheld from seedlings until water potentials were < -5.0 MPa. The primary difference in water relations between varieties was in the relative water content of the apoplast (RWC_a) and in needle cellulose content. Most water relations parameters of the shaded seedlings differed significantly from those of the unshaded seedlings; the shaded seedlings had lower symplastic osmotic concentration, higher cell volume-to-mass ratio, lower water content of the apoplast, and less

capacitance of the cell walls. These differences may have contributed to lower drought stress tolerance of the shaded seedlings; under increasing drought stress apparent tissue damage occurred in shaded seedlings (RWC < 60%). The shaded seedlings also had significantly less cellulose in the needle tissue than the unshaded seedlings which was attributed to reduction in photosynthate and which may have contributed to differences in water relations and to reduced tolerance of dehydration.

Introduction

It is known that woody plant species differ in drought tolerance (Brix, 1979); however, it is not clear how drought-adapted species tolerate extreme water deficits. In particular, little is known about the physiological or structural mechanisms that protect the leaf tissue of coniferous gymnosperms from dehydration injury.

Characteristically, plant cells adapted to desiccating environments are relatively small and have thick walls (Cutler, Rains and Loomis, 1977). In shade-adapted leaf tissue, the reduced amount and altered allocation of photosynthate may reduce dehydration tolerance physiologically and structurally. Shaded leaves increase photosynthetic surface relative to tissue mass at the expense of cell-wall structure (cellulose and lignin), resulting in larger volume-to-mass ratios (Esau, 1977), which may render the cells less resistant to desiccation. In addition, the limited photosynthate may not adequately meet the demand for respiratory substrate and compatible solutes which normally increases under water deficits (Ackerson, 1981).

Tyree and Hammel (1972) assessed several water relations parameters in plants by using pressure-volume (P-V) curves to analyze the functional relationship

between relative water content (RWC) and xylem water potential (Ψ_x). P-V analysis has demonstrated that the relationship between RWC and Ψ_x, including its components, osmotic potential (Ψ_o) and pressure potential (Ψ_p), are altered by the plant's ontogeny and water stress history (Joly and Zaerr, 1987). Kyriakopoulos and Richter (1981) and Turner (1976) found that injury to leaf tissue also altered this relationship within an individual isotherm. P-V analysis has been used to detect changes in water relations due to drought stress (Kandiko, Timmis & Worrall, 1980) and differences between intra-specific populations (Ladiges, 1975). This study adapts the P-V technique to analyze independent data sets of Ψ_x and RWC in drought stressed and shaded plants of two varieties of ponderosa pine (Pinus ponderosa Dougl. ex Laws.).

Ponderosa pine is drought tolerant. Seedlings have survived water deficits indexed by water potentials as negative as -6.0 MPa (L.J. Heidmann, Principal Research Physiologist, USDA Forest Service, personal communication). Since ponderosa pine seedlings maintain viability over a prolonged period of drought and under relatively severe water deficits, they provide a model plant system for studying the physiological effects of drought stress and the factors that may contribute to desiccation tolerance. Read

(1980) delineated the variation in 24 traits of two varieties of ponderosa pine, (Pinus ponderosa var. scopulorum and var. ponderosa); however, none of the traits were related to drought resistance.

This study compares the water relations of drought-stressed seedlings of P. ponderosa var. ponderosa and P. ponderosa var. scopulorum. The study also examines the influence of light starvation on drought-stressed seedlings of var. scopulorum. We hypothesize that the limited photosynthate available in shade-adapted leaves will reduce tolerance of dehydration.

Materials and Methods

Plant Material

The seed source for Pinus ponderosa var. scopulorum was the Kaibab Plateau in northern Arizona, and for Pinus ponderosa var. ponderosa, the Ochoco National Forest in central Oregon. These sources represent, respectively, the Southern Rockies and the North Plateau, and have the greatest genetic and geographic divergence within the species (Read 1980). Seeds were donated by the USDA Forest Service: Rocky Mountain Forest and Range Experiment Station, Forestry Sciences Laboratory, Flagstaff, Arizona and Pacific Northwest Forest and Range Experiment Station, Forestry Sciences Laboratory, Corvallis, Oregon.

All seed was surface sterilized in 10% sodium hypochlorite and sown in plastic tubes (165 cm^3, Ray Leach, Aurora, OR) in a medium of potting soil, vermiculite and perlite (2:1:1). Seedlings were reared in a greenhouse maintained at day/night temperatures of approximately 25/15^{o}C, watered daily and fertilized twice weekly with Peters 20-20-20 (Grace and Co., Allentown, PA). Irradiance measurements taken in the greenhouse with a quantum sensor (Li-Cor 190S) indicated an average photosynthetic photon flux density of about 700 μmol photons $m^{-2}s^{-1}$ with a range of approximately 300-1200 μmol photons $m^{-2}s^{-1}$.

Treatments

In 1986 200 seedlings, and in 1987, 400 seedlings from Oregon (var. ponderosa) and Arizona (var. scopulorum) were used for the study. Drought, imposed by withholding water, commenced when seedlings were approximately 15 weeks old. Ten days before drought began, a block of 100 seedlings in 1986 and 200 in 1987 of var. scopulorum was shaded to approximately 10% irradiance with commercial shade cloth and remained shaded for the duration of the study.

In 1986, 100 seedlings of var. ponderosa reared in smaller containers (Ray Leach pine cells) were subjected to two drought cycles (two weeks each cycle) during the same period as that of the major study. Measurements were taken during the second cycle.

Seedlings from each treatment were sampled every two days for the first 10 days, then daily until watering was restored. The drought was terminated when predawn $\Psi_x \leq 5.0$ MPa of sampled seedlings for two successive days, or after approximately 30 days. Of the remaining 1987 seedlings, non-destructive water potential (Ψ_x) measurements (using fascicles and terminals) were taken before seedlings were returned to a normal watering regime. Survival data were taken 3 months later. Seedlings sampled for starch,

cellulose and lignin were selected from unstressed seedlings (predawn Ψ_x > -1.0 MPa).

Measurements

Randomly selected seedlings were kept in the dark until predawn Ψ_x was taken to maintain comparable sampling conditions. Each seedling was cleanly severed 1 cm below the cotyledons and carefully wrapped in plastic. Ψ_x was measured with a pressure chamber (PMS Instruments, Corvallis, OR) by the method of Cleary and Zaerr (1980). RWC was calculated by the method of Clausen and Kozlowski (1965) with minor modifications. Two needles from each of three equal intervals above the cotyledons were weighed to obtain fresh weight (FW). They were placed in a 5 ml vial with 1 ml of water, sealed and placed in a refrigerator for 48 hr at 5°C. Needles were then blotted dried and promptly weighed. Preliminary trials showed that this procedure produced a reliable measurement of weight at full turgor (TW). Needles were placed in a drying oven at 70°C for 48 hr, brought to room temperature in a desiccator, and weighed to obtain dry weight (DW). All weights were to the nearest mg.

RWC was calculated from the formula:

$$RWC = \frac{FW - DW}{TW - DW} * 100$$

P-V Analysis

Analysis of water potential isotherms (P-V curves) is facilitated by transforming one of the variables, usually Ψ_x to $1/\Psi_x$. A linear regression was initially calculated from the transformed data points visually determined to be in the linear region (RWC below turgor loss). The final regression was determined by maximization of the coefficient of determination (r^2) Joly and Zaerr (1987). Osmotic potential at full turgor (Ψ_{oft}), was determined by extrapolating the linear region of the P-V curve to the Y axis. Ψ_{oft} is dependent upon the solute concentration of the leaf cells and may be related to desiccation tolerance. Another parameter, relative water content of the apoplast (RWC_a), is determined by extrapolation to the X axis. RWC_a is the residual cell water after the vacuolar water is depleted. The individually sampled data pairs for each population were used to develop the water potential isotherms in this P-V analysis.

To compare data obtained by more typical P-V analysis with the other data, in late August, four seedlings watered to saturation from each seed source were brought into the laboratory after taking an initial predawn Ψ_x. They were allowed to dehydrate on the bench by transpiration (DT). Ψ_x was repeatedly measured over the range of decreasing Ψ_x. Before each measurement, three needles were selected from the

epicotyl as described previously, and quickly weighed to obtain FW. TW and DW were also obtained for determination of RWC. The measurements were pooled for each population.

Physiological Measurements

Physiological analyses were performed on the seedlings studied in 1987. Approximately 0.2 to 0.3 g of needles harvested from a randomly selected subsample of seedlings was plunged into liquid nitrogen and stored at -80^{o}C. Needle cellulose and lignin were analyzed by the method of Van Soest (1963). Starch was analyzed by the method of Hansen and Moller (1975).

Results

Parameters derived from typical P-V analysis are based on a family of curves calculated from an inverse transformation of Ψ_x plotted against RWC; each curve represents the change in RWC and Ψ_x of a sampled individual. In this study, the plotted data represented independent sample points each lying on a hypothetical P-V curve. Collectively they constituted a sampled population that reflected the change in water status as dehydration occurred. The true regression line represented a least squares best fit of independent measurements where Ψ_{oft} is an estimate of the Y intercept of the regression. Detectable differences in intercepts were determined by multiple regression analysis. Because insufficient data points from the 1986 shade treatment fell in the linear region, results are not shown.

The relation between Ψ_x and relative water loss (RWL = 100 - RWC) (Fig. 1.1A, B and C, respectively) and transformed data that include the regression slope (Fig. 1.1D, E and F) are similar for var. ponderosa and unshaded var. scopulorum seedlings. The spread in Ψ_x, RWL data pairs of the ponderosa and unshaded scopulorum seedlings (Fig. 1.1A, D and B, E) occurs at a RWL of approximately 16%. Variability in this region is not apparent in the shaded seedlings. However, at a RWL

greater than 40% the data points show increasing divergence from linearity above the regression line.

Estimates of osmotic potential at full turgor (Ψ_{oft}) and relative water content of the apoplastic fraction (RWC_a) varied among populations (Table 1.1). Ψ_{oft} of ponderosa seedlings were more negative than those of scopulorum seedlings. The lowest Ψ_{oft} was estimated in the ponderosa seedlings that received DT in the laboratory and the highest was estimated in the shaded seedlings.

RWC_a was also lower in the var. ponderosa than in the unshaded var. scopulorum seedlings; however, RWC_a was lowest in the shaded var. scopulorum seedlings (Table 1.1). Statistical anlysis of Ψ_{oft} and RWC_a indicates significant differences between shaded and unshaded seedlings at $P = 0.05$. Within each seed origin, the seedlings that dehydrated in the laboratory by transpiration (DT) had the largest RWC_a.

The differences in the TW/DW ratios corresponded to differences in W_{oft} estimates among populations with the exception of var. scopulorum seedlings treated to DT (Table 1.1). The shaded seedlings had the highest W_{oft} and the lowest RWC_a of all populations; they also had the highest TW/DW ratio. The var. ponderosa seedlings stressed for two cycles had one of the lowest TW/DW ratios and one of the lowest W_{oft} of all populations.

Analysis of needle content of cellulose, lignin and starch in the needles indicates significant differences in the amount of cellulose (g/g) among the Oregon and Arizona shaded and unshaded seedlings (Table 1.2). The needle tissue cellulose of the shaded seedlings was approximately 0.67 that of the unshaded Arizona seedlings and 0.8 that of the Oregon seedlings. Lignin content did not vary among the treatments or sources. Although needle starch concentration was higher in the unshaded than in the shaded Arizona seedlings, the low values were typical for needle tissue.

The Mann-Whitney test was applied to compare the Ψ_x of seedlings that did not survive after re-watering in 1987. Mortality was not detected in Oregon and unshaded Arizona seedlings until Ψ_x reached -4.0 and -4.5, respectively; however, mortality occurred in shaded seedlings at Ψ_x as high as -1.85 MPa. The differences were significant among the three groups. It should be noted that unusually high Ψ_x were measured in the shaded seedlings dehydrated to RWC < 60% and that no shaded seedling recovered from a RWC < -4.0 MPa.

Discussion

A P-V curve plotted with independent data points from many sampled individuals will naturally show greater variability than one plotted with non-independent data from one individual. However, in this study, variability increased when Ψ_x approached approximately -1.6 MPa and RWC, 84% in the unshaded seedlings. This variability may be due to several events that occur together and may be related: stomatal closure, loss of turgor ($\Psi_p = 0.0$), cavitation of the tracheids and a transitory rehydration of needle tissue (Dixon, Grace and Tyree, 1984). Stomatal closure on ponderosa pine seedlings from an adjacent greenhouse occurred at Ψ_x = -1.6 MPa (S. Omi, personal communication). Lopushinsky's (1969) estimates of stomatal closure at -1.65 MPa and 84% RWC agree closely with the above.

The extreme variability in Ψ_x of shaded seedlings at RWC below 60% was distributed above the regression slope (Fig. 1.1C and F), and suggests loss of cellular integrity (Turner, 1976; Kyriakopoulos and Richter, 1981). At RWC of 50%, Ψ_x was as high as -1.5 MPa. The shaded seedlings appeared severely wilted even at relatively low water deficits; however, there were no visible lesions. In the needle tissue of the shaded seedlings, cells with a high volume-to-mass ratio and

relatively thin cell walls may have collapsed under the stress of increasing xylem water tension caused by dehydration. Structural failure could cause a redistribution of symplastic and apoplastic water, which would affect the slope of the P-V curve and the extrapolated water relations parameters Ψ_{oft} and RWC_a. Turner (1976), however, found that partial tissue damage did not alter the linearity of the P-V curve, though lesions were visible. This may be because linearity is strongly related to lack of independence of the data points. In a typical P-V curve, slope and magnitude of the coordinates may be a more appropriate indicator of tissue damage.

The significantly lower TW/DW ratios of the DT seedlings measured in late August and those receiving two cycles of drought suggest that conditioning or seasonal change in cell tissue may influence the ratio of cell volume to dry matter. Cutler et al. (1977) observed that cell size was less, and cell wall thickness greater, in cotton leaves that had been water-stressed. In the present study, the ratio of TW/DW did not change significantly over the course of a single drought. Joly and Zaerr (1987) found no difference in the TW/DW ratio between irrigated and non-irrigated Douglas-fir seedlings, but did note a seasonal decrease in the ratio.

The greater RWC_a of the DT seedlings may be related to their lower TW/DW ratios, discussed above; and together suggest greater cell wall volume. Joly and Zaerr (1987) suggested that changes in cell wall elasticity and RWC_a may be influenced by alterations in cell wall structure and composition, and that the plant cell wall may provide a small but significant capacitance against intra-cellular dehydration. Gaff and Carr (1961) suggest that the larger water fraction of the cell wall may act as a buffer against protoplastic dehydration and therefore be a factor in drought hardening. Of the seedlings that received two drought cycles, however, no significant increase in RWC_a was detected.

In addition, RWC_a may be a function of the amount of cavitation and water released from xylem in the leaf (Sperry, 1986). The DT seedlings' stems were cut at the beginning of the P-V measurements, creating a greater potential for cavitation in the xylem as they air-dried. Hardegree (1987) demonstrated an alteration in the Ψ_x/ RWC relationship of seedlings air-dried for P-V analysis, and suggested that it was due to increased cavitation. The adjacent cell walls of needle tissue may absorb water released from cavitating xylem (Gaff and Carr 1961). Dixon et al. (1984) suggest that cavitation is important in the relocation of water reserves. RWC determined by weighing the

entire seedling for P-V analysis would not reflect changes in the distribution of water and could therefore alter the slope of the Ψ_x/RWC curve.

In the var. scopulorum, redistribution of water may explain the higher W_{oft} and RWC_a; however, cellulose content was also higher in the needles of the unshaded scopulorum seedlings. The water relations differences between the ponderosa and scopulorum populations may be ascribed to the more frequent development of fascicled needles in var. scopulorum. Therefore, the apparent varietal differences may be related to needle anatomy. The structural relationship of the two needle types to water relations parameters relating to drought tolerance merits further investigation.

Larsen (1927) compared leaf structure among eight conifer species which ranged in adaptation from shade and high moisture, to high light and low moisture. The author found that a major adaptive characteristic was the ratio of xylem area to cross sectional leaf area. In ponderosa pine needles, the ratio was 1/30, the highest ratio of all species studied; in contrast, the lowest ratio (1/253) was in needles of the high moisture- and shade-adapted western hemlock (Tsuga heterophylla Sarg.). The larger xylem-to-leaf ratio of ponderosa pine suggests a greater potential for releasing water by cavitation. Combined with

structural strength and the capacitance of cell walls, cavitation may delay dehydration in the needle tissue. However, if structural strength is reduced because of decreased cellulose in cell walls, no emboli could form since, in these cavitated tracheids, an early collapse of the vascular tissue would occur. Rewatering would fail to restore the water-conducting capacity of these cells, resulting in tissue death.

The significantly higher TW/DW ratio and lower RWC_a and cellulose content of the shaded seedlings suggest that shading causes a limitation and reallocation of photosynthate, affecting the structure of plant tissue and its capacity to withstand dehydration. The higher volume-to-mass ratio is also typical of plant cells not exposed to desiccation (Pettersson et al., 1957). Parker (1952) described anatomical changes in needles of two Pinus spp. at lethal levels of dehydration (near 50% of original weight). Cells collapsed or were distorted, and after rehydration membrane and cellular integrity failed to recover. This suggests that cell-wall reinforcement, in addition to elasticity, is important in resisting lethal structural stress imposed by dehydration.

The higher Ψ_{oft} and the lower starch content in the shaded seedlings suggest that the production of metabolites contributing to dehydration tolerance is also limited by light starvation. Starch is normally

converted to sugars under increasing water deficit, providing energy and compatible solutes that may contribute to osmotic adjustment or macromolecular stability. Turner and Jones (1980) reported that reduction in photon flux density by approximately one-half reduced osmotic adjustment from 0.6 MPa to 0.3 MPa. In leaves of ponderosa pine seedlings the osmotic concentration of the symplast was reduced by shading; however, this dilution effect could be a result of increased water volume rather than reduced solute production.

This study demonstrates the negative effect of severe light limitation on tolerance of drought. Several water-conserving mechanisms normally contribute to drought tolerance: increased symplastic osmotic concentration; release of water to adjacent cells by cavitation; lower cell volume-to-mass ratio; greater water content in cell walls; and greater cell wall capacitance. Seedlings of Pinus ponderosa var. scopulorum, by virtue of their architecture, may have an advantage during drought. The results merit further investigation. It may be that the structural integrity of the plant in withstanding the physical stress on cells and tissues imposed by dehydration is critical to its survival under severe water deficit.

Figure 1.1 Plots of xylem water potential (Ψ_x) vs relative water loss (RWL) of 4-month-old ponderosa pine seedlings sampled in 1987 (A) var. ponderosa, (B) var. scopulorum and (C) var. scopulorum (shaded). D,E and F same as A,B and C except plots are of $1/\Psi_x$ vs RWL. Lines determined by least squares best fit of the regression of $1/\Psi_x$ on RWL. For shaded seedlings the regression was performed for data points in the linear region < 41% RWC.

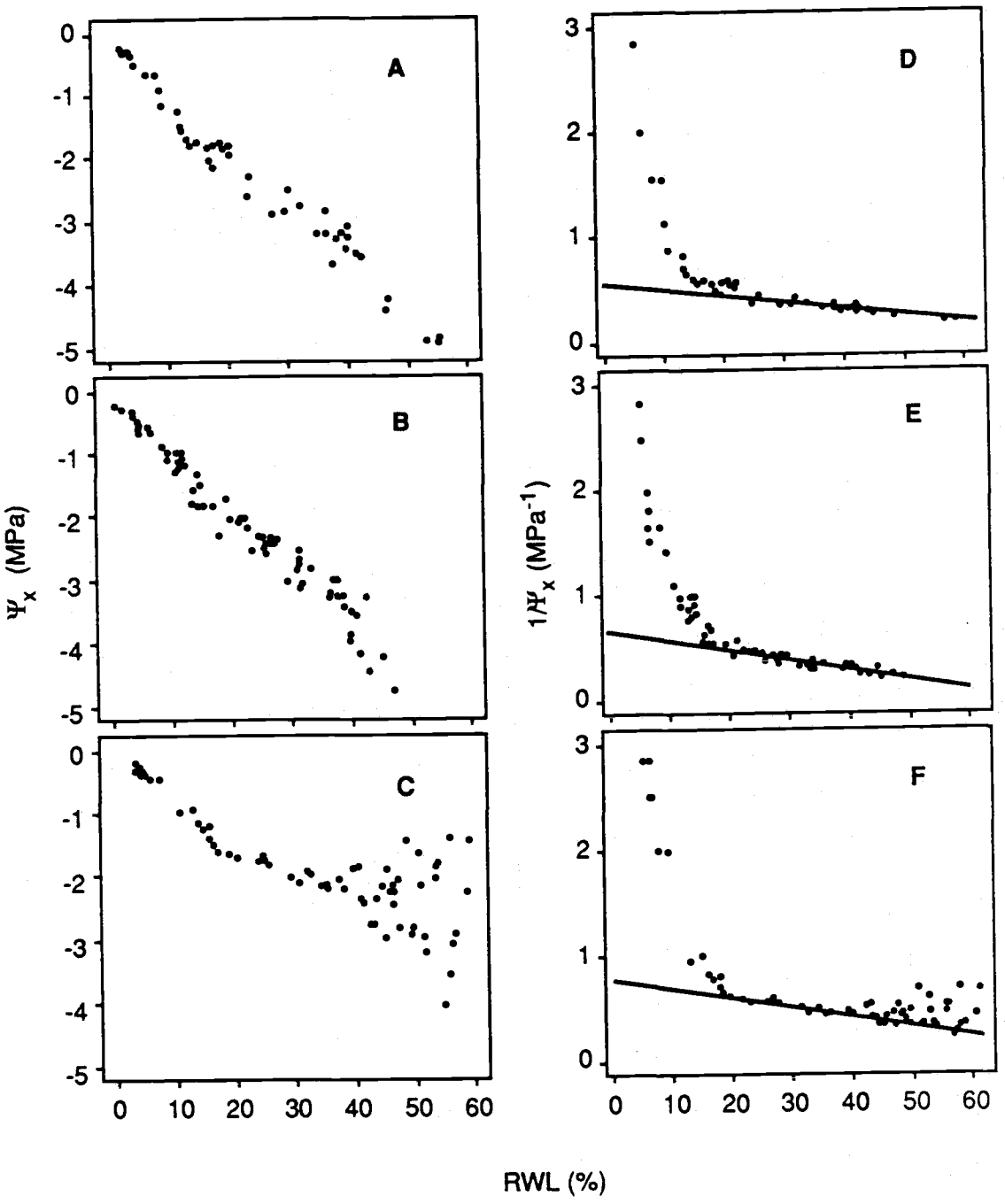

Figure 1.1

Table 1.1 Water relations parameters of greenhouse-grown ponderosa pine seedlings dehydrated by drought. Inverse transformation of Ψ_x vs RWC and extrapolation of linear region of water potential isotherm to obtain osmotic potential at full turgor (Ψ_{oft}).*

Variety / Treatment	Ψ_{oft}	Ψ_{oft}^{-1}	+ SE*	RWC_a	r^2	TW/DW
	MPa	MPa^{-1}		%		
Ponderosa (1987)	-1.80	-0.56b	0.03	14.0b	0.86	3.80bc
Ponderosa (1986)	-1.68	-0.59b	0.02	17.9b	0.90	3.69b
Ponderosa (2 cycle)	-1.91	-0.52ab	0.02	14.1b	0.89	3.11a
Ponderosa (DT)	-2.03	-0.49a	0.02	24.8c	0.89	3.05a
Scopulorum (1987)	-1.51	-0.66c	0.02	30.0c	0.88	3.92c
Scopulorum (1986)	-1.60	-0.63c	0.04	20.4bc	0.88	3.81bc
Scopulorum (DT)	-1.42	-0.70cd	0.02	41.9d	0.96	3.12a
Scopulorum Shade	-1.34	-0.75d	0.02	7.0a	0.91	4.42d

* Abbreviations: Ψ_{oft}, Osmotic potential at full turgor; r^2, coefficient of determination; TW, full turgor weight; DW, dry weight; RWC_a, relative water content of the apoplast; DT, bench dehydration by transpiration.

* Multiple regression analysis using indicator variables for differences between intercepts on estimates of Ψ_{oft} and RWC_a, and Scheffe's range test on TW/DW,were used to detect significant differences at P = 0.05 denoted by different letters.

Table 1.2 Structural constituents of ponderosa pine needles comparing seedlings of var. ponderosa, and of shaded and unshaded var. scopulorum.*

	varieties		
	ponderosa	scopulorum	scopulorum shaded
Cellulose (g/g)	0.491 + 0.015b	0.584 + 0.018a	0.405 + 0.009c
Lignin (g/g)	0.306 + 0.010a	0.311 + 0.009a	0.310 + 0.009a
Starch (mg/g)	N/A*	38.500 + 3.37a	26.810 + 4.12b

* Data presented are means (+SE) of measurements based on oven dry wt. The Scheffe's test was used to detect significant differences at P = 0.05 indicated by letters (n=8).
* Abbreviation: N/A, not analyzed.

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CHAPTER 2

ANALYSIS OF FREE AMINO ACIDS IN PLANT EXTRACTS BY DANSYLATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

N. C. Vance and J. B. Zaerr

Abstract

To better understand the role free amino acids play in plant physiological processes, a convenient method was developed for separating and quantitating amino acids in plant tissue extracts using derivatization with dansyl chloride and reverse phase high-performance liquid chromatography (HPLC). Free amino acids extracted from needles of Pinus ponderosa Doug. ex Laws.) were dansylated overnight. Twenty-one amino acids were separated on an Ultrasphere ODS C18 column and detected with a fluorescence spectrophotometer. Good peak separation was achieved and retention times were reproducible with a variability of approximately 6 s. As little as 10 pmol of each amino acid were detected. To test the effect on amino acids of a common step in purifying plant extracts before dansylation, samples were eluted from a cation exchange column with 4N NH_4OH prior to dansylation. The strong base may have hydrolyzed a

proportional amount of arginine to ornithine which produced an artifactual shift in the relative concentrations of arginine and ornithine on the chromatographs. The results demonstrate that without additional sample preparation, dansylation coupled with HPLC provides a rapid and useful method for accurately separating and quantitating free amino acids extracted from plant tissue .

Abbreviations used:

RP, reverse phase; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; PTH, 3-phenyl-2-thiohydantoin; OPA, o-phthalaldehyde, NH_4OH, ammonium hydroxide; Na_2CO_3, sodium carbonate; $NaHCO_3$, sodium bicarbonate; GABA, γ-aminobutyric acid.

Introduction

Analysis of free amino acids is necessary to many studies of nitrogen assimilation and metabolism (Brunk and Rhodes 1988), N nutrition (Kim et al. 1987) and metabolic responses to stress (Meza-Basso 1986, Pulich 1986, Rhodes et al. 1986) in plants. Because free amino acids form a critical linkage between protein, nitrogen and carbon metabolism, a fast, convenient and accurate method for analyzing amino acids in crude plant extracts is needed.

The most common methods of quantitative or qualitative analysis of free amino acids in plant extracts use OPA- or PTH-derivatives with thin-layer chromatography (TLC), or an amino acid analyzer. Each of these methods has drawbacks in complexity, time, cost, or inability to detect specific amino acids. In some cases, an additional treatment is required to detect the imino acids proline and hydroxyproline (Ford 1984), or to distinguish between the amide forms of aspartic acid and glutamic acid (Malhotra and Sarkar 1979).

The highly fluorescent 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl) forms a derivative which can be detected in pmol amounts with a fluorescence spectrophotometer (Wilkinson 1984). The yield and specificity of the Dns-amino reaction depends on

controlling reaction conditions that favor formation of the product and inhibit by-products and multiple derivatives (Neadle and Pollitt 1965). With favorable pH, product yield is independent of the Dns-Cl:amino acid ratio over a 1000-fold range (Tapuhi et al. 1981). A previous study demonstrated that pre-column derivatization can be carried out at room temperature with the resulting derivatives stable over a 96 hour period (Wiedmeier et al. 1982).

Dansyl derivatization has been used with TLC to detect free amino acids in plant tissue (De Los Angeles Barcelon 1982, Kim et al. 1987), and with high performance liquid chromatography (HPLC) to detect and quantify proline in callus cultures (Newton et al. 1986). Dansyl derivatization with reverse phase HPLC was also successfully applied to analyzing polyamines in lichen (Escribano and Legaz 1988).

A problem in the biochemical analysis of plants by HPLC is the interference of secondary compounds which contaminate plant tissue extracts. To avoid the interference of contaminants, a common procedure prior to chromatography is to concentrate or purify the extract by passing the solution through a cation exchange column, followed by eluting the amino acids with NH_4OH at high molar concentration (Moreno and Garcia-Martinez 1984, Fukutoku and Yamada 1984). However, this procedure may

result in the hydrolysis of arginine to ornithine (Clarke 1947, Jones et al. 1979). The loss of arginine is detrimental to a clear interpretation of the physiological significance of free amino acids especially in coniferous gymnosperms because arginine plays a significant role as an N-storage compound (Durzan 1971) and may accumulate to high levels (Kim et al. 1987, Vance and Zaerr, unpublished). The high reactivity of Dns-Cl with amino groups, the stability of the derivatives and the sensitivity of fluorescence detection (Negro et al. 1987), makes this preparative step unnecessary.

With the increasing use of non-dedicated HPLC systems in biological laboratories, it is desirable to have a procedure for analyzing physiological compounds that is relatively simple, reliable and inexpensive. The procedure described in this paper can be used to acquire a large data set in a reasonable length of time for relatively low cost in a laboratory equipped with an HPLC system. We report a method of separating and quantitating free amino acids of physiological significance in plant leaf tissue by using precolumn dansyl derivatization and reverse phase HPLC without requiring preparative cleanup of samples. The procedure was used for analyzing free amino acids extracted from needles of Pinus ponderosa (Doug. ex Laws.).

Materials and Methods

Chemicals and Materials

Crystalline Dns-Cl, dansyl amino acids, free amino acid standards, and analytical grade $NaHCO_3$ and Na_2CO_3 were purchased from Sigma (St. Louis, Mo.). The original concentration of each of the 17 amino acids in the standard solutions used for calibration dilutions was 2.5 µmol/ml except cystine which was 1.25 µmol/ml (AA-S-18), and of each of the standards used for routine peak verification was 25.0 nmol/ml except that of cystine which was 12.5 nmol/ml (A-2161). HPLC-grade acetonitrile and methanol, and reagent grade ethanol (95%), ammonium hydroxide and glacial acetic acid were purchased from J.T.Baker Inc. (Phillipsburg, NJ). Triethylamine (TEA) was purchased from Sigma Chemical Company (St. Louis, MO). Dowex cation exchange resin (AG 50W-X8, 100-200 mesh) and disposable Poly-Prep columns were purchased from Bio-Rad (Richmond, CA).

Extraction of Amino Acids

Approximately 200 mg of needle tissue were removed from each sampled seedling of 5-month-old Pinus ponderosa, plunged in liquid nitrogen and stored at

-80°C. The extraction procedure of Bieleski and Turner (1966) was followed with some modifications. The needle tissue was ground in liquid nitrogen and added to 4 ml of methanol/chloroform/water, (12:5:3, v/v/v). The mixture was vortexed for 2 min and centrifuged for 10 min. The pellet was reextracted with 2 ml of methanol/ chloroform/ water, vortexed and centrifuged at top speed with a desk top centrifuge for 5 min; the procedure was repeated with 2 ml of 80% ethanol. The supernatants were combined, and phase separation was achieved by adding 2 ml of chloroform and 1.5 ml of deionized water followed by centrifugation. Two 1 ml aliquots of the aqueous extract were dried under vacuum. The amino acids were resolubilized in 500 µl of 0.01N HCl and stored at -20°C until needed.

To test the effects of a strong base on arginine, replicates of three crude needle-extract samples additionally were subjected to a preparative cleanup step. Each sample was applied to a column of cation exchange resin (AG 50W-8X, 100-200 mesh), washed with 80% ethanol and eluted with 8 ml of 4N NH_4OH. Two 1-ml aliquots of the eluate were dried under vacuum. The dried extract was resolubilized in 500 µl of 0.01N HCl and stored as reported above.

Reagent Preparation

Reagents were prepared by a procedure of Bongiovanni et al. (1981) with minor modifications. Small glass tubes (5 cc) were buffered with 300 µl of 0.5M $NaHCO_3$ that had been adjusted to pH 9.0 with 0.5M Na_2CO_3. The buffer was dried under vacuum at 50°C. The buffered tubes were covered with parafilm and stored until used for derivatization. Dns-Cl was prepared at a concentration of 3 mg/ml in HPLC-grade acetonitrile and stored at 4°C; a fresh solution was made every 3 days. Individual amino acid standards were made up in 0.1N HCl at a concentration of 10 µmol/ml. These stock solutions were stored at 4°C, and diluted as needed.

Derivatization

Six to eight samples and a standard mixture were derivatized one day prior to chromatography. Into the buffered test tubes, 200 µl of the dansyl chloride solution were added to 200 µl aliquots of sample or amino acid standard solution. After vortexing, the tubes were tightly covered and stored overnight in the dark at room temperature. The following day, the dansylated samples and standards were pipetted into micro-centrifuge tubes and briefly centrifuged. Samples were queued in a darkened autoanalyzer (Perkin-Elmer ISS-10, Germany) to prevent photodegradation of the dansylated samples.

Chromatography

Each dansylated sample or standard (10 µl) was injected into a Beckman reverse phase column (Ultrasphere ODS C18, 5-µm particle diameter, 250 X 4.6 mm i.d; San Ramon, CA) fitted to a varian model 500 HPLC (Palo Alto, CA). Detection was by a Perkin-Elmer model 650-10LC fluorescence spectrophotometer equipped with an 8-µl flow-through cell. The fluorescence detector, operating at one-third maximum sensitivity for Dns-amines, was set at λ ex.360/ em.480 nm.

A mobile phase of 10 mM sodium acetate buffer (0.01% TEA), pH 3.8, in pump A and acetonitrile in pump B was used for eluting the dansylated solutions. The solvents were degassed by sparging with helium before and during the chromatography. A 60 min program consisted of a flow-rate of 1 ml/min and a solvent gradient beginning at 10% B, rising to 40% B over 30 min, held at 40% for 15 min, followed by a gradient of 40% to 100% B over 10 min. The column was recycled for 18 min and initial conditions were held for 12 min before the next injection. Detector output was processed by a Keithley DAS 500 analog/digital signal acquisition system coupled to an IBM-PC with Waters (MAXIMA) software, Millipore Corp. (Milford, Massachusetts, USA).

Results and Discussion

Individual amino acids were verified by derivatizing and chromatographing each amino acid standard individually and with the standard mixture. Four amino acids routinely added to the standard mixture were asparagine, glutamine, γ-aminobutyric acid (GABA) and tryptophan. Each peak of asparagine, glutamine, tryptophan and GABA represented 0.125 nmol; each of the other standard peaks represented 0.0625 nmol. By using a solvent buffer of relatively low pH (pH 3.8), the Dns-amino acids eluted well after Dns-OH which appeared to elute at approximately 10 min and near the elution front (Fig. 2.1).

The variation in retention time among eight chromatograms produced on separate days, for 10 representative amino acids was low (Table 2.1). Dns-leucine varied the most in retention time, about 13 s. The average variability in retention time of the dns-amino acids listed in Table 2.1 was less than 6 s.

A set of standard mixtures was serially diluted over a range of concentrations (0.05-0.25 nmol/10 μl) and chromatographed so that calibration curves could be developed for calculating the unknown concentrations of amino acids in the needle extracts. Known concentration

(C) of each amino acid standard was regressed against peak response (R), where

$$R = \text{peak area } (x\ 10^{-4}),\ n = 3,$$

by the computer software programmed for concentration determination. The relationship between concentration of the standards and peak response was linear for all standards with the exception of Dns-lysine and Dns-NH2, for which an exponential model provided the best fit (Table 2.2).

The stability of the Dns-amino acids permitted derivatization the day before samples were to be injected and the queueing of many samples with an autosampler. The data for the eight samples and one standard chromatographed each day were stored on the computer for further analysis. The chromatography-dedicated computer software speeded up processing by identifying retention times, integrating peak areas, developing calibration curves of the standards and calculating concentrations of samples based on known standard concentrations. The information was stored in files that could be exported to other programs for further statistical analysis.

A chromatogram of a sample of free amino acids extracted from ponderosa pine needles (Fig. 2.2a) demonstrates that without a predansylation cleanup

procedure, amino acids were easily identifiable and eluted separately from unknown compounds. The chromatogram of the dansylated crude needle extract (Fig. 2.2a) compares favorably with that of the same sample receiving predansylation cleanup (Fig.2.2b.) The proportional differences in peak heights between chromatographs of identical physiological samples are due to slightly greater final concentration of amino acids in the sample that received the pretreatment.

The extent of arginine hydrolysis among different amino acid samples when they were eluted from the cation exchange column with a strong base was determinined by using three replicated sample extracts from needles of Pinus ponderosa seedlings each representing a different physiological condition: no stress, light starvation and water stress. In light starved and water-stressed plants, concentrations of arginine were found to increase (N.C. Vance and J.B. Zaerr, unpublished); therefore, response levels of arginine relative to ornithine varied among the three replicated samples. Nevertheless, the shift in arginine and ornithine occurred in all three samples as demonstrated by the representative chromatograms in Fig. 2a and 2b.

The mean arginine:ornithine integrated peak area ratio of the three samples was 14.16 without pretreatment and 0.17 with pretreatment. In the sample (light

starvation) in which the peak area ratio of arginine to ornithine was highest (29.17), the change in ratio after the pretreatment was least (0.34), indicating proportionally less was converted to ornithine. Note that the peak area of proline in the chromatogram of the treated sample (Fig 2.2b), appears to be less than that of proline in the untreated sample (Fig 2.2a), indicating that the pretreatment using 4N NH_4OH may affect other amino acids as well.

For many plant tissues, and particularly those of coniferous gymnosperms, preparative steps taken to purify or concentrate extractions before chromatography may increase time, loss of compounds and the possibility of introducing artifacts. The strong reactivity of Dns-Cl with amino groups and the sensitivity of fluorescent detection permitted the analysis of pine needle extracts without the necessity of additional ion-exchange techniques to concentrate and purify samples. Twenty-one amino acids were separated and identified by this method; as little as 10 pmol of each were detected and quantified. In the case of free amino acids in needle tissue of Pinus ponderosa, all the amino acids of physiological interest were separated and quantified. Because arginine is of metabolic and physiological significance in plants (Miflin and Lea 1977, Pawashe and Srivastava 1987), the method may be particularly useful

in providing a means for accurately quantitating relative changes in the concentration of this amino acid.

Table 2.1

Means and Variability in Retention Times of 10 Dansyl-Amino Acids Chromatographed Over a Span of 3 Weeks[a]

Amino Acid	Retention Time[b] (min)	SE[c]
Asn	18.11	0.045
Ser	20.41	0.039
Asp	20.90	0.146
Arg	21.49	0.058
Gly	25.89	0.106
Ala	29.84	0.109
Pro	35.89	0.069
Val	37.42	0.072
Leu	42.68	0.219
Lys	53.28	0.033

[a] Amino acids extracted from ponderosa pine needles. Conditions are described under Materials and Methods.
[b] Values represent the mean of retention times (n=8) for each amino acid randomly sampled from the chromatography data.
[c] Standard error of the mean.

Table 2.2

Regressions of Amino Acid Concentration on Fluorescence Response[a]

Amino Acid	Regression[b]	Coefficient of Determination (r^2)
Asn	C = 0.129 + 6.27R	0.999
Gln	C = 0.070 + 3.59R	0.999
Ser	C = -0.160 + 6.98R	0.999
Asp	C = -0.160 + 8.11R	0.999
Arg	C = -0.020 + 4.12R	0.999
Glu	C = 0.044 + 3.77R	0.996
Thr	C = -0.104 + 7.07R	0.991
Gly	C = -0.040 + 2.69R	0.999
Ala	C = -0.037 + 2.09R	0.999
-NH2	C = 0.059e 0.34R	0.999
Met	C = -0.034 + 1.42R	0.999
GABA	C = -0.097 + 0.41R	0.999
Pro	C = 0.028 + 0.86R	0.999
Val	C = -0.048 + 1.10R	0.998
Phe	C = 0.043 + 1.70R	0.997
Trp	C = -0.042 + 1.50R	0.984
Leu	C = -0.034 + 1.01R	0.994
Ise	C = -0.054 + 1.14R	0.999
Orn	C = 0.008 + 0.40R	0.996
Lys	C = 0.345e 0.60R	0.999
Tyr	C = 0.223 + 2.67R	0.999
His	C = 0.000 + 4.47R	0.997

[a] Calibration curves developed from serial dilutions of amino acid concentrations over a range of 0.05 nmol/10 µl to 0.25 nmol/10 µl. Conditions described under Materials and Methods.

[b] Concentration (C) regressed on response (R) where R = peak area (x 10-4), n = 3.

Figure 2.1 A chromatogram showing the elution order of dansylated amino acid standards separated by reverse phase HPLC. The Ultrasphere ODS C18 column was injected with a 10 μl mixture containing approximately 0.06 nmol each of serine (Ser), aspartic acid (Asp), arginine (Arg), glutamic acid (Glu) threonine (Thr), glycine (Gly), alaninine (Ala), methionine (Met), proline (Pro), valine (Val), phenylalanine (Phe), leucine (Leu), isoleucine (Ise), lysine (Lys), tyrosine (Tyr), and histidine (His) and 0.13 nmol of asparagine (Asn), glutamine (Gln), γ-aminobutyric acid (GABA), tryptophan (Trp) and ornithine (Orn).

FLUORESCENCE INTENSITY
Dns-OH
Asn
Gln
Ser
Asp
Arg
Glu
Thr
Gly
Ala
-NH2
Met
GABA
Pro
Val
Phe
Trp
Leu
Ise
Orn
Lys
Tyr
His
20
30
40
50
TIME (min)

Figure 2.1

Figure 2.2 Chromatograms of dansylated samples extracted from needles of ponderosa pine and generated under conditions described in Fig. 2.1 showing the elution pattern of (a) a sample that did not receive preparative cleanup (application to a cation exchange column and elution with 4N NH4OH) before dansylation, and (b) the elution pattern of a replicate sample after it received the predansylation cleanup.

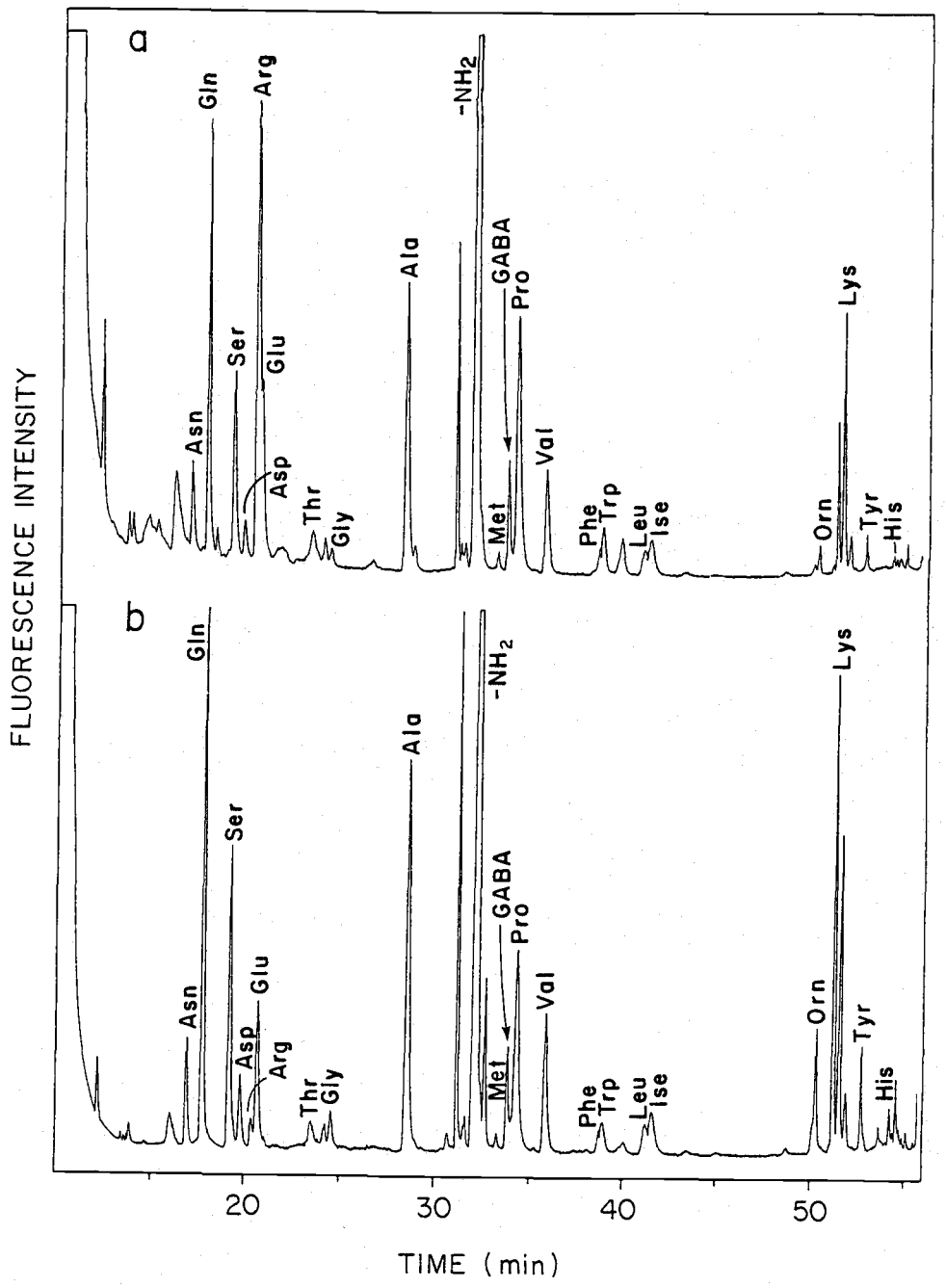
a
b
Asn
Gln
Ser
Arg
Glu
Asp
Thr
Gly
Ala
-NH2
GABA
Pro
Met
Val
Phe
Trp
Leu
Ise
Orn
Lys
Tyr
His
FLUORESCENCE INTENSITY
20
30
40
50
TIME (min)

Figure 2.2

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CHAPTER 3

INFLUENCE OF DROUGHT STRESS AND LIGHT STARVATION ON FREE AMINO ACID METABOLISM IN PINUS PONDEROSA SEEDLINGS

Nan C. Vance and Joe B. Zaerr

Abstract

Free amino acid concentrations in needles of drought-stressed and shaded seedlings of ponderosa pine (Pinus ponderosa Dougl. ex Laws.) were analyzed to determine the influence of low irradiance on the biochemical response to drought stress. Also, drought-induced varietal differences in free amino acid metabolism were examined in P. ponderosa, var. scopulorum and P. ponderosa var. ponderosa. Free amino acids were extracted from needles of four-month-old seedlings, derivatized, and separated on an Ultrasphere ODS C18 column by HPLC. Drought and shading induced an increase in total amino acid concentrations (μmol/g dw) with shading having the greatest effect. Drought stress induced the greatest increase in concentrations of arginine and proline in shaded and unshaded seedlings, respectively. In unstressed, shaded seedlings, glutamine accumulated most. Aspartic acid decreased significantly in drought-stressed seedlings. The effect of shade was

accumulation induced by drought stress. The large increase in arginine in light-starved (shaded) and drought-stressed seedlings suggests that NH_3 assimilation and nitrogen storage are active processes under both conditions. Shaded seedlings did not survive severe drought stress. That amino acids accumulated to a lesser extent in var. ponderosa than in var. scopulorum, had no apparent effect on relative dehydration tolerance as the two varieties were equally tolerant of drought stress.

Introduction

Changes in free amino acid concentrations as dehydration increases, coupled with a sharp increase in proline at a critical level of water stress, have been observed in a variety of plants (reviewed by Stewart and Larher 1980, Hanson and Hitz 1982). The accumulation of specific amino acids is thought to be an adaptation to water stress; however, their role in osmotic adjustment, ammonia detoxification, or as compatible solutes is not clear.

With the exception of proline, the effects of low irradiance on amino acid metabolism in leaves of drought-stressed plants have not been examined. One effect of low irradiance is to alter the carbon/nitrogen ratio as well as reduce the supply of energy and photoassimilates to the plant (Kosuge and Kimpel 1981). Durzan (1971) demonstrated that severe shade significantly altered amino acid metabolism in jack pine (P. banksiana) and white spruce (Picea glauca). The accumulation of the nitrogen storage compound arginine was greater in leaves and buds of jack pine, which responded more to light deprivation than did white spruce. Conversely, the synthesis of proline in water-stressed tissues may depend on light for carbohydrates and reducing power since its

synthesis is inhibited by light deprivation (Stewart 1978, Hanson and Tully 1979, Joyce et al. 1984).

Few studies have reported the effects of drought on the biochemistry of coniferous gymnosperms, although important species of this major plant group grow in regions of low rainfall and high evaporative demand. In coniferous gymnosperms, changes in the concentrations of free amino acids over a day, a season, and under various stresses have been well documented (Durzan 1971, Durzan 1968, Malhotra and Sarkar 1979, Kim et al. 1988). Cyr (1983) reported that total and specific free amino acids, including arginine and proline, accumulated in water-stressed shoots and roots of several coniferous species. The increase in proline induced by osmotic stress was observed in cultured callus of Pinus Taeda (Newton et al. 1986).

Ponderosa pine (Pinus ponderosa Dougl. ex Laws.) is a drought-tolerant coniferous gymnosperm that predominates in arid, forested regions of western North America. Read (1980), by analyzing 13 traits in provenance tests of seedlings, found significant genetic differences in the two major varieties of ponderosa pine, var. ponderosa and var.scopulorum. Differences in traits were greatest between seedlings of var. scopulorum originating in the Southern Rockies and those of var. ponderosa originating in the North Plateau. Seedlings

originating from the North Plateau demonstrated a strong growth vs. environmental response: the tallest seedlings showed the greatest winter damage. No trait, however, directly related to tolerance of drought stress. Houpis et al. (1988) reported that the two varieties having widely separated seed sources (California and Colorado) demonstrated a biochemical difference in pigment concentrations under long term elevated CO_2. Comparison of the varietal response to drought has not been reported.

The primary objective of this study is to compare the altered amino acid metabolism in light-limited and drought-stressed plants of a drought tolerant species in order to clarify the relative importance of specific amino acids to tolerance of drought stress. This paper reports on changes in free amino acids in unshaded and shaded needles of ponderosa pine seedlings during progressive drought stress and compares the response to drought stress of the two varieties, var. ponderosa and var. scopulorum.

Materials and Methods

Plant Materials

Seed of P. ponderosa var. ponderosa (Oregon) originated in the Ochoco National Forest of central Oregon, and seed of P. ponderosa var. scopulorum (Arizona) originated in the Kaibab Plateau of northern Arizona. The Oregon and Arizona seed sources represent, respectively, the North Plateau and Southern Rockies regions. Seed from Oregon was stratified for 30 days. All seed was surface sterilized and sown in plastic tubes (165 cm^3, Ray Leach, Aurora, OR) in a growing medium of potting soil, vermiculite and perlite (2:1:1). Seedlings were reared in a greenhouse during the spring and summer of 1987 under a day/night temperature of approximately 25/15^oC, watered daily and fertilized twice weekly with Peters 20-20-20 (Grace and Co., Allentown, PA) for 14 weeks.

Shading and Drought Treatments

A block of Arizona (var. scopulorum) seedlings were shaded 10 days before the commencement of drought and for the duration of the study. Irradiance measurements taken in the greenhouse with a quantum sensor (LiCor 190S) indicated an average photosynthetic photon flux density of 700 μmol photons $m^{-2}s^{-1}$ PAR with a range of approximately 300-1200 μmol photons $m^{-2}s^{-1}$ PAR. Shading

reduced the irradiance to 10% of that received by the unshaded seedlings.

The drought study commenced by withholding water when seedlings were approximately 15 weeks-old. Seedlings were sampled every two days over the course of the drought. To determine the water status of the seedling at the time of sampling, measurements of xylem water potential (Ψ_x) were taken with a pressure chamber (Cleary and Zaerr 1980). The drought ended when Ψ_x was -5.0 MPa in all seedlings sampled for two consecutive days. The seedlings were rewatered, and 24 and 48 hours later, recovery in water status was measured. Survival data was taken 3 months after the drought ended.

Needle Sampling and Amino Acid Analysis

Two needles from three intervals on the epicotyl (six total) were harvested for the determination of relative water content (RWC) of sampled individuals. For the amino acid analysis, approximately 200 mg of needles were harvested from the upper two-thirds of the epicotyl, placed in small plastic bags and immediately frozen in liquid nitrogen. Samples were stored in a freezer at -80^{o}C.

Most of the var. scopulorum seedlings developed only primary needles, whereas, the var. ponderosa seedlings

had developed secondary (fascicled) needles as well. Both types were sampled from the seedlings.

The extraction, derivatization and analysis of amino acids from needle tissue is by the method of N. C. Vance and J. B. Zaerr (unpublished). Briefly, amino acids were extracted from needles in methanol, chloroform and water (15:5:3 v/v/v) and 80% ethanol, then dried under vacuum. Samples were resolubilized in 0.01 N HCl and stored at -20^{o}C until chromatographed.

For chromatography by HPLC, samples were derivatized overnight in the dark with 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl) and injected into a Beckman Ultrasphere ODS C18 reversed-phase column (San Ramon, CA). Amino acids were separated by HPLC (Varian, Palo Alto, CA) with a programmed gradient of sodium acetate (pH 3.8) buffer and acetonitrile. Detection was by a fluorescence spectrophotometer (Perkin-Elmer, model 650-10S, Germany). Analysis was by Maxima computer software (Waters WIRC, Millipore Corp., Milford, MA).

Results

The total concentration of amino acids changed significantly under the influence of water deficit and shade (Table 3.1). ANOVA and analysis of means by Fisher's protected LSD indicated a significant interaction among the two varieties and the drought-stressed, shaded seedlings.

Shading increased the total amino acid concentration whether or not seedlings were drought stressed (Table 3.2). However, the drought-induced increase in total amino acid concentration was greater in the unshaded Arizona (var. scopulorum) than in the shaded Arizona, or the Oregon (var. ponderosa) seedlings. The concentration of specific amino acids was also altered by drought stress and low light (Table 3.2). Asparagine, serine, arginine,glutamic acid, isoleucine and tyrosine concentrations were significantly higher in the shaded Arizona seedlings under unstressed and drought-stressed conditions. A significant increase in the concentration of alanine, γ-aminobutyric acid (GABA), proline, valine, phenylalanine, leucine and histidine was detected in the unstressed but shaded plants.

Concentrations of specific amino acids also changed under increasing water deficits. Asparagine, arginine, γ-aminobutyric acid, proline, phenylalanine and

The trends among specific amino acids that changed under drought stress were indicated by plotting their concentrations over increasing water loss (RWL, RWL = RWC - 100). Proline and arginine concentrations increased as RWL increased; however, the amount of increase differed among treatments and varieties (Figures 3.1A, 1B). Proline did not increase until RWL reached 40% or higher in the unshaded var. ponderosa seedlings, but then increased dramatically. Proline accumulated in the var. ponderosa seedlings but to a lesser extent. Glutamic acid decreased among treatments and varieties until the RWL reached approximately 40%; then it increased Figure 3.2A). Glutamine concentrations increased over increasing RWL until RWL reached about 55%, at which point, glutamine decreased (Figure 3.2B). Aspartic acid concentrations decreased with increasing water deficit among treatments and varieties (Figure 3.3A). The relative increase in total free amino acids over increasing RWL was greatest in the unshaded var. scopulorum seedlings (Figure 3.3B). The higher variability in the total amino acid concentrations of the shaded seedlings, particularly those unstressed, is evident in the spread of data points. The amino acids that contributed most to the variability were arginine and glutamine (Figures 3.1B, 3.2B).

Analysis three months after the termination of drought indicated differences in seedling survival. Of the seedlings that had been stressed to Ψ_x = -4.0 MPa, 53% of the unshaded var. scopulorum, 52% of var. ponderosa, and none of the shaded var. scopulorum seedlings survived.

Discussion

Drought stress resulted in the accumulation of total free amino acids. However, this increase was due primarily to the accumulation of proline and arginine in the unshaded seedlings. The abrupt increase in proline in the unshaded scopulorum seedlings when RWC reached 60% occurred well below the point of turgor loss and at a severe stage of water stress. It seems unlikely that this accumulation so late in the progress of water loss constitutes a primary function of osmotic adjustment. However, accumulations of proline to high levels do not suggest irreversible damage leading to death. Forty-eight hours after the scopulorum seedlings were rewatered, their water status recovered to that of unstressed seedlings (mean Ψ_x = -0.6 MPa, n = 4). Concentrations of proline remained elevated in needles of rewatered seedlings 24 hours (21.86 µmol/g dw) and 48 hours (9.74 µmol/g dw) later. The concentrations were apparently declining over time indicating proline was being oxidized. Quantitative and qualitative analysis of proteins synthesized in these seedlings also indicated recovery had occurred (N. C. Vance, J. B. Zaerr and D. W. Copes, unpublished).

Arginine and glutamine, the other principle amino acids to accumulate under increasing water deficit,

together with proline accounted for over 89% of the free amino acids in stressed needles of the unshaded var. scopulorum seedlings. These three amino acids are derived from α-ketoglutarate and belong to the glutamate family. Arginine and glutamine are important in nitrogen storage and transport having a N:C ratio of 1:2 or better (Miflin and Lea 1977). Kim et al. (1987) reported that arginine, the amides and proline increased significantly with increased nitrogen fertilization in jack pine (Pinus banksiana) and black spruce (Picea nigra). However, nitrogen uptake is not a factor under conditions of drought; therefore, the increase in these amino acids may reflect the refixation of endogenous NH_3 that would otherwise accumulate to toxic levels in stressed needles possibly as a result of photorespiration or protein catabolism. High concentrations of arginine may decrease the stability or activity of enzymes (Hochachka and Somero 1984). The accumulation of proline along with arginine may have a counteracting effect on the latter's destabilizing potential, thus, proline may act as compatible solute.

The drought-stressed var. ponderosa seedlings accumulated less proline and arginine, but more glutamine than did the stressed, unshaded var. scopulorum seedlings; however, the var. ponderosa seedlings recovered upon rewatering and had similar survival rates

three months later. Although the data suggest a metabolic difference between the two varieties, it is not apparently sufficient to affect the role of individual amino acids in drought tolerance, since both varieties had high survival well after relief of the drought. The lower accumulations of the amides and arginine in ponderosa than in scopulorum seedlings could imply lower pools of NH_3. The lower accumulation of proline and arginine in the var. ponderosa seedlings also suggests lower photoassimilate and, therefore, lower substrate pools. Glutamate, the common precursor of the three amino acids, is the primary NH_4+ acceptor as well as product of ammonia assimilation (Miflin and Lea 1977). Enzymes in the biosynthetic pathways of arginine and proline may be in competition for substrate; if so, accumulations may be dependent, in part, on a delicate balance of enzyme activity and substrate availability. Needles of var. ponderosa were reported to be more metabolically sensitive to CO_2, having lower pigment concentrations and growth rates, than were those of var. scopulorum; (Houpis et al. 1988). Thus, there may be real metabolic differences in response to stresses, a subject that warrants more detailed study.

Proline accumulation did not occur to any significant level in the shaded seedlings until they were severely drought-stressed (RWC was less than 50%).

Stewart (1972) and Joyce et al. (1984) noted that light is necessary for proline accumulation, although irradiance below the compensation point will not prevent proline from accumulating to a lesser extent. The authors interpret the minimal light requirement as a requirement for reducing power or photoassimilate to provide substrate for proline synthesis in the leaves.

Although shading reduced the accumulation of proline in the study seedlings, altered concentrations of amino acid apparently had no direct effect on reducing drought tolerance. The altered concentrations do suggest, however, that carbohydrates and reducing power may have been in short supply. No shaded seedlings recovered at levels of drought stress comparable to those of unshaded seedlings. However, protein synthesis, including synthesis of the CO_2 assimilating enzyme ribulose 1,5-bisphosphate carboxylase, declined drastically in the stressed, shaded seedlings (N. C. Vance, D. W. Copes and J. B. Zaerr, in review). Concentrations of cellulose were reduced in shaded seedlings; they may have died partially because of failure in the structural integrity of needle tissue (N. C. Vance and J. B. Zaerr in review).

Although arginine accumulated in the needles of drought-stressed, shaded seedlings, glutamine did not similarly increase. Arginine, proline and glutamine contributed to 59% of the total amino acids in the

stressed, shaded seedlings. The proportionally lower concentrations of these amino acids in the drought-stressed, shaded seedlings suggest a diminished response to drought stress, ie., a consequence of limited photoassimilates and energy, or the significant accumulation of amino acids that has already occurred. The accumulation of arginine may also be controlled through the inhibition of its catabolism by NH_3, a catabolic end-product. In this case, arginine would serve not only as a nitrogen storage compound but as a detoxifier of ammonia as well. That glutamic acid increases and glutamine decreases when drought stress is severe, may indicate a disruption in the activity of the enzymes glutamine synthetase and glutamate synthase. Analysis of changes in these amino acids, both of which play a key role in NH_3 assimilation, coupled with measures of enzyme activity and levels of NH_3 might provide insight on how stress affects nitrogen metabolism.

Table 3.1 Results from analysis of variance of total amino acid concentrations in needles of *Pinus ponderosa* seedlings. ANOVA was performed on log-transformed data of unstressed and drought-stressed, unshaded and shaded seedlings of var. *scopulorum* and drought-stressed seedlings of var. *ponderosa*. RWC > 84% and < 69% of unstressed and drought-stressed seedlings, respectively.

Source of Variation	DF	F Value	Prob. > F
A. Drought stress	1	28.50	0.0001
B. Varieties and shading	2	27.59	0.0001
C. Interaction of A x B	2	4.75	0.0161

Table 3.2. Free amino acids extracted from needles of drought-stressed Pinus ponderosa seedlings and separated by HPLC. Comparisons are among unshaded and shaded var. scopulorum and unshaded var. ponderosa seedlings.

Amino Acid	Amino acid concentration (μmol g^{-1} dw of needles)[1]						
	------- unstressed ------- varieties				---- drought-stressed ---- varieties		
	Ponderosa Unshaded	Scopulorum Unshaded	Scopulorum Shaded		Ponderosa Unshaded	Scopulorum Unshaded	Scopulorum Shaded
Asparagine	0.49 a	0.34 a	2.69 b	*	0.90 a	0.85 a	4.27 b
Glutamine	1.74 a	1.91 a	5.91 a		4.96 a	3.61 a	5.48 a
Serine	1.18 a	1.74 a	6.01 b		1.67 a	1.07 a	5.49 b
Aspartic acid	1.93 a	1.46 a	1.26 a	*	0.87 b	0.28 a	0.59 b
Arginine	3.67 a	3.57 a	16.21 b	*	7.84 a	15.15 b	22.12 ab
Glutamic acid	1.86 a	1.70 a	2.59 a		1.29 a	1.62 a	4.14 b
Glycine	0.20 a	0.11 a	0.19 a		0.16 a	0.11 a	0.24 a
Alanine	0.70 a	0.59 a	3.07 b		0.86 a	1.19 a	1.69 a
Methionine	0.05 a	0.05 a	0.11 a		0.03 a	0.04 a	0.01 a
$-NH_2$	1.20 a	1.32 a	1.29 a		1.03 a	0.71 a	0.94 a
γ-aminobutyric acid	0.12 a	0.13 a	0.26 b	*	0.28 a	0.87 a	0.75 b
Proline	0.43 ab	0.24 a	1.15 b	*	1.97 a	12.43 c	7.69 b
Valine	0.16 a	0.11 a	0.61 b		0.25 a	0.15 a	0.32 a
Phenylalanine	0.10 a	0.08 a	0.21 b	*	0.20 a	0.22 a	0.15 a
Tryptophan	0.16 a	0.16 a	0.35 a	*	0.34 a	0.40 a	0.96 a
Leucine	0.04 a	0.05 a	0.20 b		0.08 a	0.05 a	0.10 a
Isoleucine	0.13 a	0.16 a	0.35 b		0.13 ab	0.12 a	0.21 b
Ornithine	0.19 a	0.05 a	0.27 ab		0.08 a	0.21 b	0.16 ab
Lysine	0.36 a	0.36 a	0.50 a		0.44 a	0.66 a	0.57 a
Tyrosine	0.36 a	0.42 a	0.92 b		0.43 a	0.54 a	1.19 b
Histidine	0.22 a	0.21 a	0.54 b		0.19 a	0.43 a	0.38 a
Total	16.79 a	14.98 a	45.98 b	*	23.86 a	35.84 bc	59.77 c

[1] Each value is the mean of 6 to 8 samples. Asterisks (*) indicate significant differences between unstressed and drought-stressed seedlings. Within each of those groups, values bearing the same letter do not differ significantly ($P < 0.05$) by Fisher's protected LSD test.

Figure 3.1 Changes in concentration over increasing relative water loss (RWL) of (A.) proline and (B.) arginine in needle extracts from unshaded Pinus ponderosa var. ponderosa (◇), var. scopulorum (●) and shaded var. scopulorum (+) seedlings.

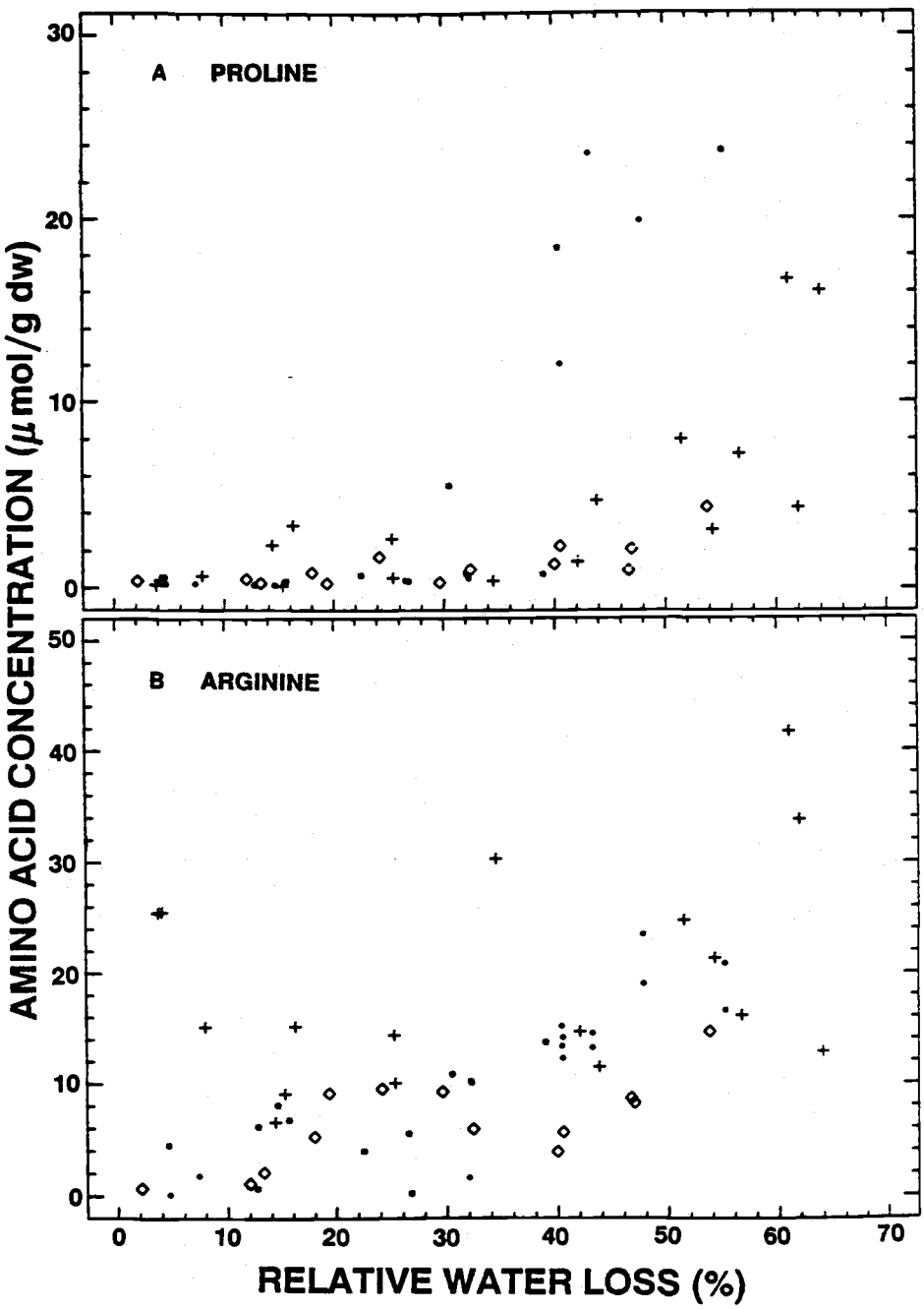
A PROLINE
B ARGININE
AMINO ACID CONCENTRATION (μmol/g dw)
RELATIVE WATER LOSS (%)
0
10
20
30
40
50
60
70

Figure 3.1

Figure 3.2 Changes in concentration over increasing relative water loss (RWL) of (A.) glutamic acid and (B.) glutamine in needle extracts from unshaded Pinus ponderosa var. ponderosa (◇), var. scopulorum (●) and shaded var. scopulorum (+) seedlings.

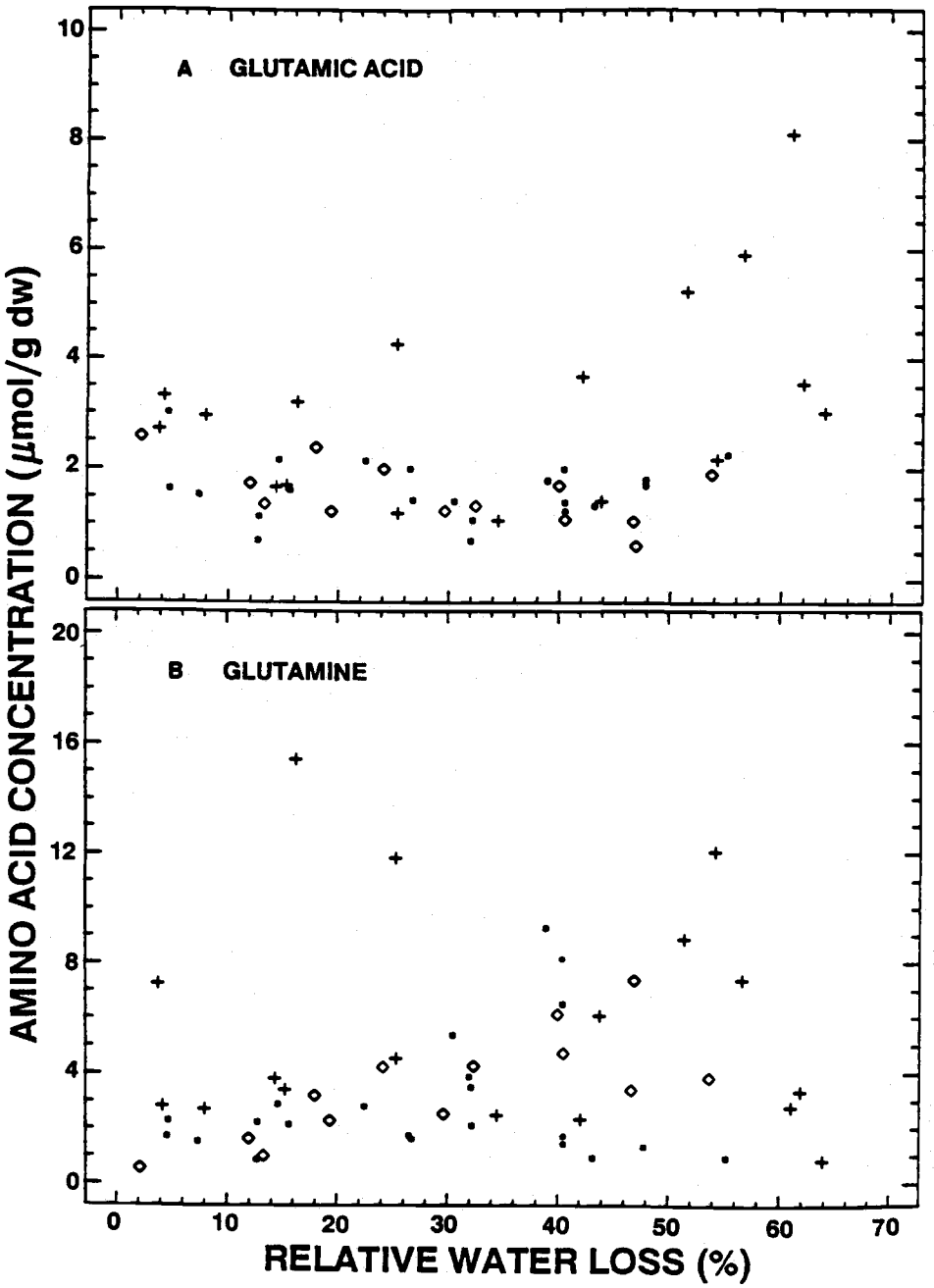
A GLUTAMIC ACID
B GLUTAMINE
AMINO ACID CONCENTRATION (μmol/g dw)
RELATIVE WATER LOSS (%)
0
2
4
6
8
10
12
16
20
0
10
20
30
40
50
60
70

Figure 3.2

Figure 3.3 Changes in concentrations over increasing relative water loss (RWL) of (A.) aspartic acid and (B.) total amino acids in needle extracts from unshaded Pinus ponderosa var. ponderosa (◇), var. scopulorum (●) and shaded var. scopulorum (+) seedlings.

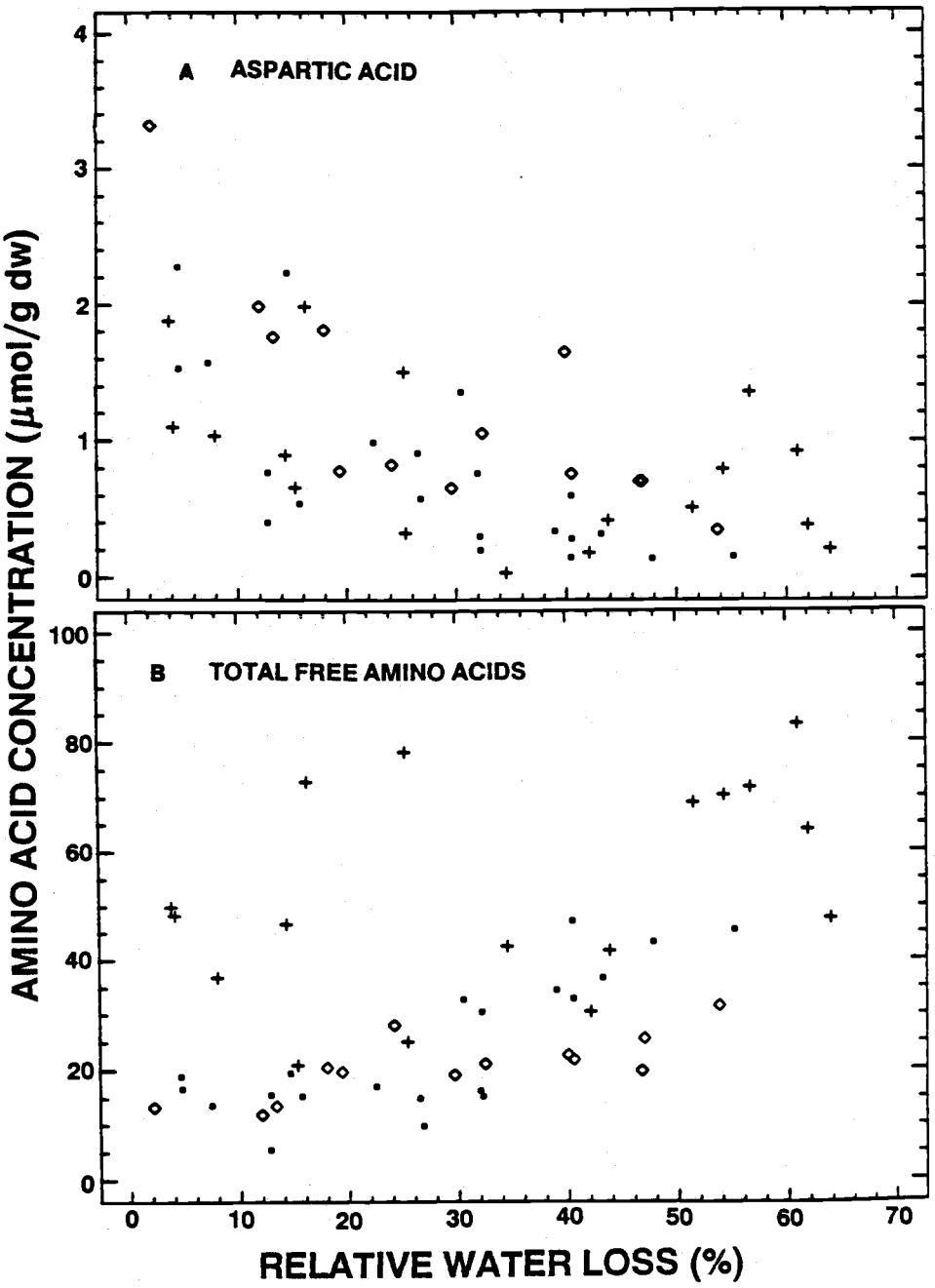
A ASPARTIC ACID
B TOTAL FREE AMINO ACIDS
AMINO ACID CONCENTRATION (µmol/g dw)
RELATIVE WATER LOSS (%)
0
1
2
3
4
0
20
40
60
80
100
0
10
20
30
40
50
60
70

Figure 3.3

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CHAPTER 4

INFLUENCE OF DROUGHT STRESS AND LOW IRRADIANCE ON PROTEIN SYNTHESIS IN NEEDLES OF *PINUS PONDEROSA* SEEDLINGS

Nan C. Vance, Donald O. Copes and Joe B. Zaerr

Abstract

Soluble proteins extracted from needles of unwatered 15 week-old seedlings of *Pinus ponderosa* (Dougl. *ex* Laws.) were analyzed by 1- and 2-D gel electrophoresis for changes in patterns of protein synthesis induced by drought stress. *P. ponderosa* var. *ponderosa* and var. *scopulorum* were compared to detect varietal differences. Detection of polypeptides was by silver stain, in vivo labeling with [^{3}H]-leucine and fluorography. Immunodetection was used to determine if specific proteins synthesized under heat, osmotic or salt (NaCl) stress were similar to those induced by drought stress. Decline in the synthesis of ribulose 1,5-bisphosphate carboxylase and changes in the patterns of polypeptides were observed as drought stress progressed. A set of low M_r proteins were synthesized in stressed, unshaded seedlings (water potentials < 3.0 MPa and relative water contents < 69%). These proteins were not detected, or

appeared at very low levels in the light-starved seedlings. When drought-stressed seedlings were rewatered, seedlings recovered and protein synthesis returned to that of the unstressed seedlings. However, when light-starved seedlings, similarly stressed, were rewatered, they did not recover. Antibodies against the 72-83 kDa heat-shock proteins in maize cross-reacted to 68-82 kDa proteins in needles of control, heat- and drought-stressed seedlings. Antibody against a 26 kDa osmotic stress-induced protein in tomato cross-reacted to 27 and 28 kDa proteins in salt-stressed, but not in drought-stressed, seedlings. Drought stress induces the synthesis of a set of proteins which are associated with plants that recover and differs from osmotic stress in not inducing the synthesis of at least one protein, prominent in osmotic-stressed plant tissue.

Introduction

Investigations of the effects of various environmental perturbations on gene expression in higher plants have demonstrated stress-induced alteration in gene expression resulting in qualitative as well as quantitative changes in synthesized proteins (reviewed by Adams and Rinne 1982, Sachs and Ho 1986). However, there is relatively little information on the effects of drought stress on gene expression, although drought is one of the primary environmental limitations to crop productivity.

Although water stress-induced changes in gene expression have been studied, few studies use drought to impose water stress. Bewley et al. (1983) reported a decrease in the synthesis of constitutive proteins and enhanced production of other proteins in maize mesocotyls that were water stressed by PEG. Singh et al. (1985) reported that several novel proteins were synthesized in cultured tobacco cells undergoing osmotic adjustment, including a 26 kDa protein that increased under salt, PEG, or desiccation stress (Singh et al. 1987). This 26 kDa protein was also induced in osmotic-stressed roots and leaves of whole plants (King et al. 1986). Changes in protein patterns of drought-stressed Brassica napus roots was reported by Vartanian et al. (1987) who

detected the appearance of 13 new low M_r polypeptides on silver-stained, two-dimensional gels. The authors suggested that the appearance of these polypeptides was related to an adaptive response to drought.

The effect of drought on the metabolism and gene expression of Pinus species is of interest because this genus of coniferous gymnosperm has adapted successfully to drought in semi-arid regions, world-wide. Hulbert et al. (1988) noted the inhibition of protein synthesis in mannitol-stressed hypocotyls of Pinus taeda, and Valluri et al. (1987) reported the effects of mannitol-induced water stress on protein synthesis in Pinus elliottii. Studies have demonstrated that osmotic and drought stress do not elicit the same metabolic responses (Mexal et al. 1975, Jacomini et al. 1987); therefore, it is likely that protein synthesis in response to the two types of water stress would not be the same.

Comparing mosses that are tolerant and intolerant of dehydration was an approach successfully applied to characterize effects of dehydration on plant ultrastructure, metabolism (Krochko et al. 1978) and protein synthesis (Dhindsa and Bewley 1977). Reducing a plant's adaptivity to a specific stress by altering its environment, may also allow comparisons to be drawn. Extremely low irradiance, by limiting available photosynthate and energy, introduces additional stress to

the plant and reduces its tolerance of drought stress (N.C. Vance and J.B. Zaerr, unpublished). It is not known, however, if light starvation will affect the amount and kinds of proteins normally synthesized in drought-stressed plants.

In this paper, we examine qualitative and quantitative changes in patterns of synthesized proteins in needles extracts of drought-stressed seedlings of the drought tolerant conifer (Pinus ponderosa Dougl. ex Laws.). We compare proteins synthesized in two varieties of P. ponderosa and in light-starved seedlings of one variety to determine if the appearance of stress proteins may be related to dehydration tolerance. Drought-induced gene expression is characterized in part by comparing proteins synthesized under increasing drought stress with proteins synthesized under heat and osmotic stress.

Materials and Methods

Plant Material and Culture

Seed of Pinus ponderosa, vars. scopulorum and ponderosa, were donated by the USDA Forest Service, Rocky Mountain Forest and Range Experiment Station and Pacific Northwest Forest and Range Experiment Station, respectively. The seed sources were the Southern Rocky Mountains (var. scopulorum) and the Pacific Northwest (var. ponderosa). Ponderosa pine germinants were reared in a greenhouse under 25/15°C (± 5°C) day/night temperatures, watered daily and fertilized twice weekly. Unshaded seedlings received average photon flux density (PPFD) under full light in the greenhouse of 700 μmol photons $m^{-2}s^{-1}$. Light-starved seedlings were shaded to 10% full light when they were 15 weeks old and 10 days before the commencement of drought. Drought was imposed by withholding water from the seedlings until severe plant-water deficits were achieved. During the period of drought, increasing water deficit was monitored by measuring predawn xylem water potential (Ψ_x) with a pressure chamber (Cleary and Zaerr 1980). Drought was discontinued when Ψ_x of four consecutively sampled seedlings was < -5.0 MPa. The unshaded and shaded seedlings were rewatered after 31 days and 40 days,

respectively. In addition to measuring Ψ_x, relative water content (RWC) of the needles was determined.

Radioactive Labeling

After predawn Ψ_x was measured in a sampled seedling, radioactivity was incorporated into leaf tissue by introducing L-[3,4,5-^{3}H]leucine (37 MBq/ml; New England Nuclear, Boston) into the conducting tissue of the stem severed just above the cotyledons. The seedling was placed in the pressure chamber; pressure was applied until sap appeared on the surface of the cut stem. Pressure was slowly increased until approximately 6 μl of sap was expressed. The same volume of 10x concentrated 3H-leucine (2.22 MBq/μl) was applied to the stem and infiltrated as pressure was slowly released. The seedling was removed from the chamber and secured in a plastic container in 100% relative humidity and placed overnight in the greenhouse. Approximately 18 hours later, predawn Ψ_x was re-measured. If Ψ_x changed by + 0.2 MPa the sample was rejected. Otherwise, needles were stripped from the epicotyl, quickly weighed to the nearest mg, placed in plastic bags and plunged into liquid nitrogen. The samples were stored at -80^{o}C until proteins were extracted.

Heat and Osmotic Stress Experiments

To compare the effects of heat and drought stress on protein synthesis, four unstressed seedlings were labeled with [^{3}H]leucine, as previously described. Approximately 15 hours after label was introduced, the seedlings were placed in a heated chamber in high humidity and subjected to 48^{o}C for an additional 3 hours. At the termination of the heat treatment, Ψ_x was measured, needles weighed and stored at -80^{o}C.

To compare osmotic and drought stress, twelve 3-month-old seedlings were used. Four unstressed seedlings were watered daily and eight were watered 10 days with 200 mM NaCl. Ψ_x measurements and growth reduction indicated the salt-treated seedlings were osmotic stressed. Needles were harvested from the seedlings, weighed, and stored at -80^{o}C until proteins were extracted.

Protein Extraction and Electrophoresis

Approximately 300 mg of frozen leaf tissue were ground in liquid nitrogen with mortar and pestle. The powder was rapidly suspended in 15 ml of an extraction buffer consisting of 50 mM Tris-HCl (ph 6.8), PVPP (0.125 g/ml), 2% w/v SDS, 20 mM sodium metabisulfite, 20 mM DIECA, and 2% v/v β-mercaptoethanol. The slurry was expressed through Miracloth and centrifuged at 12,000 g

for 20 min. Proteins were precipitated in 4 volumes of acetone. Acetone was evaporated under N_2, and the pellet suspended in sample buffer (Laemmli 1977) and boiled for 2 min. Samples were stored at -80°C. Total proteins were quantitated by the the Coomassie Brilliant Blue dye-binding technique of Bradford (1976) with a commercial preparation (Bio-Rad). Amino acid incorporation was determined by TCA precipitation on filter-paper disks (Mans and Novelli 1961) and by counting with a scintillation spectrophotometer.

SDS-Page was performed according to Laemmli (1977) on 14% (w/v) acrylamide gels. Two dimensional SDS-PAGE was performed by the method of O'Farrell (1975) with some modifications. Samples for IEF in the first dimension were prepared by adding lysis buffer in a ratio of 2:1 to an aliquot of sample in Laemmli buffer. Total ampholytes, (pH 5-7 and pH 3.5-10, Serva), were increased to 4% in sample and gel solutions. The effective focusing range was determined to be from pH 4.7 to 7.9. Approximately 2.0×10^4 cpm for SDS-PAGE in one dimension, or 3.5×10^4 cpm for IEF were loaded per sample on 1.5 mm gels. Molecular weight standards were co-electrophoresed with samples. Proteins separated in one dimension were detected by Coomassie blue stain and fluorography. Detection of proteins on 2-D gels was by silver stain (Bio-Rad) and fluorography. Fluorography

was performed on replicates of silver-stained gels that had been dried, fixed, soaked in EnHance (New England Nuclear), apposed to Kodak XAR-5 film and stored at -80°C from 6 to 13 weeks.

Immunodetection of Proteins

Proteins from heat-, drought- or salt-treated tissue were separated by 1-D SDS-PAGE and subjected to electrophoretic transfer (Trans-Blot cell, Bio-Rad) onto nitrocellulose paper (NCP). Immuno-assays were performed with horseradish peroxidase detection kits (Vector Laboratories, Burlingame, CA) containing biotinylated secondary antibodies against the primary polyclonal antibodies raised in rabbits. Polyclonal antibodies to high molecular weight hsps (73-89 kDa) were donated by Dr. C.L. Baszczynski, Department of Plant Sciences, University of Western Ontario. Polyclonal antibody to the 26 kDa osmotic-stressed induced protein was donated by Dr. G.J. King, NPI, Salt Lake City. Immunodetection was also used to verify identification of large subunit (LSU) and small subunit (SSU) of ribulose 1,5-bisphosphate carboxylase (Rubisco). Antibody against the holoenzyme of Rubisco prepared in rabbits was donated by Dr. E.J. Pell, Department of Plant Pathology and Center for Environmental Studies, Pennsylvania State University.

Results and Discussion

As water deficits increased in the needles of unshaded, drought-stressed seedlings, the incorporation of amino acids into proteins rose, declined slowly until about 60% RWC then more rapidly at <60% RWC (Figure 4.1). Surprisingly, needles of light-starved seedlings consistently incorporated radioactive label with water deficits below 60% RWC. As long as there was uptake, incorporation occurred. In most cases, incorporation was limited by failure of the seedling to take up labelled precursor which probably was the result of hydraulic failure in the conducting tissue.

Little difference is detectable in patterns of polypeptides among the Coomassie-stained gels of proteins extracted from increasingly drought-stressed seedlings of var. scopulorum and resolved by SDS-PAGE (Figure 4.2A). Prominent bands are the large subunit (LSU) and small subunit (SSU) of Rubisco at 53 and 14 kDa, respectively. Differences in intensity among protein bands begin to show in the corresponding fluorographs (Figure 4.2B). Lanes 1 through 5 represent synthesized proteins from unstressed to moderately drought-stressed seedlings (95-70% RWC). Lanes 6 through 8 represent increasingly severe drought stress (68-61% RWC). The synthesis of most polypeptides including LSU and SSU (marked with

arrows) decreased as drought stress progressed. At RWC < 68%, the appearance of a set of new, or enhanced, polypeptide bands ranging between 14 and 30 kDa is evident. One low M_r polypeptide band that noticeably increased in intensity as RWC decreased, appears adjacent and below SSU at approximately 13.5 kDa. Polypeptides (marked by darts) ranging from 68-88 kDa and two higher relative M_r polypeptides (90,92 kDa, not marked) apparently accumulated with increased drought stress. These polypeptides correspond in M_r to the high M_r heat shock proteins (hsps) characterized by Key et al. (1981) and Baszczynski et al. (1982). Twenty-four hours after the termination of drought stress, the pattern of protein synthesis in severely stressed seedlings (Lane 9) returned to nearly that of the unstressed seedling (Lane 1) which demonstrates that severely drought-stressed seedlings (approximately 60% RWC and Ψ_x < 4.0 MPa) can resume normal protein synthesis and suggests that cellular structures and translational apparatus are intact or rapidly repaired. Analysis of translational events over the first 24 hours after rewatering and studies of the cell's ultrastructure may elucidate further the mechanisms of conservation of protein synthesis with tissue dehydration and rehydration.

Representative fluorographs of electrophoresed proteins extracted from needles of var. ponderosa (Figure

4.3A) demonstrate that patterns of protein synthesis are similar to those of var. scopulorum (Figure 4.2B). Fluorographs of 1-D gels show little varietal difference in protein synthesis over progressive drought stress. However, the synthesis of LSU in var. ponderosa appeared to decrease at higher values of RWC than in var. scopulorum. Reduction in band intensity in other fluorographs, as well as the ones shown here, indicate that relative synthesis of LSU is less in var. ponderosa than in var. scopulorum at comparable levels of stress. Increasing intensity of a polypeptide band just below SSU with the progression of drought stress confounds its interpretation on 1-D gels.

Disappearance of the band corresponding to LSU. occurred to an even greater extent in the light-starved seedlings of var. scopulorum (Figure 4.3B). In addition, as drought stress progressed, the severely shaded seedlings demonstrated a distinctly different pattern of protein synthesis from that of the unshaded seedlings. The drought-stress induced low M_r polypeptide bands detected in the unshaded seedlings appear fewer and relatively less intense. Synthesis of polypeptides in the range of 68-82 kDa appeared to increase relative to others, appearing as prominent bands in Lanes 6 and 7 which represent proteins in needles of severely drought-stressed seedlings (58-52% RWC).

The greater resolution of polypeptides on 2-D gels enhanced the identification of changes in specific polypeptides. In Figure 4.4, fluorographs of 2-D gels of proteins extracted from needles of unshaded (Figure 4.4A,B, E,F) and shaded (Figure 4.4C,D) seedlings at various stress levels correspond to fluorographs of 1-D gels in Figure 4.2 and Figure 4.3. Only those polypeptides between 18 and 30 kDa that were unambiguously identified as increasing, or newly synthesized, are marked. Two polypeptides (marked with open arrows), are representative of constitutive proteins that decreased under progressive drought stress. A high M_r polypeptide that increased in stressed plants is clustered with polypeptides of the same M_r as the high molecular weight hsps; however, the M_r and isoelectric points of the hsps in the 2-D gels were not determined.

Fluorographs of unshaded seedlings of var. scopulorum that were unstressed (95% RWC), moderately (67% RWC) and severely (60%) drought stressed, and recovered (92% RWC) are shown in Figure 4.4A and B, and Figure 4.5A and B respectively. The fluorographs in Figure 4.4A and B are comparable to fluorographs of 1-D gels in Figure 4.3, Lanes 1 and 6, respectively. The IEF dimension of 2-D PAGE further resolved drought-stress induced polypeptides into seven major polypeptides of low M_r (marked with darts), henceforth referred to as dsps.

LSU and SSU are synthesized relatively less under severe water deficit (67% RWC), but surprisingly, are still synthesized at 61% RWC, although, at much reduced levels (Figure 4.5A). In the recovered seedling, the pattern of protein synthesis has returned nearly to that of the controls (Figure 4.5B). The association of stress-induced proteins in needles that retain the ability to synthesize Rubisco and the recovery of comparably drought-stressed seedlings suggest a relationship between the strongly synthesized dsps and a mechanism for tolerating drought stress at the cellular level.

On a representative fluorograph of proteins extracted from light-starved and moderately stressed (75% RWC), or severely stressed (59% RWC), seedlings of var. scopulorum and separated by 2-D PAGE, low M_r dsps are faintly visible or are not detectable (Figure 4.4C and D, corresponding to Lanes 4 and 8, Figure 4.3B). The dsps appear not to be synthesized, or synthesized much less than the dsps in the unshaded seedlings (Figure 4.4,B,D and F). When rewatered, light-starved seedlings did not recover; however, the light-starved seedlings were lower in RWCs than the unshaded seedlings at similar Ψ_x (N.C. Vance and J.B. Zaerr, unpublished). There is no way of predicting that the seedling represented by the fluorographs presented here would, or would not, recover upon rewatering. Nevertheless, examination of 32

fluorographs of 2-D gels provides evidence that the synthesis of stress-induced proteins did not preclude recovery. The dsps proteins extracted from stressed seedlings were sampled from a population with high recovery and survival rates.

Conversely, during progressive drought stress, dsps accumulated less, or were not synthesized in the light-starved seedlings. Concurrent water relations analyses which included seedlings used in this study, demonstrated that the light-starved seedlings were less drought tolerant having higher mortality rates at comparable Ψ_x (N.C. Vance and J.B. Zaerr, unpublished). The polyeptides detected in fluorographs of light-starved seedlings under relatively severe dehydration suggest that protein synthesis may continue while cells are severely drought stressed. However, light-starved seedlings have thinner cell walls which are more fragile than their unshaded counterparts. The result is a loss of cellular integrity when severely stressed (N.C. Vance and J.B. Zaerr, unpublished) and may explain why rehydration was lethal. It should be noted that because each seedling was sampled at a point over the course of progressive water loss and because samples are points on a continuum, the RWC at which dsps are first synthesized in any one seedling can only be estimated. However, in this study no dsps were detected in fluorographs of

unshaded seedlings at >70% RWC; whereas, out of eight, 1- and 2-D fluorographs of light-starved seedlings, the greatest accumulation of dsps detected was in one fluorograph presented in Figure 4.4C.

The fluorographs presented in Figures 4.4E and 4.4F are of representative 2-D gels of proteins extracted from unstressed (92% RWC) and severely stressed (60% RWC) seedlings of var. ponderosa (corresponding to Lanes 1 and 8, Figure 4.3). Synthesis of dsps at equivalent levels of stress appears similar to that of var. scopulorum: two polypeptides of the dsp cluster in var. ponderosa are not synthesized under severe drought stress, and are missing in the dsp cluster in var. scopulorum at a comparable level of stress (Figure 4.5A).

The greater resolution of 2-D PAGE gels reveals the drastic reduction in synthesis of LSU and SSU under conditions of severe stress in all seedlings. Surprisingly, synthesis of LSU does occur in a severely drought stressed seedling of var. scopulorum (Figure 4.5A). Examination of all the gels indicate that there is a more rapid relative decrease in the synthesis of LSU in var. ponderosa (Figure 4.3A) than in var. scopulorum (Figure 4.2B), and total incorporation of precursor appears slightly less at comparable RWC (Figure 4.1); nevertheless, seedling recovery and survival were similar.

The difference between the two varieties in synthesis of Rubisco, which constitutes the major portion of leaf protein, may be attributed to different needle growth characteristics. Young seedlings of var. ponderosa characteristically grow primary needles; whereas, similarly aged seedlings of var. scopulorum also develop secondary (fascicled) needles. Needles of the two varieties differed in sensitivity of needle pigments to elevated CO_2 (Houpis it al. 1988) and may differ also in sensitivity of Rubisco to stress. Differences between the two needle types should be investigated before conclusions can be made about genetic differences in leaf protein metabolism of the two varieties.

A representative silver-stained gel compared with its fluorographic replicate provides evidence of protein turnover; SSU is still prominent in the silver-stained gel (Figure 4.5C); whereas, synthesis of the protein in the corresponding fluorograph (Figure 4.5A) appears to have almost ceased. Conversely, after 24 hours of recovery, the residual low M_r dsps are still detectable in the silver-stained gel (Figure 4.5D), but no longer appear in a comparable fluorograph (Figure 4.4B). Several dsps that stained pale yellow are not distinct. It is notable that polypeptides with this altered color on silver-stained gels previously have been determined to be glycoproteins (Goldman et al. 1980).

Because the induction of high M_r hsps by water stress has been demonstrated in other plants, characterizing such ubiquitous stress proteins may provide insight on the commonality and differences in functional responses of plants to drought and heat stress. In Figure 4.7 the Coomassie-stained gels (A), and corresponding fluorographs (B) and Western blots (C) are representative of proteins extracted from heat-stressed (Lanes 2 and 3) unstressed (Lane 1) and severely drought-stressed (Lane 4), needle tissue subjected to SDS-PAGE. Polypeptide bands at 68-71 and 80-82 kDa are visible in protein gels of unstressed needle tissue, and increase in intensity as water deficit increases. Antisera against the 73-to 76 kDa and 84- to 89-kDa hsps in maize plumules cross-reacted with the 68-72 and 80-82 kDa polypeptides in Pinus needles. Antibody reaction to this set of proteins in unstressed needles was positive, but weaker (Figure 4.7C, Lane 1). These results support previous studies that suggest several of these highly conserved proteins are constitutive (Bazczynski 1986) and induced by drought, as well as heat, and other stresses (Heikkila et al. 1984).

To further characterize response to drought stress, immunodetection with polyclonal antibody against a 26 kDa osmotic-stress induced protein was used to compare proteins from salt- and drought-stressed tissue. Lane 1

represents electrophoresed and NCP-blotted proteins that had previously reacted positively to antibody directed against lyophilized leaf tissue of salt- and PEG-stressed tomato seedlings. Antibody against the 26 kDa protein apparently reacted with polypeptides at 24 and 26 kDa. The 26 kDa protein is susceptible to proteolysis. Proteolysis of the 26 kDa protein has been demonstrated by the appearance of protein bands of several kDa lower molecular weight (Singh et al. 1987), and may explain the higher positive reaction to a polypeptide at 24 kDa than at 26 kDa. Lyophilized tissue was shipped, stored at -20^{o}C, added directly to Laemmli sample buffer with 1mM EDTA and boiled just before electrophoresis. After crude extract was used once for electrophoresis and stored at -20^{o}C, browning of sample was visible indicating and sample degradation and possible proteolysis.

Polypeptides extracted from roots (Lane 2) and needles (Lane 3) of salt-stressed Pinus ponderosa seedlings were electrophoresed, transferred to NCP and probed with antibody. Proteins in the roots at 27 kDa and in the needles at 28 kDa cross-reacted to the antibody. Western blots of proteins extracted from unstressed roots (Lane 4), moderately (Lane 5) and severely (Lane 6) drought-stressed needles showed no detectable antibody reaction. The results suggest osmotic stress will induce specific protein(s) in

coniferous tissue that are not induced by drought. Although not demonstrated here, osmotic stress by PEG induced the 26 kDa protein in other plants (King et al. 1986) and, presumably, would have similar effects on Pinus seedlings.

Electrophoretic techniques and fluorography were used to characterize alterations in protein synthesis in response to drought stress in needles of two varieties of Pinus ponderosa. The study demonstrated that novel proteins were synthesized under increasing drought stress, and that their synthesis was inhibited by light starvation. The induction of full gene expression in response to drought stress may require light or energy, and warrants further investigation. Synthesis of Rubisco decreased but still occurred in unshaded seedlings that were severely drought stressed. A varietal difference in the synthesis of this enzyme under severe water deficit may be based on needle differences. Failure to detect the 26 kDa protein induced by osmotic stress in drought-stressed tissue suggests there are distinct differences in the induction of gene expression by the two types of water stress. Future studies comparing gene expression induced by PEG-imposed stress and drought stress may further distinguish between the respective responses. Differences in response would have important implications

in evaluating the physiology and adaptivity of water-stressed plants.

Figure 4.1 The trend in incorporation of [^{3}H]leucine into leaf protein over increasing water deficits in unshaded (●) and shaded (+) Pinus ponderosa var. scopulorum, and unshaded var. ponderosa (□) seedlings. Approximately 13.2 MBq were vacuum infiltrated into the stem of each seedling. Period of uptake was 18 hrs.

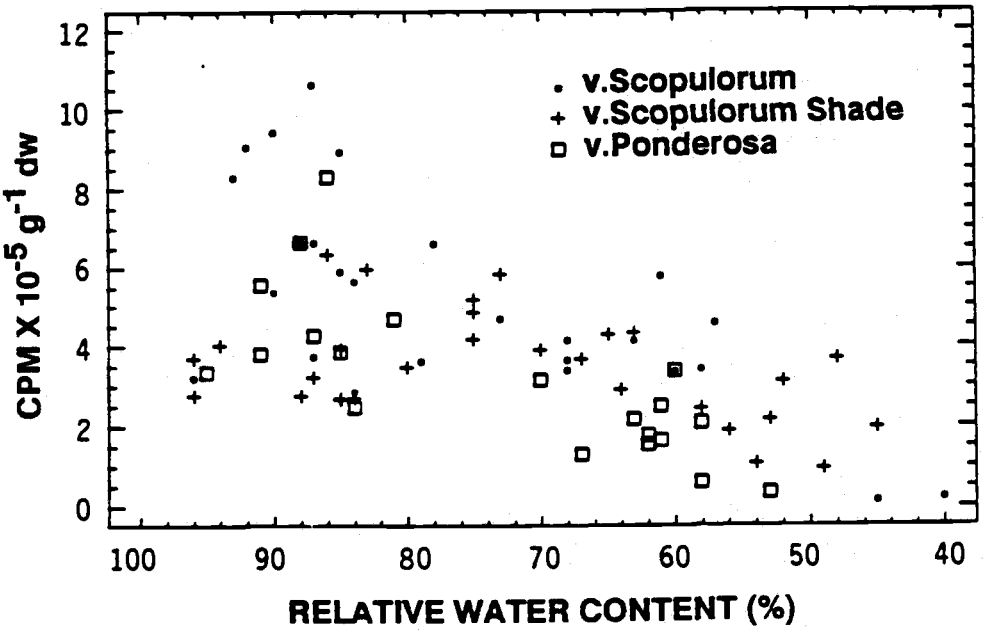
v.Scopulorum
v.Scopulorum Shade
v.Ponderosa
CPM X 10-5 g-1 dw
RELATIVE WATER CONTENT (%)
0
2
4
6
8
10
12
100
90
80
70
60
50
40

Figure 4.1

Figure 4.2 Electrophoretic separation of polypeptides synthesized in unshaded seedlings of Pinus ponderosa var. scopulorum used in Fig. 4.1. by 1-D SDS-PAGE. Aliquots (30 μg) were electrophoresed on 14% gels, Coomassie blue stained (A) and fluorographed (B). Lanes 1-9 in A and B correspond to relative water contents of 95, 87, 81, 75, 70, 67, 60, 93% representing progressive drought stress and recovery, respectively. Darts indicate position of protein bands that appear, or visibly increase with drought stress. Protein standards $M_r \times 10^{-3}$; phosphorylase b 93, BSA 66, ovalbumin 45, carbonic anhydrase 31, soybean trypsin inhibitor 22, lysozyme 14. Gels were loaded with approximately 2.5×10^4 cpm of TCA insoluble radioactivity and exposed for 10 to 13 weeks at $-80^{\circ}C$.

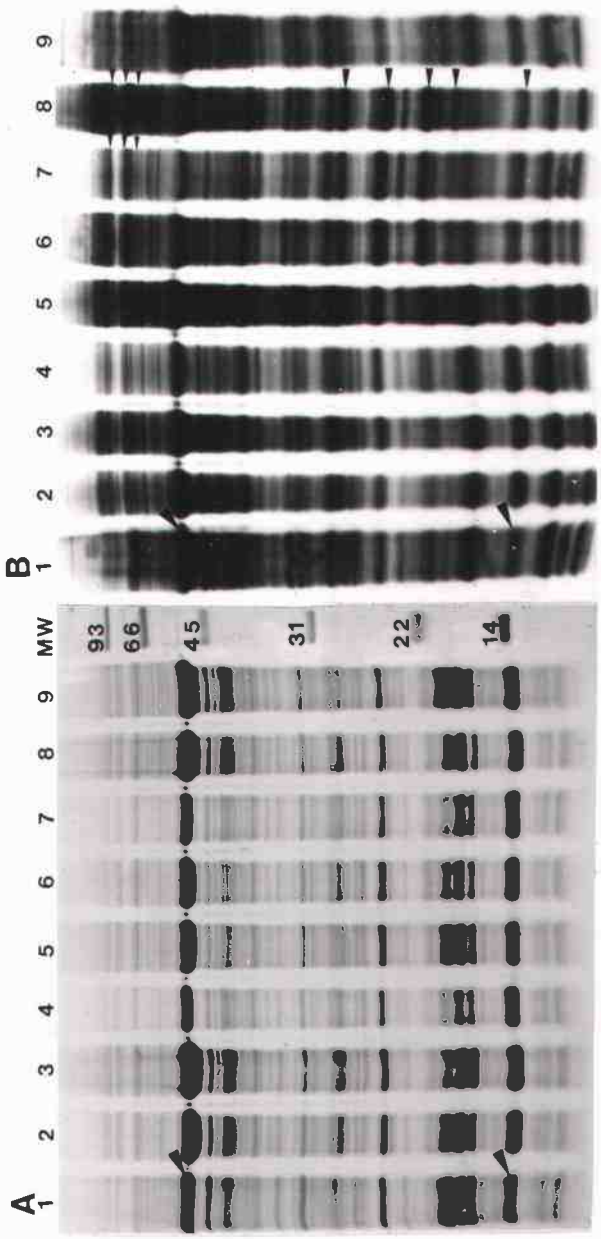

Figure 4.2

Figure 4.3 Fluorographs of proteins from radiolabeled, unshaded Pinus ponderosa var. ponderosa (A) and shaded var. scopulorum (B) seedlings drought stressed as described in Fig. 4.1, electrophoresed and fluorographed as described in Fig. 4.2. Lanes 1-8 in A correspond to 92, 87, 79, 74, 65, 62, 59% and in B to 87, 82, 78, 75, 68, 58, 52% relative water content. Darts indicate position of protein bands that appear or increase with drought stress.

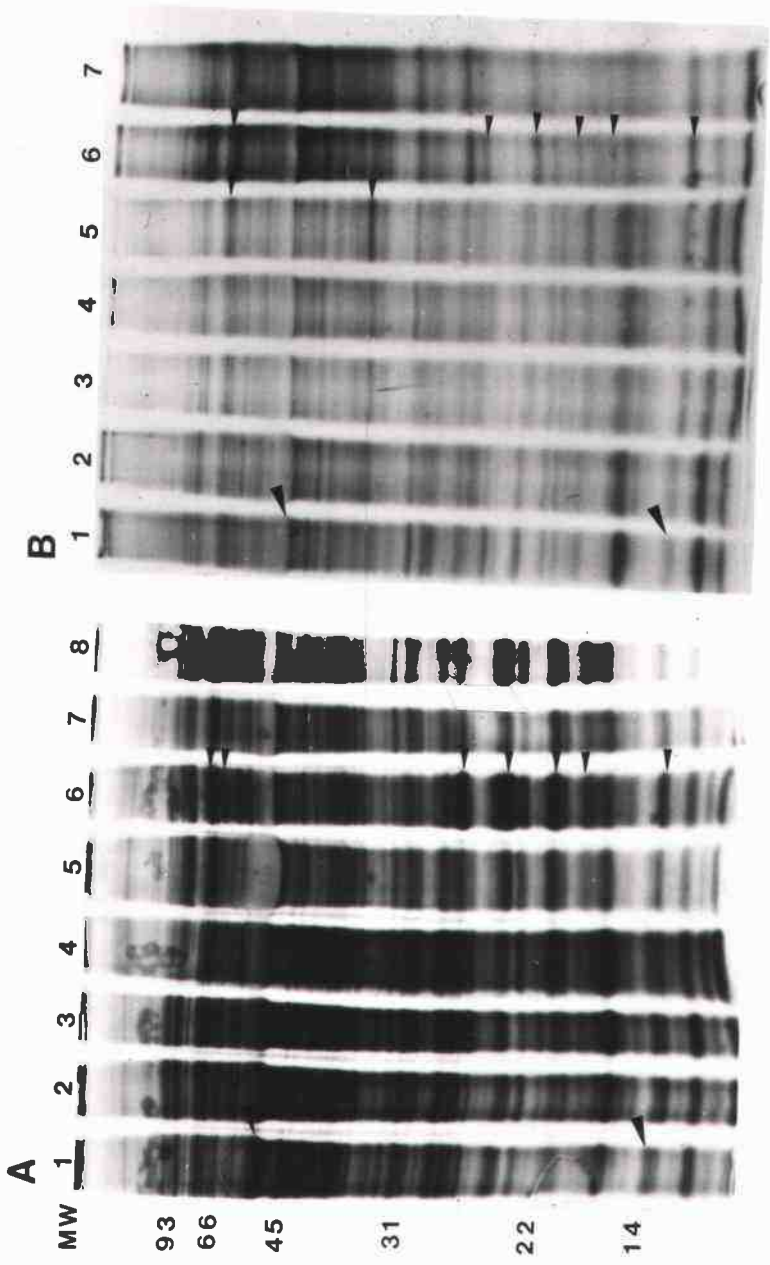

Figure 4.3

Figure 4.4 Fluorographs of protein extracts separated by 2-D gel electrophoresis from radiolabeled, progressively drought-stressed seedlings. Aliquots of 4 x 10^4 cpm were loaded per gel; effective pH gradient in the IEF dimension 4.7-7.9. Unshaded seedlings of Pinus ponderosa var. scopulorum at 95% (A) and 67% (B) RWC; shaded var. scopulorum at 75% (C) and 58% (D) RWC; unshaded var. ponderosa at 87% and 59% RWC. Polypeptide spots that changed with stress indicated: newly appeared or increased in intensity (darts), representative spots that decreased in intensity (open arrows), and spots that increased in intensity only in shaded seedlings (wide, closed arrows). The large and small subunit of Rubisco indicated by narrow arrows.

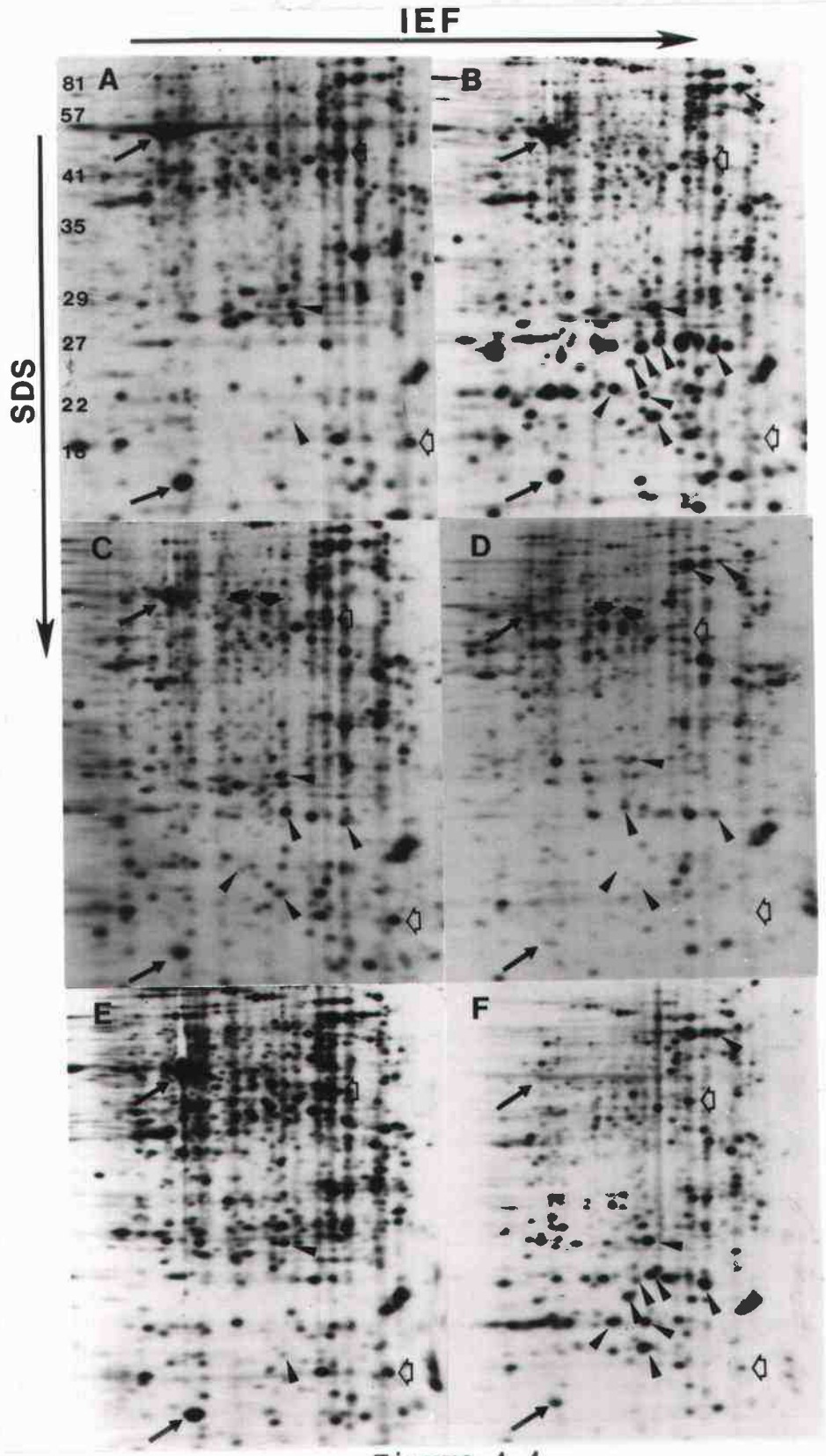

Figure 4.4

Figure 4.5 Fluorographs of 2-D electrophoresed proteins extracted from (A) extremely stressed (60% RWC) and (B) recovered (93% RWC) Pinus ponderosa var. scopulorum seedlings. Corresponding silver stained replicates of protein extracts under conditions of A and B above, represented in C and D. Darts and arrows indicate protein spots as described in Figure 4.4.

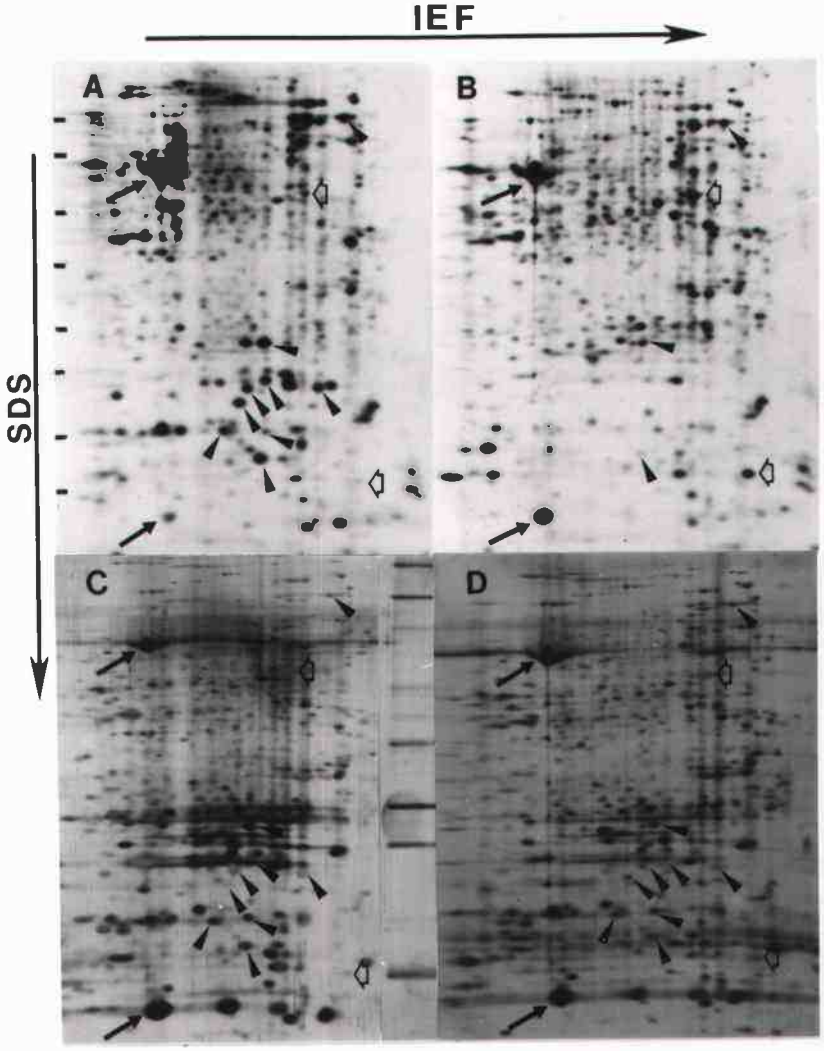

Figure 4.5

Figure 4.6 Coomassie blue stain (A) fluorograph (B) and Western blot (C) detection of high molecular weight proteins extracted from Pinus ponderosa var. scopulorum needles and separated by SDS-PAGE. Seedlings represented by Lane 1, unstressed (94% RWC); Lanes 2 and 3, heat stressed (3 hr at 48^{o}C); and Lane 4, drought stressed (68% RWC). Immunodetection was with polyclonal antibodies raised in rabbits against 73-89 kDa heat-shock proteins in maize.

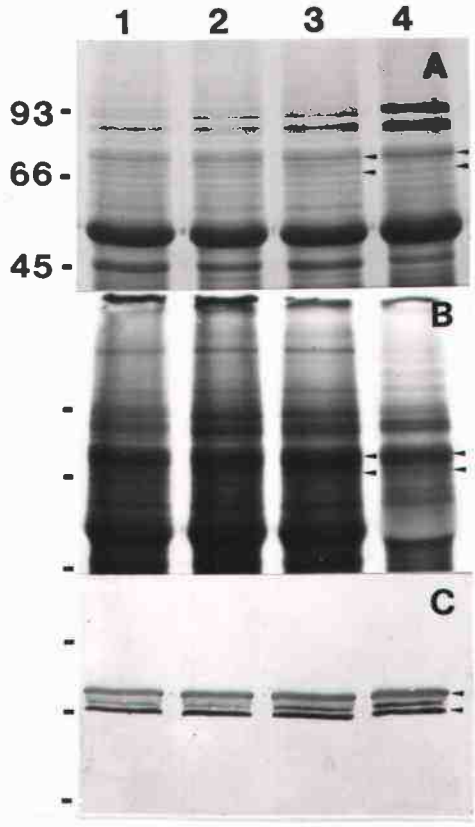

Figure 4.6

Figure 4.7 Western blots of proteins separated by SDS-PAGE, transferred to NCP and probed with a polyclonal antibody raised against a 26 kDa osmotic-stress induced protein in tomato. Lane 1 represents antibody reaction to proteins from osmotic-stressed tomato leaf tissue. Lanes 2-6 in Pinus ponderosa seedlings: lanes 2 and 3, antibody reaction to salt-stressed roots and needles respectively; lanes 4-6, no reaction to (4) unstressed roots, (5) moderately and (6) severely drought-stressed needles.

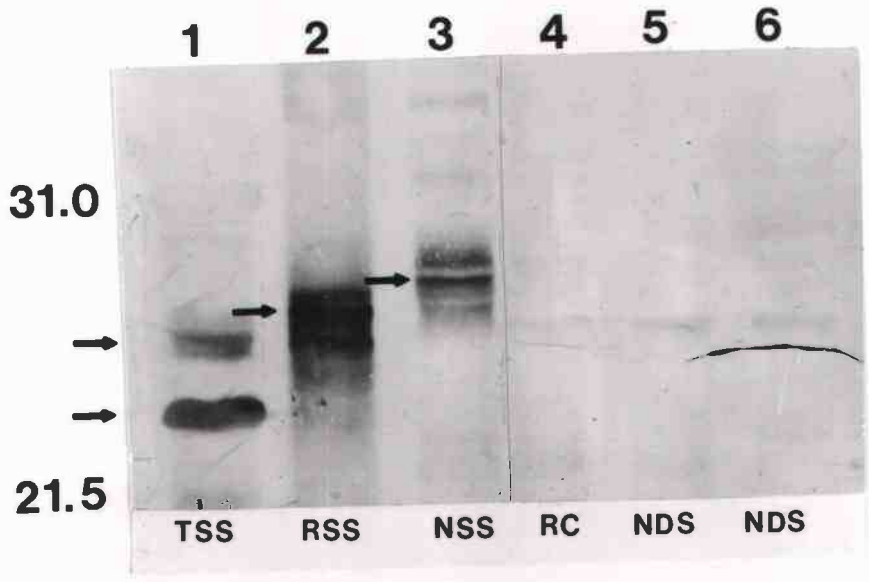

Figure 4.7

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CONCLUSIONS

In the first study (CHAPTER 1) which reported the water relations of drought-stressed ponderosa pine seedlings, difficulties arose in relying on two traditional water relations parameters (Ψ_x and RWC) to directly associate water status with a physiological response. In the water relations study, Ψ_x of the light-starved seedlings did not vary normally with RWC. Non-specific loss of structural integrity in the cells may have caused Ψ_x to increase relative to RWC, but, in fact, the loss may have been confined to xylery tissue. Correspondingly, RWC may have been lower in the light-starved seedlings than in unshaded seedlings of equivalent Wx because of the greater proportion of water volume to dry matter which was evidenced by the larger TW/DW ratios. The two parameters do not adequately provide a basis for comparing the water status of the shaded and unshaded seedlings used for assessing associated physiological processes.

Until an accurate and non-destructive way is found to measure the water status of seedlings, or their tissues, there is no way of knowing if a sampled seedling would survive the level of stress measured. Thus, adaptivity or tolerance of drought stress had to be inferred. Evidence that a specific set of physiological

conditions were adaptive rather than a deleterious consequence of dehydration was circumstantial.

Plotting independent data points for the P-V analyses was less precise; therefore specific parameters, such as the turgor-loss point, could not be satisfactorily calculated. However, when independent data sets in a water potential isotherm are plotted, the variability between individuals as it changes along the isotherm may be informative. Specific departures from the regression line may indicate other physiological events or perturbations in individuals. In the traditional P-V curve, each successive data point is dependent on the preceding one, yielding a high precision (r^2) which is useful for determining water relations parameters. However, the high degree of linearity under certain conditions may mask real variation, which could lead to unrealistic interpretations.

The study of effects of drought and shading on free amino acid concentrations demonstrated that shading altered the accumulation of free amino acids that typically occur in drought stressed seedlings of ponderosa pine. However, the reduced tolerance of severe water deficits in the light-starved seedlings could not be directly related to altered amino acid metabolism. It also demonstrated that amino acid accumulation in ponderosa pine probably has little effect on maintenance of turgor.

Accumulation of arginine suggested an adaptive response in preventing NH_3 toxicity. However, it is not known if arginine was accumulated and concentrated in the cytoplasm or the vacuole. If it accumulated to high concentrations in the cytoplasm, proline might function as a compatible solute, because arginine is an incompatible species and is strongly inhibitory of specific enzyme activities (Hochachka and Somero 1984). The technique for analysis of free amino acids described in CHAPTER 2 provided for accurately measuring this important amino acid. Its accumulation under stress should be examined in conjunction with the activities of arginase, urease and the accumulation of polyamines, all of which are intricately related to arginine metabolism.

One indication of irreversible damage and loss of enzyme activity in the chloroplasts may have been the reversal in concentrations of glutamate and glutamine metabolism under severe stress in needles of the shaded seedlings. Analysis of key enzymes involved in NH_3 assimilation, ammonia, and amino acids derived from glutamate would shed more light on this critical aspect of amino acid metabolism in plants under stress conditions.

Electrophoretic techniques and fluorography were used to characterize alterations in protein synthesis in response to drought stress in needles of two varieties of Pinus ponderosa (CHAPTER 4). The study demonstrated that

novel proteins were synthesized under increasing drought stress, and that their synthesis was inhibited by light starvation. The induction of full gene expression in response to drought stress may require light or energy, and warrants further investigation. Synthesis of Rubisco decreased but still occurred in unshaded seedlings with severe water deficits. There was a varietal difference in the synthesis of this enzyme under severe water deficits, but may be based on needle differences. Failure to detect the 26 kDa protein induced by osmotic stress in drought-stressed tissue suggests there are distinct differences in the induction of gene expression by the two types of water stress. Future studies comparing gene expression induced by PEG-imposed stress and drought stress may further distinguish between the respective responses. Differences in response would have important implications in evaluating the physiology and adaptivity of water-stressed plants.

Ponderosa pine seedlings of both varieties demonstrated their drought tolerance in surviving water potentials as negative as -4.0 MPa and RWC below 65%. Metabolic responses to drought became distinguishable at RWC below 70%, eg. the synthesis of dsps and sharp increases in proline. The dsps appeared earlier in the progression of drought stress than did the accelerated accumulation of proline; they did not appear to be directly related, although both occurred to a greater

extent in seedlings receiving adequate light. Light starvation reduced drought tolerance and altered metabolic response and gene expression induced by drought. Most differences between the two varieties may be attributable to differences in needle type, ie. the early development of secondary needles in var. scopulorum.

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