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Title: Environmental and Physiological Control of Stomates in Douglas-fir and Other Species

Abstract approved: Signature redacted for privacy.
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This research dealt with the direct environmental influence of atmospheric vapor pressure on stomates of *Pseudotsuga menziesii*, *Picea engelmannii*, and *Fragaria X ananassa*, and the indirect physiological control of stomates by abscisic acid in *Pseudotsuga menziesii*.

Two ventilated porometers (diffusion and steady-state) were compared on four broadleaf and five coniferous species. The diffusion porometer gave consistently lower conductance values for both types of species, reflecting a direct stomatal response to low chamber humidity. At high conductance values, the porometers produced nearly equal response, but the diffusion porometer was less sensitive at low conductance values. This was due to lower wind velocity (20% of the velocity in the steady-state porometer) and water vapor sorption by its acrylic plastic chamber.

The rapid response of stomates to changes in atmospheric vapor pressure and its interaction with plant water stress was characterized in the three species above. All of the species showed similar curvilinear response of enhanced conductance at low vapor pressure deficit and depressed conductance at high vapor pressure deficit. The response time ranged from 30 seconds at high VPD to 2 minutes at low VPD. Engelmann spruce was more sensitive than either Douglas-fir or strawberry, which were equally sensitive.

Plant water status significantly altered stomatal response to humidity. The relationship of conductance to xylem water potential was linear under ambient conditions (15 mb), but became curvilinear when conductance was measured at a vapor pressure above (20 mb) and below (7 mb) ambient. Between -0.5 MPa and -2.0 MPa, the stomates were sensitive to vapor pressure deficit, but below -2.0 MPa, this sensitivity lessened. This desensitization was attributed to the increase in abscisic acid overriding the influence of atmospheric humidity.

The photoisomerization kinetics of the geometric isomers of abscisic acid were studied in solution and in seedlings of Douglas-fir. *In vitro*, 250-nm light caused photolysis of both isomers. The 350-nm light and sunlight were comparable in isomerizing ABA and 2-trans-ABA with a half-time of about 3 minutes, whereas isomerization under fluorescent light took longer (half-time = 7 minutes). Methylation of the isomers caused the photoisomerization half-time to increase in all of the light sources. The equilibrium concentration of 55% ABA and 45% 2-trans-ABA was consistent for the free acid and the methyl ester

under all radiation treatments. The absorption spectra of the isomers provided evidence for the higher concentration of the cis-isomer at photo-equilibrium.

In vivo, no significant conversions in ABA, 2-trans-ABA or their saponifiable conjugates were observed in either 350-nm or fluorescent light. However, the presence of 2-trans-ABA and its conjugate in the needle extracts suggests that isomerization occurs, *in vivo*.

The changes in ABA and its metabolites in Douglas-fir needles were followed through two drought cycles and its effect on stomatal conductance was determined. Leaf conductance showed the typical water potential threshold, decreasing abruptly at -2.0 MPa. This corresponded to the simultaneous increase in ABA level, from 500 to 850 ng g^{-1}. No adjustment to stress was observed in any of the relationships examined, but stress progressed at a slower rate during the second cycle.

Metabolism of ABA was found to differ from previous studies. A linear relationship between ABA and its conjugate strongly implicated the importance of the interconversion of the two compounds for storage and supply of the free acid. This may have been due to tissue age and water stress preconditioning. Phaseic acid and epi-dihydrophaseic acid were the primary metabolites. Changes in trans-dihydrophaseic acid paralleled ABA providing evidence for concentration-dependent photoisomerization, either of ABA or one of its metabolites.

Environmental and Physiological Control of Stomates in Douglas-fir and Other Species

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Environmental and Physiological Control of Stomates in Douglas-fir and Other Species

CHAPTER 1

Introduction

The characteristic summer drought of the Pacific Northwest causes water stress in the vegetation. Growth reduction in established Douglas-fir trees, one of the area's important timber producers, is a result of this water stress as is increased mortality in young regeneration on logged areas. A better understanding of the water stress physiology of Douglas-fir may help eliminate or reduce these and other stress effects.

The induction of plant water stress is primarily a result of transpirational water loss from the needles exceeding water uptake by the roots. The stress progressively intensifies as the soil dries. Numerous physiological changes accompany this stress dehydration and have been reviewed by Hsiao (1973). The principal means of retarding the dehydration is stomatal regulation. Stomatal closure allows a rapid, and reversible, reduction in transpiration. Consequently, a more favorable water status can be maintained during periods of low precipitation.

In the field, stomata are controlled by a myraid of factors as a result of complex interactions of biotic and abiotic elements. The importance of each factor appears to change as soil moisture becomes limiting. Environmental variables that influence stomata have received much attention due to their role in controlling CO_2 influx and water vapor efflux. Physically, stomata are situated in the leaf at the interface between plant and atmosphere, and have developed sensitivity to

atmospheric variables (light, CO_2 concentration, temperature, humidity and wind) as well as to plant variables (nutrition, hormones and water status). Optimizing stomatal aperture under a given set of conditions would benefit the plant by maximizing photosynthesis while at the same time minimizing transpiration. Cowan and Farquhar (1977) constructed a mathematical model of stomatal response to environmental variables using this premise. As with most models, this one was limited to examining stomatal response to one variable while the others were held constant. This allows for only tenuous extrapolation to field conditions.

There is growing evidence that stomata are capable of responding rapidly to various environmental stimuli. The presence of stomatal oscillations has been known to occur in several species (Barrs, 1971; Brun, 1962; Farquhar and Cowan, 1974; Lange *et al.* 1969). In these studies, the oscillations were initiated by a rapid change in either vapor pressure, CO_2 concentration or light intensity. These oscillations have been attributed to root resistance in the transpirational stream (Meidner and Mansfield, 1968), but there may be a better explanation in light of recent evidence for peristomatal transpiration, i.e., the direct stomatal sensing of the atmosphere by varying transpiration from the guard and subsidiary cells (Cowan, 1977; Landsberg and Butler, 1980; Lange *et al.* 1971; Lange *et al.* 1975; Maercker, 1965; Maier-Maercker, 1979a,b,c,d). Lange *et al.* (1971) visually observed that stomatal closure could be intiated in detached epidermal tissue by passing jets of dry air over it. Closure began in two minutes, and was complete within five minutes. Conversely, opening was

actuated by humid air, but it took longer, from eight to ten minutes. They also found that the stomata under the air stream responded independently of adjacent stomata which were exposed to the ambient atmosphere.

During water stress the stomata of some plants have been found to be controlled primarily by the plant hormone, abscisic acid (ABA) (Cummins *et al.* 1971; Hiron and Wright, 1973; Kriedemann *et al.* 1972; Milborrow, 1974; Walton, 1980). The presence of ABA in Douglas-fir was first demonstrated by Webber *et al.* (1979) and was implicated in stomatal control during water stress by Blake and Ferrell (1977) and again by Newville and Ferrell (1980).

Increase in water stress has been shown to result in a rapid rise in leaf ABA levels (Aharoni, *et al.* 1977; Beardsell and Cohen, 1974; Bengston *et al.* 1977). The increase in foliar ABA appears to be dependent upon the magnitude and duration of the stress (Dörffling *et al.* 1977) as well as the individual plant species (Cummins, 1973; Lancaster *et al.* 1977). Other investigators have reported the presence of water potential thresholds beyond which ABA levels were found to rapidly increase (Beardsell and Cohen, 1974; Zabadal, 1974). The reported thresholds varied between -0.6 and -1.2 MPa and the ABA increased from 3 to 20 times the initial concentrations. The occurrence of such a water potential threshold has been recently reported in Douglas-fir (Blake and Ferrell, 1977; Newville and Ferrell, 1980). The ABA content increased fourfold at a water potential of about -0.8 MPa.

The objective of this research was to characterize some of the processes involved in stomatal regulation as it related to outplanted seedlings. Two areas of stomatal regulation were investigated:

1) the rapid response of stomata to changing atmospheric humidity under non-limiting and limiting water supply; and 2) the role of ABA and its metabolism in controlling stomata during and after water stress.

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CHAPTER 2

Two Types of Ventilated Porometer Compared on Broadleaf and Coniferous Species[1,2]

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ABSTRACT

Two ventilated porometers (diffusion and steady-state) were compared on four broadleaf and five coniferous species. The diffusion porometer gave consistently lower conductance values for both types of species, reflecting a direct stomatal response to low chamber humidity. At high conductance values, the porometers produced a linear and nearly equal response, but the diffusion porometer was less sensitive at low conductance values. This was due to lower wind velocity (20% of the velocity in the steady-state porometer) and water vapor sorption (by its acrylic plastic chamber). The broadleaf species had less variation (R^2 = 0.81) than the coniferous species (R^2 = 0.61), but with the latter there was better correspondence between the two porometers, possibly due to sampling technique. Conductance values were clustered by species.

In the last decade, porometry has become a widely used technique in both laboratory and field research on water relations. Until recently, only diffusion porometers have been commercially available -- a passive diffusion model (4) and a turbulent diffusion (ventilated) model (8) -- and so have been the basis for much of the published data on leaf conductance. Since 1972, however, a steady-state (null balance) porometer (1) has gradually supplanted the diffusion porometers.

The two types of porometer differ primarily in their theoretical approach to measuring conductance. With the diffusion porometer leaf resistance (r_ℓ) is related to the transit time between two predetermined humidities in the chamber. The diffusion porometer usually uses a narrow-range LiCl sensor, so r_ℓ must be measured at a chamber humidity between 20 and 30% RH (vapor pressure deficit between 18.7 and 16.4 mb, respectively, at 20°C), an unusual range under most natural conditions. Turner and Parlange (7) discuss the theory and calibration of the ventilated diffusion porometer more thoroughly. In contrast, the steady-state porometer is based on the determination of dry air flow into the chamber to offset increased humidity due to transpiration; stomatal conductance (g_ℓ; $g_\ell = 1/r_\ell$) is calculated from the flow rate and the steady-state humidity, which can range from 0 to 100%, allowing measurements under ambient conditions. Beardsell *et al.* (1) address the theoretical model and the operational theory of the steady-state porometer.

This study compared the performance of commercially available, ventilated propometers -- a diffusion type (Wren Instruments, Hamden, Connecticut) and a steady-state type (Interface Instruments, Corvallis,

Oregon) -- to document their g_ℓ values on broadleaf and coniferous species, to determine the effect of foliage shape (broadleaf versus needles), and to explain discrepancies in the literature.

MATERIALS AND METHODS

Four broadleaf species [*Alnus japonica* Sieb. & Zuc., *Alnus rubra* Bong., *Celtis occidentalis* L., and *Frageria X ananassa* Duch.] and five coniferous species [*Abies procera* Rehd., *Larix occidentalis* Nutt., *Picea engelmannii* Parry, *Pseudotsuga menziesii* (Mirb). Franco, and *Tsuga heterophylla* (Raf.) Sarg.] were selected to ensure a full range of g_ℓ and to allow comparison among species and between foliage shapes. Throughout the study, the seedlings were watered and maintained in a growth room at 20°C, 70% RH with a 16-hr photoperiod. Cool white fluorescent lamps provided radiation of 200 $\mu E\ m^{-2}\ s^{-1}$ at seedling level.

The diffusion porometer was calibrated prior to the study by the method of Turner and Parlange (8) and with a perforated plate (4). The humidity sensor was calibrated at 20°C by mixing air bubbled through two saturated salt solutions. The sensor in the steady-state porometer was calibrated from 0 to 100% RH with five saturated salt solutions and dry nitrogen gas. The wind velocity profile within each chamber was characterized with an Alnor hot-wire anemometer.

For each coniferous species, the needles were pulled off 1- to 2-mm strips about 1 cm from the tips of four branches. At these strips, the branches were then sealed to split rubber stoppers with modeling clay. For the diffusion porometer, the branch and stopper were inserted into

the larger chamber aperture until the stopper was seated (the spring-loaded clamp was removed). After g_ℓ was measured, the leaf area of the needles was estimated with LICOR surface area meter (Model LI-3000) and g_ℓ was calculated with projected (one-sided) leaf area. Two leaves per broadleaf species were measured with the fixed aperture on the two porometers.

Conductance of the same branch or leaf was measured sequentially with the two porometers, and the sequence was reversed every time. Separate analysis using two regression equations for each sampling sequence did not show statistically different ($P = 0.05$) chamber effects, so the data were pooled by species type and averaged by branch or leaf.

RESULTS AND DISCUSSION

The diffusion porometer gave consistently lower g_ℓ values (higher r_ℓ) for both species type (Fig. 1 and 2) largely because of its lower wind velocity (maximum of 60 cm s^{-1} versus 300 cm s^{-1} in the steady-state porometer). Tan and Black (6) showed a 20% decrease in transit time when the fan speed in the diffusion porometer was increased from 1,000 to 2,000 rpm.

The low humidity in the diffusion porometer chamber also may have caused the stomates to partially close. Significant stomatal closure has been observed within 2 min after exposure to low humidity (5), and more recently, stomates have been found to close 30 sec after humidity reduction (3). Furthermore, reducing chamber humidity from 60 to 20% depressed g_ℓ from 20 to 30% of that measured at ambient humidity (3), similar to g_ℓ values reported here in the diffusion porometer.

Although the steady-state porometer was more sensitive at lower conductances (Fig. 1 and 2), the two porometers gave nearly equal readings. The nonlinearity at low conductance may be due to the acrylic plastic chamber of the diffusion porometer, a material that reportedly can adsorb and desorb a significant amount of water vapor (2). Presumably, this is accounted for in the calculated porometer resistance (Rp) and subtracted (7); however, Rp is assumed to be constant with time and independent of lead conductance. More likely, Rp varies, since it depends on the rate of vapor supply and the adsorption capacity of the walls. Adsorption, in turn, depends on prior humidity and present temperature. If Rp varies, the sorption by the walls would be very slow at low conductances, causing the observed curvilinear response. At high vapor supply, i.e., high leaf conductance, the sorption requirement would be fulfilled faster. In contrast, the aluminum-machined chamber of the steady-state porometer should not have this sorption problem.

The coniferous and broadleaf species responded differently to the two porometers. Although the data for the broadleaf species gave a better statistical fit (R^2 = 0.81), they also showed a greater discrepancy between the two porometers. The broadleaf species were measured with the fixed aperture attachement on both porometers. In the steady-state porometer, air flow at a velocity of 300 cm s^{-1} hits the leaf surface at an angle that minimizes the boundary layer. However, in the diffusion porometer, the leaf remains several millimeters above the main air flow within the chamber, which diminishes the effectiveness of ventilation and inadvertently increases the boundary-layer resistance. At

this point in the chamber, wind velocity was 25 to 30 cm s^{-1} (40 to 50% of maximum).

The coniferous species, in contrast, were measured with their needles in the center of the air flow of both porometers, which may explain the better agreement between the two porometers for these species. An unventilated diffusion porometer consistently gave higher g_ℓ for beans than a ventilated model (6). Thus, the geometry of the sensor in relation to the leaf also appears critical. The sampling technique for the conifer foliage positioned the needles closer to the sensor than the broadleaf technique. The high dispersion of the conifer data (R^2 = 0.61) is attributed (A) to differences within and between species in the presentation of the various shapes and sizes of needles to the air flow, and (B) to the possibility of an incomplete seal of the branch with the diffusion porometer.

The g_ℓ values clustered by species. For the broadleaf species, g_ℓ increased from *A. rubra* to *C. occidentalis*, *S. japonica* and *F. ananassa* (Fig. 1). In the coniferous species, the ranking was *T. heterophylla*, *P. menziesii*, *A. procera*, *P. engelmannii*, then *L. occidentalis* (Fig. 2).

This comparison of the two types of ventilated porometers showed some shortcomings in the design, construction, and use of the porometers. In particular, wind velocity within the porometer chamber must be adequate to minimize the boundary layer, humidity in the ambient atmosphere and the chamber must be the same or very similar, and the chamber should be constructed of hydrophobic materials.

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FIGURE CAPTIONS

Figure 1. Leaf conductance as measured by a ventilated diffusion porometer and a ventilated steady-state porometer on broadleaf species where ● = *Alnus japonica*, ■ = *Alnus rubra*, ▲ = *Celtis occidentalis*, and ★ = *Fragaria X ananassa* (each point is the mean of at least four samples).

Figure 2. Branch conductance as measured by a ventilated diffusion porometer and a ventilated steady-state porometer on coniferous species where ● = *Abies procera*, ■ = *Larix occidentalis*, ▲ = *Picea engelmannii*, ★ = *Pseudotsuga menziesii*, ○ = *Tsuga heterophylla* (each point is the mean of two samples).

Figure 1

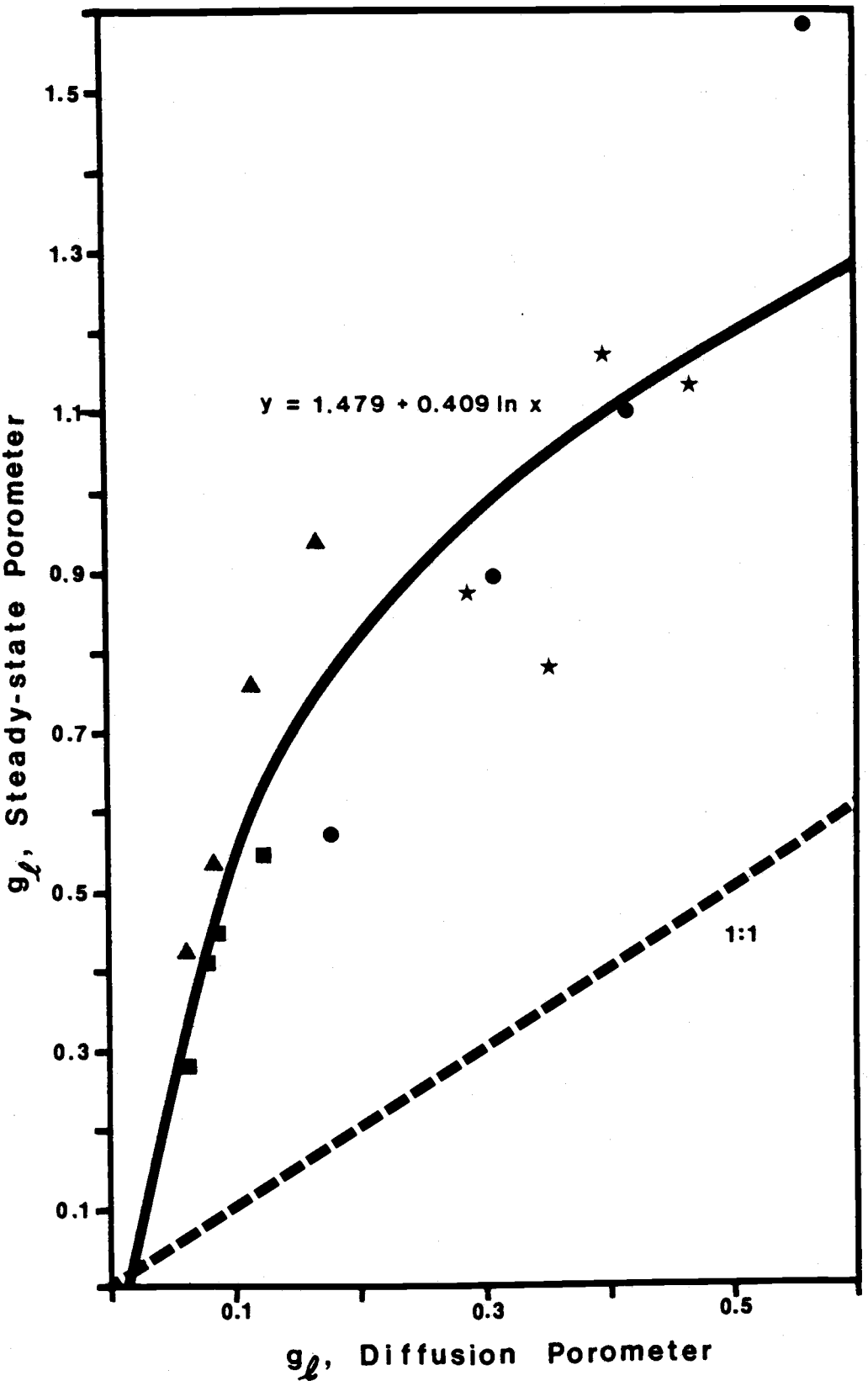
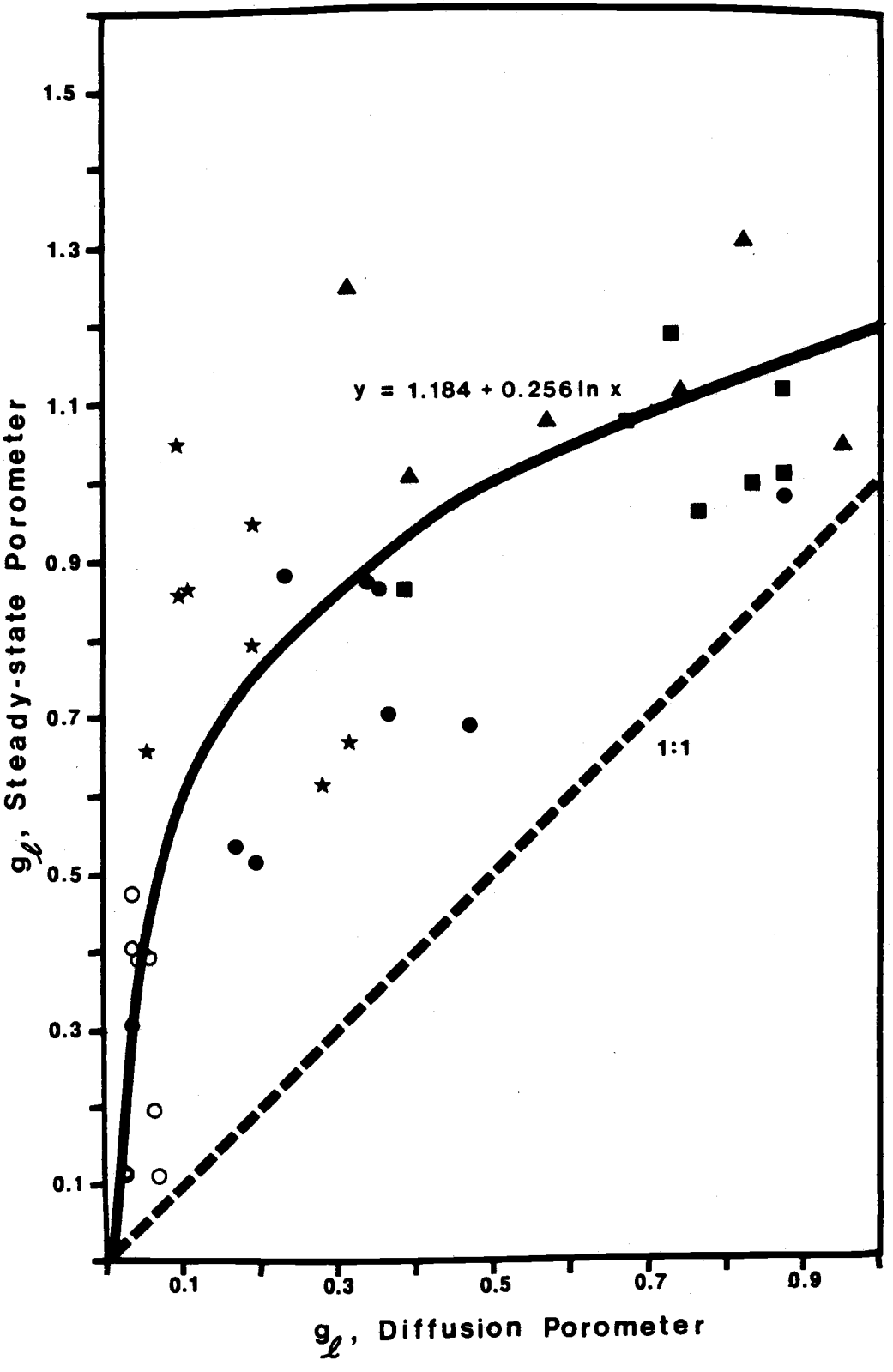

1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
y = 1.479 + 0.409 ln x
1:1
0.1
0.3
0.5
g_ℓ, Steady-state Porometer
g_ℓ, Diffusion Porometer

Figure 2


1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
y = 1.184 + 0.256 ln x
1:1
0.1
0.3
0.5
0.7
0.9
g_ℓ, Steady-state Porometer
g_ℓ, Diffusion Porometer

CHAPTER 3

A Rapid Stomatal Response to Vapor Pressure Deficit and the Influence of Plant Water Status[1,2]

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[2]To be submitted to Plant, Cell and Environment

Abbreviations: VPD, vapor pressure deficit; g_ℓ, branch or leaf conductance; ψ_x; xylem water potential.

ABSTRACT

The rapid response of stomates to changes in atmospheric humidity was investigated in *Fragaria X ananassa*, *Picea engelmanni*, and *Pseudotsuga menziesii*. Furthermore, the interaction of plant water stress on this rapid response was determined in *Pseudotsuga menziesii*.

The plants were grown under three ambient conditions of temperature and vapor pressure deficit: 35 C- 35 mb; 35 C- 5 mb; 20 C- 15 mb. Branch (stomatal) conductance was measured with a steady-state porometer, first at ambient humidity and then at one of four treatment humidities. The treatment was achieved by increasing or decreasing the humidity within the porometer cuvette. All three species showed similar stomatal response of enhanced conductance at low vapor pressure deficit and depressed conductance at high vapor pressure deficit. Response time ranged from 30 seconds at high VPD to 2 minutes at low VPD. Engelmann spruce was more sensitive than either Douglas-fir or strawberry which were comparably sensitive. This difference in sensitivity can be attributed to species habitat; Engelmann spruce occupies a cool, humid habitat whereas the other two are found in an environment of warm, dry atsmophere.

Plant water status significantly altered stomatal response to humidity. The relationship of conductance to xylem water potential was linear under ambient conditions, but became curvilinear when conductance was measured at a vapor pressure deficit above and below ambient. Between -0.5 MPa and -2.0 MPa, the stomates were sensitive to vapor pressure deficit, but below -2.0 MPa, this sensitivity lessened. This desensitization was attributed to the increase in abscisic acid overriding of the influence of atmospheric humidity.

Environmental influence on stomates has received much attention in the past, including the effects of CO_2 concentration, light intensity and temperature (23). In the last decade evidence has been accumulating which suggests that stomates of, at least, some species are also sensitive to atmospheric humidity (1,4,6,10,14,15,16,18,22,33,34). Such a response has large ramification in the environmental control of photosynthesis and transpiration, especially under field conditions.

Two theories of the controlling mechanism of stomatal sensitivity to atmospheric humidity have been proposed (5). Both consider the supply and demand of water to the epidermis and stomatal complex, and its subsequent effect on guard cell turgor potential (23). Stomatal aperture is known to be a function of guard cell turgor (8) and stomatal movements are due to a turgor potential difference between the guard and subsidiary cells (7). The theories diverge in the interpretation of the process as feedback or feedforward (5).

In the feedback process, the guard cells are presumably 'sensing' humidity via the transpiration rate from the substomatal cavity. Increased transipration due to higher VPD results in water movement out of the guard cell, a reduction in turgor and stomatal closure. The converse is believed to cause stomatal opening when VPD decreases.

The feedforward model includes an additional transpirational pathway which independently controls guard cell turgor potential by direct water loss through the outer epidermal wall. This transpiration, termed peristomatal, would allow for simultaneous stomatal closure and reduced bulk transpiration in response to increasing VPD (17).

The objectives of this research were to investigate these mechanisms by determining the rapid response of stomates to changes in VPD and the influence of plant water potential on the stomatal-VPD relationship.

MATERIALS AND METHODS

Experiment 1

Three species (*Pseudotsuga menziesii* (Mirb.) Franco, *Picea engelmanni* Parry and *Fragaria X ananassa* Duch.) were measured under three environments to investigate the rapid stomatal response to VPD. The watered, soil-potted conifer seedlings and strawberry plants were rotated through growth chambers with air temperature and VPD of 20 C-15 mb, 35 C-35 mb, and 35 C-5 mb. Light was provided by cool-white fluorescent lamps (light intensity at foliage was 200 uE m^{-2} s^{-1}) with a 16 h photoperiod. At least one week was allowed for acclimation when the plants were moved to a new environment.

Stomatal response was measured with a steady-state porometer (Interface Instruments, Corvallis, Oregon) (3). Humidity within the porometer cuvette was decreased or increased from ambient with N_2 gas or with air bubbled through distilled water. Two branches per conifer seedling were sampled on four seedlings of each species. For strawberry, two leaflets were measured on each of the two plants. All branches and leaflets were initially measured for g_ℓ at ambient VPD, then g_ℓ was measured at one of four cuvette humidities; 20, 40, 60, or 80% (one humidity always corresponded to the ambient environment).

Several minutes were allowed for cuvette equilibration when the humidity was changed. Only one pair of measurements were made on a branch per day to lessen chamber effects on the stomates. Sampling continued until each branch and leaflet was measured at all four cuvette humidities. Surface area of the needles was determined after the completion of the experiment with a surface area meter (LICOR, Model LI-3000, Lincoln, Nebraska), and g_ℓ was computed on the basis of projected (one-sided) leaf area.

The data were expressed as the ratio of treatment g_ℓ to ambient g_ℓ to reduce some of the seedling and branch variability. Errors associated with leaf area determination were eliminated by this procedure. For clarification, the ratios were multiplied by the mean g_ℓ at ambient humidity. The data were statistically analyzed by analysis of variance.

Experiment 2

Two sets of six *Pseudotsuga menziesii* (Mirb.) Franco seedlings were water stressed to study the influence of plant water status on the g_ℓ-VPD relationship. The seedlings were grown in the controlled environment of 20 C, 15 mb VPD. The light environment was similar to Experiment 1. Water stress was imposed by allowing the soil to dry naturally. Two consecutive water stress cycles were sampled; however, there were no differences between the data, so they were pooled. One set of seedlings was used to investigate low VPD effects (5 mb) and the other to determine the response to high VPD (20 mb).

The steady-state porometer described in Experiment 1 was used to measure g_ℓ first at ambient humidity and then at treatment humidity. Xylem water potential (ψ_x) was simultaneously measured on an excised branch with a pressure chamber (27). The seedlings were rewatered when ψ_x was between -3.0 and -4.0 MPa, and were then allowed to recover for at least one week. Leaf area of the needles was estimated as in Experiment 1, but the values were multiplied by 2.38 to give total surface area (9).

The data were analyzed with linear regression. The equations for ambient and treatment VPD were first tested to establish statistical difference and then were separately analyzed.

RESULTS

Experiment 1

The stomates of all three species responded rapidly to changes in VPD. Response time averaged 30 sec at high VPD and 2 min at low VPD. The response, in general, was curvilinear with high g_ℓ decreasing first rapidly, then slower as VPD increased, but species differences were evident (Fig. 1, 2 and 3). Englemann spruce stomates were more sensitive to VPD than either Douglas-fir or strawberry, which exhibited comparable sensitivity.

Growth room environment affected stomatal response to VPD. The g_ℓ-VPD relationships were similar at 35 C (Fig. 1 and 2), but differed at 20 C (Figure 3) with both Douglas-fir and strawberry stomates closing at low VPD, and Engelmann spruce stomates partially closing at moderate

VPD and then increasing as VPD decreased. The g_ℓ values also differed by environment with the highest being recorded in 35 C-5 mb (fig. 2), and the decreasing from the 35 C-35 mb (Fig. 1) to the 20 C-15 mb environment (Fig. 3).

Sampling the same branch or leaflet twice affected the stomates in all three environments; however, the effect was most prominent under humid conditions (Fig. 2) with a decrease of 1.5 cm s^{-1} for Douglas-fir, 0.7 cm s^{-1} for Engelmann spruce and 0.5 cm s^{-1} for stawberry. This chamber effect diminished with drier ambient air.

The analysis of variance for the different species and environments are summarized in Table 1. Only the Engelmann spruce data in the 20 C-15 mb environment failed to be significant by these analyses.

Experiment 2

Stomatal sensitivity to VPD was found to be affected by plant water status (Fig. 4 and 5). The response was consistent through the two stress cycles. Treatment VPD above and below ambient gave similar exponential relationships of g_ℓ to ψ_x whereas the response at ambient VPD was linear. The low VPD treatment never caused an enhancement of g_ℓ as found in the first experiment, even though plant water status was adequate at the beginning of each stress cycle. The maximum difference between the ambient and treatment lines in both cases was about 0.025 cm s^{-1} at -2.0 MPa. This deviation between ambient and treatment response indicates the involvement of several controlling factors.

The difference in g_ℓ values between the two data sets (Fig. 4 and 5) are attributed to a phenological effect because they were sampled a

month apart. This is supported by the nearly equal slopes and dissimilar intercepts.

The regressions equations accounted for better than half of the variation in the data. For the two ambient VPD equations, R^2 = 0.65 for Fig. 4 and R^2 = 0.57 for Fig. 5. The equations describing the treatment response explained 0.66 and 0.69 of the variation for low and high VPD, respectively.

DISCUSSION

The rapid response of g_ℓ to VPD was generally curvilinear (Fig. 1, 2, and 3), similar to measurements taken at equilibrium (4,10,12,17, 18,33,34). In Sitka spruce, g_ℓ decreased with increasing VPD between 0.5 and 10 mb, but sensitivity decreased at higher VPD (34). Similarly, stomatal response in several species was found to be negligible between 8 and 27 mb (24). Stomatal response in our study showed varying linearity between 5 and 35 mb, and in some instances up to 50 mb, but sensitivity on the whole declined at the higher values (Fig. 1 and 2).

The stomates rapidly responded to VPD changes, but the response time varied between 30 sec for closing (the lower limit for the instrument) and 2 min for opening. A similar, but slower response pattern was reported for *Polypodium* epidermal tissue (15). Stomatal closure in response to low humidity was initiated within 2 min whereas opening under humid conditions took 8 to 10 min. Aston (1), and Watts and Neilson (34) reported transient stomatal movements within 4 to 5 min of a reduction in ambient humidity, but equilibrium in their system was not achieved until after 40 min. These discrepancies in response time can

be attributed to instrument time constant. The time constant for the steady-state porometer in our study is on the order of one second or less.

Stomates respond dynamically to VPD presumably through the effect VPD has on the demand for water flowing through the epidermis and stomatal complex (5), but water supply and varying plant hydraulic resistance can also signficantly influence stomates. Evaporation occurs equally from the inner and outer epidermal cell walls, affecting guard cell turgor and hence stomatal aperture (28). Thus, under constant supply, less water is available to the cells of the stomatal complex under high evaporative demand which reduces guard cell turgor potential. Conversely, when VPD is decreased, more water can move into the guard and subsidiary cells increasing turgor potential and causing stomatal opening.

This scheme is only partially complete because water supply and osmoticum flux interact with water demand to control turgor relations. Water supply to the epidermis appears to be dichotomous, flowing through the vascular system and the cell walls of the epidermis (20,30). The latter is believed to be more efficient in resupplying water lost from the epidermal tissue (30) which explains why the turgor relations of the guard and subsidiary cells are only loosely coupled with bulk leaf turgor potential (2,18,33). Also, variable resistance to water flow through the plant influencues the rate of supply (11,13). Ion flux between the guard and subsidiary cells modify supply and demand effects through changes in cellular osmotic potential. The influence of osmotic re-adjustment may, however, by secondary under these conditions since

K^+ flux is relatively slow, at least 20 min for photoactive endosmosis and 5 min exosmosis (5). This is supported by the reported lag in changes in guard cell K^+ content following humidity-induced stomatal aperture movement (19). Additionally, subsidiary cells possess a 'mechanical advantage' over the guard cells that results in disproportionately large changes in guard cell turgor for small changes in subsidiary cell turgor (7,21). Thus, changes in supply or demand will have a larger effect on guard cell turgor than on the turgor of the adjacent subsidiary and epidermal cells.

Consequently, at any given moment, guard cell turgor and stomatal aperture are the product of a quasi-equilibrium between water supply and demand as modified by the osmotic and matrix potenials of the stomatal complex. A rapid change in demand (VPD) would perturb this equilibrium state and cause a change in stomatal aperture. However, the direction of the response is not uniform. Saxe (26) reported stomatal opening under decreasing humidity, and in our study and others stomatal closure was found to occur at low VPD (1; Fig. 3). Optimal guard cell responses occurs when the epidermal cells are at or near zero turgor (7) and could explain these unexpected results if the epidermal cells has positive turgor in the former case and negative turgor in the latter case.

The ambient environment affected g_ℓ as indicated in Fig. 1 and 2, which may be due, in part, to measuring only one branch instead of the whole plant (24). At all cuvette VPDs, g_ℓ was higher in seedlings from the 5 mb environment. A comparison of the g_ℓ values at 35 mb in the two environments shows that the g_ℓ measured in the 5 mb environment (Fig. 2) was double that in the low humidity environment (Fig. 1.). Enhanced

water supply to the sample branch from the stem and adjacent branches, i.e., higher stem reservoir (32), probably occurred in the plants growing under low evaporative demand. Similar differences between single-branch and whole-plant samples have been reported in other species (24).

A cuvette effect was evident in the 35 C-5 mb environment (Fig. 2), more so than in the other two environments. Consecutive measurements at ambient VPD caused a similar decrease in g_ℓ of all three species, indicating a physical rather than physiological pheonomenon such as the disruption of the boundary layer (6). Air movement within the cuvette is rapid enough to significantly reduce the boundary layer resistance, causing an increase in evaporative demand and transpiration, and a decrease in guard cell turgor. The leaf boundary layer has a greater effect as g_ℓ increases.

Bulk plant water status affected the response of stomates to VPD (Fig. 4 and 5). The exponential relationship of the treatment VPD suggests an interaction between ψ_x and VPD which influences stomatal response. Species with stomates responsive to bulk leaf water potential were found to be sensitive to VPD and thus the physiological basis for these responses were concluded to be the same (29). The data for Douglas-fir, however, indicate an interaction of the two factors and different physiological bases.

The stomates were most sensitive to VPD, i.e., g_ℓ was reduced the most, when ψ_x was higher than -2.0 MPa. This suggests that in this range the subsidiary cells are maintaining their "advantage" over the guard cells in terms of turgor potential. The changes in demand,

then, cause a larger reduction in guard cell turgor under high VPD or a greater increase in subsidiary cell turgor under low VPD. Both processes would result in stomatal closure. However, once ψ_x dropped below -2.0 MPa, the difference between these two cell types decreased and caused a dampened VPD response. This also is in the ψ_x range where abscisic acid has been found to increase and close stomates (31). ABA is known to influence the K^+ relations of the stomatal complex which could account for the depression of the VPD response.

The sensitivity of stomates to VPD has important implications in survival strategies and habitat adaptation. Engelmann spruce with its highly VPD-sensitive stomates grows in a cool, humid environment with adequate soil moisture through most of the growing season as supplied by snow melt. Sitka spruce which grows under warmer but similar conditions showed only loose coupling of g_ℓ to ψ_x above -1.6 MPa (34). In contrast, both the Douglas-fir and strawberry come from similar habitats characterized by warm, dry atmosphere with progressing soil moisture stress during the growing season. This would explain the strong association of g_ℓ to plant water stress in Douglas-fir (Fig. 4 and 5). In terms of habitat response, Douglas-fir stomates would remain open longer under favorable soil moisture and a large range of VPD. However, once soil moisture and ψ_x began to decrease, the stomates would also begin to close. Engelmann spruce, on the other hand, would respond more to changes in VPD than to plant water status. Severe soil moisture depletion would have to occur for the stomates to close due to plant water stress. Similar differences in species response were reported for bean and sunflower (1). An ecological adaptation similar

to that in Engelmann spruce has been reported for desert species (16). Under non-limiting soil moisture, midday stomatal closure was observed and was attributed to stomatal sensitivity to VPD.

Stomates under field conditions are controlled by several factors, e.g., CO_2 concentration (23), light intensity, humidity (16, 19) and plant water status. It is proposed that the influence of each factor varies in importance throughout the day. Light intensity and CO_2 concentration related to photosynthesis establish the maximum guard and subsidiary cell turgor through their influence on K^+ and organic anion synthesis. Once this maximum is established and providing light intensity does not change appreciably, atmospheric VPD through modifications of water vapor demand controls stomatal aperture for the remainder of the day until light intensity decreases at dusk. This hypothsis is supported by field measurements of g_ℓ under varying light and humidity conditions. On clear days, once the stomates have opened (photosynthesis becomes light saturated) g_ℓ has been shown to respond inversely to VPD[A/] (25). Measurements as in Experiment 1 conducted in sunlight on a clear, dry day showed an enhanced stomatal response to low VPD (g_ℓ increased by up to 10 times) and a small response to high VPD (g_ℓ was between 80 and 95% of ambient g_ℓ)[B/]. On cloudy days when photosynthesis is not light saturated, g_ℓ is lower despite lower VPD, but the stomates are still responsive to VPD changes. This is in accordance with reports of lower g_ℓ under low light and high VPD in comparision to low light or high VPD, alone (12,22). Plant water status acts as a modifier

A,B/ Unpublished data

of the process outlined above through its influence on water supply to the epidermis and ABA sysnthesis.

A rapid stomatal response to VPD has been demonstrated in three species. The g_{ℓ}-VPD relationship was significantly incluenced by ψ_x. The results indicate that stomates are extremely sensitive to changes in their environment and that utmost care is required when measuring them. The results support a comprehensive theory of stomatal relations under field conditions.

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TABLE 1. A Summary of the Analysis of Variance for Experiment 1. Variation in g_ℓ between classes (treatment VPDs) was tested and probability of incorrectly rejecting the null hypothesis (Ho: treatment has no affect on g_ℓ) is reported.

Environment	Test for Heterscedasticity	AOV	Weighted Mean AOV	Logarithm AOV
	percent probability			
30 C- 35 mb				
F. ananassa	1.0	-	2.5	-
P. engelmannii	1.0	-	2.5	-
P. menziesii	1.0	-	0.1	-
30 C- 5 mb				
F. ananassa	N.S.	2.5	-	-
P. engelmannii	1.0	-	N.S.	0.1
P. menziesii	N.S.	2.5	N.S.	0.1
20 C- 15 mb				
F. ananassa	N.S.	5.0	-	-
P. engelmannii	1.0	-	N.S.	N.S.
P. menziesii	5.0	-	N.S.	2.5

FIGURE CAPTIONS

Figure 1. The rapid stomatal response to VPD changes in an ambient environment of 35 C- 35 mb where ▰ = *Fragaria X ananassa*, ▮▮▮▮ = *Picea engelmannii*, ▮▬▮ = *Pseudotsuga menziesii*. Mean leaf conductance values at ambient conditions are denoted by an "a".

Figure 2. The rapid stomatal response to VPD changes in an ambient environment of 35 C- 5 mb. Symbols are same as Fig. 1.

Figure 3. The rapid stomatal response to VPD changes in an ambient environment of 20 C- 15 mb. Symbols are same as Fig. 1.

Figure 4. The effect of xylem water potential and high VPD on leaf conductance in *Pseudotsuga menziesii*. Ambient environmental conditions were 22 C- 15 mb. Regression equations by VPD are: 15 mb (●) -- $g_\ell = 0.0858 + 0.164\ \psi_x$, $R^2 = 0.65$; 20 mb (★) -- $g_\ell = e^{(-2.562 + 0.461\ \psi_x)}$, $R^2 = 0.69$.

Figure 5. The effect of xylem water potential and low VPD on leaf conductance in *Psuedotsuga menziesii*. Ambient environment was same as in Fig. 4. Regression equations by VPD are 15 mb (●) -- $g_\ell = 0.0582 + 0.0130\ \psi_x$, $R^2 = 0.57$; 7 mb (◆) -- $g_\ell = e^{(-3.136 + 0.829\ \psi_x)}$, $R^2 = 0.66$.

Figure 1

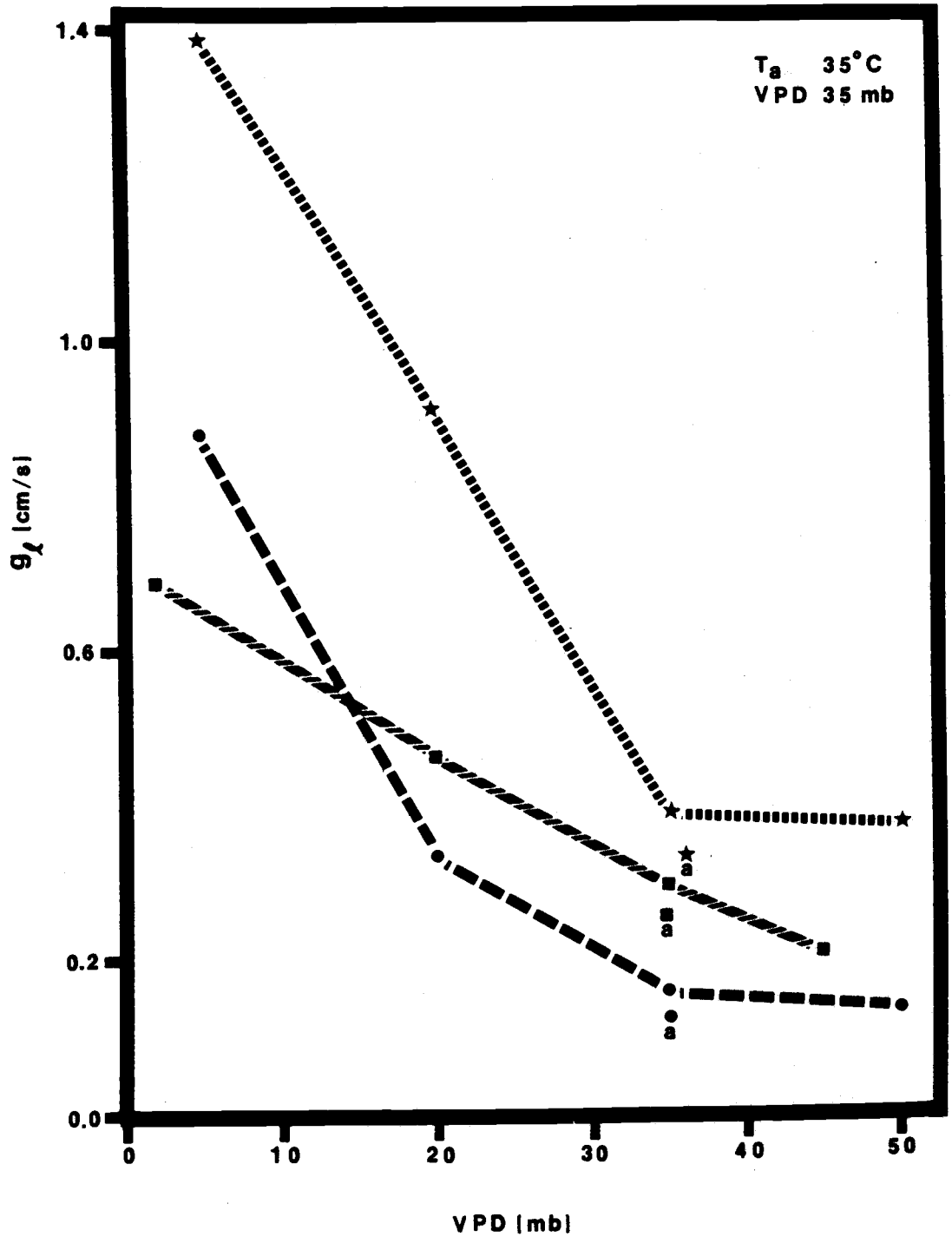


Figure 2

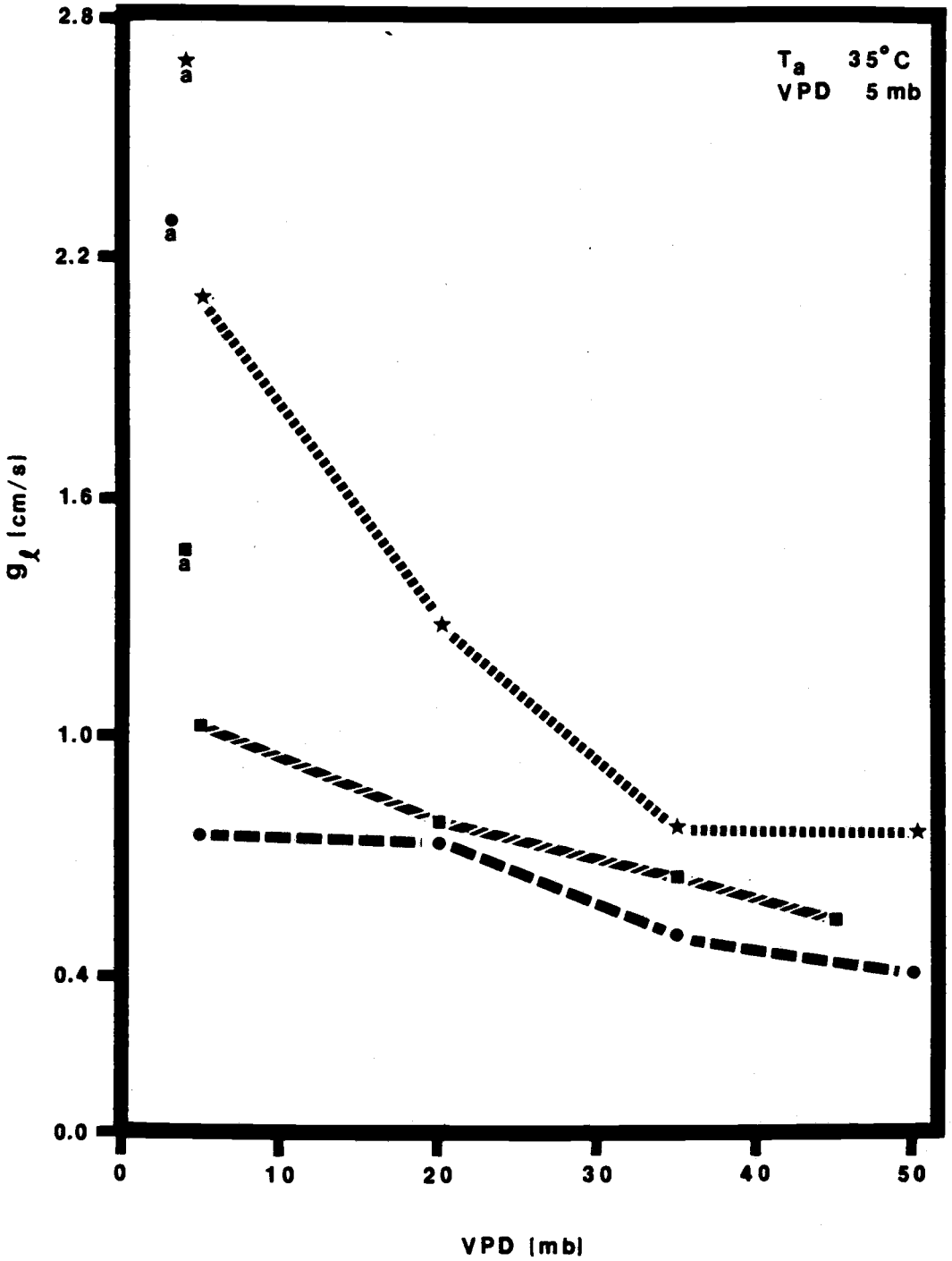


Figure 3

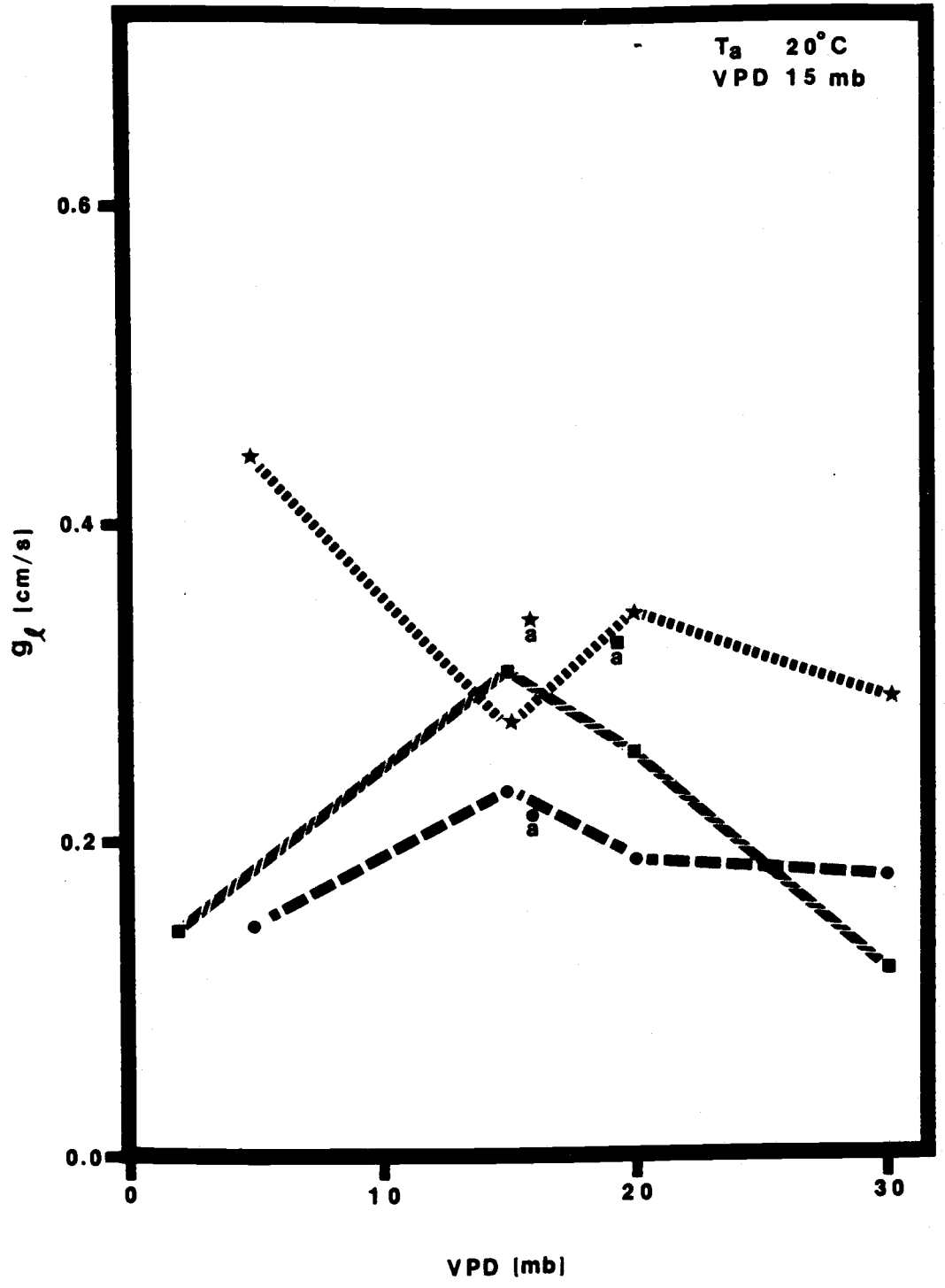


Figure 4

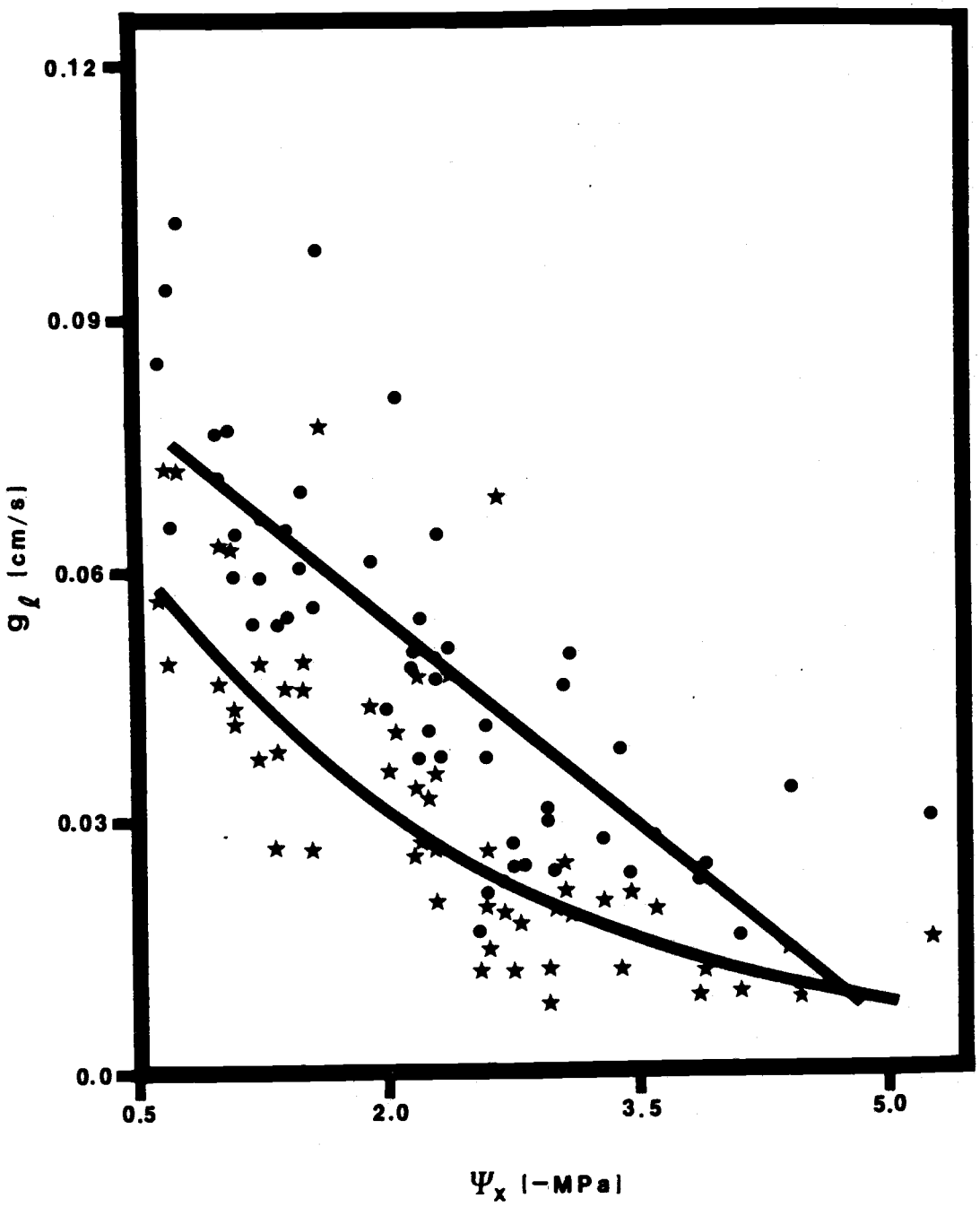
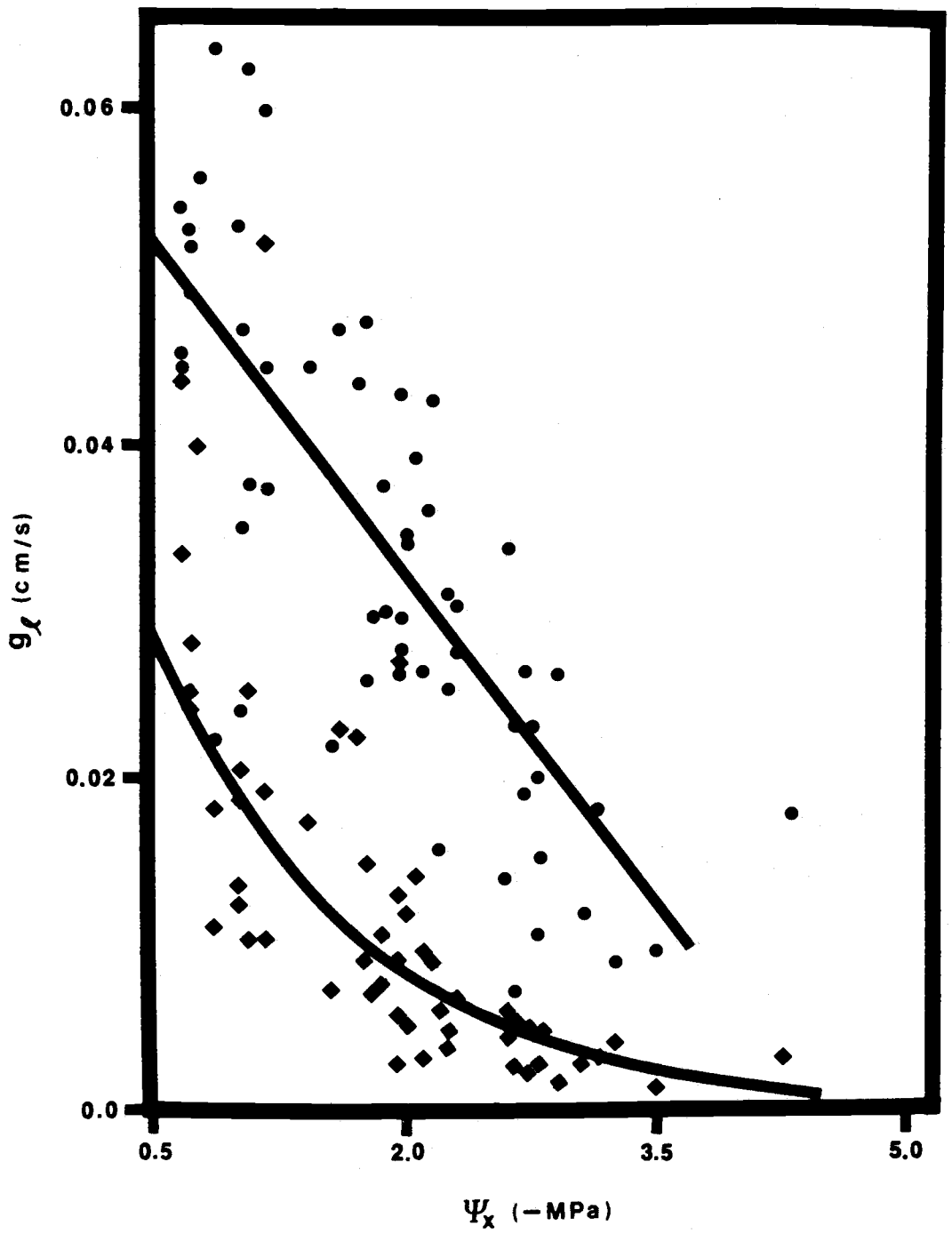


Figure 5



CHAPTER 4

The Kinetics of *In Vitro* and *In Vivo* Photoisomerization of Abscisic Acid[1,2]

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Abbreviation: ABA, abscisic acid; t-ABA, 2-trans-abscisic acid; PSS, photostationary state; ECD, electron capture detector; ε, molar absorptivity; Me-ABA, methyl ester of abscisic acid.

ABSTRACT

The photoisomerization kinetics of the geometric isomers of abscisic acid were investigated in solution and in seedlings. Aqueous solutions of ABA, 2-trans-ABA, and their methyl esters were exposed for specified time periods up to 60 minutes under 254-nanometer, 350-nanometer, fluorescent, and solar radiation. *Pseudotsuga menziesii* (Mirb.) Franco seedlings were irradiated with 350-nanometer or fluorescent lamps and the needles analyzed for endogenous changes in the concentration of ABA, 2-trans-ABA, and their saponifiable conjugates.

In vitro, the 254-nanometer light caused photolysis of both isomers. The 350-nanometer light and sunlight were comparable in isomerizing ABA and 2-trans-ABA with half-time of about 3 minutes, whereas isomerization under fluorescent lamps took longer (half-time = 7 minutes). Methylation of the isomers caused the half-time for photoisomerization to increase in all of the light sources. The equilibrium concentration, 55% ABA and 45% 2-trans-ABA, was consistent for the free acid and the methyl ester under all radiation treatments. The absorption spectra of the isomers provided evidence for the higher concentration of the cis-isomer at photoequilibrium.

In vivo, no significant conversions in any of the four compounds were observed in either light. However, the presence of 2-trans-ABA and its conjugate in the needle extracts suggests that isomerization occurs. Variation was considerable among seedlings and between branches.

Abscisic acid is an endogenous plant hormone that is ubiquitous among plants and that is implicated as a controlling agent in flowering, growth inhibition, dormancy, senescence, abscission, ion movement, and stomatal regulation (20,27,30). The biologically active form of the hormone is ABA (20). Its geometric isomer, t-ABA, found in plant extracts (9,15,19,26), has been attributed to *in vivo* photoisomerization. Reported biological activity of t-ABA has varied from 1% (3) and 3% of ABA (17) to equal activity (24). Much of the variability may be due to the sensitivity of the bioassays used for determining activity. Milborrow (17) attributed the biological activity to slow isomerization of t-ABA to ABA in the tissue.

In plant extracts, photoisomerization of the two isomers in UV light has been used to determine the presence of ABA (6,20), but little is known about *in vivo* photoisomerization. Lindoo *et al.* (16) studied the effect of UV-B radiation (280-320 nm) on the ABA status in *Rumex* leaves, but the results were inconclusive.

The objective of this research was first to characterize the *in vitro* kinetics of ABA and t-ABA photoisomerization in different types of radiation and second to determine, by comparison, possible *in vivo* isomerization in irradiated foliage of Douglas-fir seedlings.

THEORY

The photoisomerization of geometric isomers of various organic compounds has been studied extensively, and a model has been developed to explain the process (5,7,12,22,25). Ground-state isomerization, that is the thermal reaction, proceeds via a nonplaner transition state common to both cis- and trans-isomers. The collapse of the transition state produces a larger proportion of the more thermodynamically stable isomer.

usually the trans-isomer. However, photochemically-induced isomerization results in a different composition in which the cis-isomer predominates.

Irradiation of cis-isomers and trans-isomers produces an excited species common to both, the phantom state, which can be either a singlet or a triplet. The phantom state collapses to the ground state by conventional deactivation processes and affords both cis- and trans-isomers. For reasons we will discuss, the trans-isomer is more likely to be excited to the phantom state, and because collapse is a stochastic process, more of the cis-isomer will be present at equilibrium, PSS. For example, at PSS in stilbene, the proportions of the cis-isomer and trans-isomer are 92% and 8%, respectively.

The proportion at PSS depends on many factors and varies with each compound. The difference between the molar absorptivity of the cis- and trans-isomers is an important factor. The isomer with the higher molar absorptivity undergoes preferential excitation and thus occurs in lower proportion at PSS, if we assume similar quantum efficienies. In stilbene, molar absorptivity is 16,300 for the trans-isomer and 2,280 for the cis-isomer at 313 nm (7). Neckers (22) noted that the cis/trans ratio for stilbene varied with solvent, temperature, and sample purity, all of which also influence molar absorptivity.

MATERIALS AND METHODS

Radiation Source. A Raynet Photochemical reaction chamber (The Southern N.E. Ultraviolet Co., Middleton, Connecticutt) equipped with either 254-nm, 350-nm, or Sylvannia F8T5/cw lamps was used for

irradiating the solutions and the seedlings. *In vitro* isomerization in sunlight was conducted outdoors around noon on a clear day in June 1979. Photon flux density for each radiation source, summarized in Table 1, was quantified for wavelenghs below 500 nm with a Potassium Ferrioxalate actinometer (10). Photon flux density for the fluorescent lamps and sunlight was estimated by integrating the absorbance of the actinometer and the output of the source at each wavelengh up to 500 nm.

In Vitro Study. Stock solutions between 10^{-4} and 10^{-5} M of ABA and t-ABA were made in deionized, distilled water. The ABA was purchased from Calbiochem (San Diego, California) and the t-ABA was obtained by irradiation ABA in 350-nm light for 1 h. The t-ABA was separated from the cis-isomer by thin layer chromatography (toluene: ethyl acetate: acetic acid -- 50:30:4) on silica gel GF-254. Three-milliliter aliquots of the stock solutions were pipetted into quartz spectrophotometer cells and were irradiated for specified times: 254 nm -- 0.25, 0.5, 0.75, 1, 2, 5 min; 360 nm -- 0.5, 1, 2, 5, 10, 25, 60 min; fluorescent -- 0.5, 1, 2, 5, 10, 25, 60 min; and sunlight -- 10, 25, 40 min. Each exposure time was replicated twice. After exposure, the water was removed *in vacuo*, the samples were methylated with diazomethane, and the two isomers were separated and quantified by GLC-ECD.

To determine the effect of ABA esterfication on the photoisomerization, methylated ABA and t-ABA were irradiated in water and were analyzed in the same way.

The kinetic data were normalized to 100 and were fitted to equations with nonlinear regression.

$$\text{Starting compound } Y = B1 \times \exp(-B2 \times \text{time}) + B3$$

$$\text{Accumulating compound } Y = B1 \times [1 - \exp(-B2 \times \text{time})]$$

where:

B1 = PSS concentration of accumulating compound

B2 = fit by regression

B3 = PSS concentration of starting compound

The absorption spectra of ABA and t-ABA were determined in distilled 85% ethanol and in deionized-distilled water with a Hitachi Perkin-Elmer UV-Vis spectrophotometer, Model 139. Spectra of both the free acid and the methyl ester were measured. Concentrations of the solutions were determined afterward by GLC-ECD.

In Vivo Study. Three-year-old Douglas-fir seedlings [*Pseudotsuga menxiesii* (Mirb.) Franco] individually potted in soil were irradiated in the photochemical reactor with either 350-nm for 5, 10, 15, 25, 40 and 60 min or fluorescent lamps for 10, 25, 40 and 60 min. One seedling was used for each exposure time. Two branches were samples, a control branch before and another branch after the light treatment. The needles were analyzed for ABA, t-ABA, and their saponifiable conjugates, presumably their glucosides, with GLC-ECD. This *in vivo* study was repeated three times.

ABA Extraction and GLC. This extraction method was a modification of one used by Zabadal (31). Needles were stripped from the branches, plunged into liquid N_2, ground into a powder, and lyophilized. After weighing, the powdered needles were extracted twice in 75 ml acetone for

24 h in the dark at 5°C. The acetone solution was filtered, and 10^4 dpm DL - (2 - ^{14}C) - ABA (specific activity 11.1 mCi/mmol, Amersham Corp., Arlington Heights, Illinois) was added as an internal standard for extraction losses. The samples were phase-partitioned between dichloromethane and water. The conjugated forms separated from the free acids at the second partition. The esters were saponified at pH 10.8, 60°C for 1 h. The pH of the solution was adjusted to 2.5, the ^{14}C standard was added, and the solution then partitioned against dichloromethane. The samples were further purified by thin layer chromatography as we have described.

The samples were methylated with diazomethane and were separated and quantified with gas-liquid chromatography. A Hewlett-Packard Model 5750 chromatograph with a ^{63}Ni ECD was fitted with an Ultrabond 100/120 mesh (Supelco, Inc., Bellefonte, Pennsylvania) glass column (1.16 m by 3 mm) for the analysis. The carrier gas (argon:methane -- 90:10) delivered through the column at 22 ml/min, purged the detector at 30 ml/min. Temperatures were: column, 190°C; injection port, 205°C; detector, 290°C. All compounds were positively identified with a Finnigan Mass Spectrometer.

RESULTS

Absorption Spectra. Geometric photoisomerization depends not only on the energy of the radiation but also on the energy-absorbing properties of the compound. The molar absorptivities of ABA and t-ABA in ethanol and water differed (Fig. 1). Methylation also affected the spectra of molar absorptvity (Fig. 2), which was larger for the trans-

isomer than the cis-isomer in both solvents at all wavelength below 500 nm. Methylation resulted in an increase in molar absorptivity for both isomers. However, greater increases for the cis-isomer resulted in nearly equal energy absorption throughout the spectra.

In Vitro Photoisomerization. The photoisomerization of both ABA and t-ABA occurred under all of the radiation sources. However, the isomerization rate was strongly influenced by light quality. Rapid isomerization with concomitant photolysis took place in 254-nm light (Fig. 3). Measurable amounts of t-ABA appeared within 15 seconds, increasing from zero to 35 ng. Photolysis within this time span destroyed about 30 ng, and little of either isomer was detected after 2 min. (Similar results were observed when t-ABA was the starting compound.)

Longer wavelength radiation showed no photolysis but did cause photoisomerization. The kinetics of photoisomerization in 350-nm light are illustrated in Figure 4 with the initial isomers ABA and t-ABA. The figure illustrates the general kinetic curves for all longer wavelength sources as well as for the methylated ABA isomers. Notable was the PSS concentration of 55 ng ABA and 45 ng t-ABA and the characteristic crossover when starting with t-ABA. These proportions were consistently observed at PSS and account for the shorter half-times when t-ABA was the starting compound (Table 2).

The 350-nm light isomerized ABA most quickly (half-time about 2.75 min). Sunlight was nearly as efficient (half-time about 3 min.), and fluorescent light was least effective (half-time roughly 9 min). The

methyl esters of ABA were more difficult to isomerize, as indicated by their longer half times. Methylation slightly increased half-time in fluorescent light and doubled it in 350-nm light.

The quantum yield of photoisomerization for the various light sources (Table 3) help interpret the results. The photon flux densities (Table 1) were used to calculate quantum yield, and their use accounts for the value determined for sunlight.

In vivo Photoisomerization. The results for the photoisomerization of ABA and its conjugates are summarized in Table 4. In all instances, t-ABA and its conjugate were present. However, no significant trends with exposure time were apparent. Values for branches from the same seedlings as well as those from different seedlings varied widely (Table 4). To reduce variation and to compre values, we expressed the data as a percent of the sum of the four compounds.

DISCUSSION

The absorption spectra (Fig. 1, 2) correspond well to previously reported values for ABA (3,4,16,18,21). No absorption data for t-ABA have been published. The effect of solvent and methylation on the spectra provides evidence for the kinetics data (Table 2). The longer half-time for the methylated isomers reflect the nearly equal molar absorptivity observed (Fig. 2), especially at longer wavelengths.

The absorption spectra and the PSS proportion of 55% ABA and 45% t-ABA indicate that the theory for geometric photoisomerization holds for abscisic acid, at least *in vitro*. According to theory, the larger

molar absorptivity of t-ABA resulted in greater photochemical conversion to the cis-isomer. Several authors have reported 50% equilibrium concentrations for each isomer (14,16). This may be attributed to differences in solvents, temperature, sample purity (22) or to the improper assumption that 1:1 concentration should exist as equilibrium. The kinetic half-times (Table 2) further support the observation that the PSS contains more of the cis-isomer. In all instances when t-ABA was the starting compound, the half-times were shorter and the concentration of the cis-isomer ultimately exceeded that of the trans-isomer. If the solution at PSS was 1:1, then the half-times should be similar regardless of the starting isomer. The PSS concentration did not appear to be affected by methylation, though half-times increased.

The optimum wavelength for photoisomerization appeared to be within the range of UV-A (320-400 nm). The 350-nm lamps produced the quickest conversion (Table 2). They have been reported to possess fairly broad emission spectra ranging from 300 nm to 400 nm with a peak around 350 nm (13). Both sunglight and fluorescent lamps emit in this range but at lower photon flux densities (13), which accounts for their smaller quantum yields (Table 3).

The difference in half-times between the free acid and the methyl ester (Table 2) of ABA and t-ABA was attributable to the effect of methylation on molar absorptivity. Assuming that esterification increases half-time, and extrapolation suggests that the conjugated forms of ABA found in plants may inhibit *in vivo* isomerization and provide a photo-stable but sequestered source of the biologically active isomer.

The quantum yields reported for the various light sources (Table 3) are comparable to those reported by Brabham and Biggs (2) for photolysis of ABA in UV-B light and by Whillans and Johns (30) for photolysis of nucleic acids. Lindoo *et al.* (16) reported a quantum yield of 0.79 for the photoisomerization of ABA, roughly 2 to 3 orders of magnitude larger than that found in this study. However, they were using UV-B radiation that theoretically would have a larger quantum yield, and they did not account for photolysis. Figure 1 illustrates photolysis for 254-nm light, showing rapid isomerization along with the photolysis of ABA under a photon flux density lower than that of the 35-nm light (Table 1). This higher energy radiation would have a larger quantum yield. Lindoo *et al.* (16) monitored "isomerization" only by the decrease in ABA, which would not differentiate between photolysis and isomerization.

The low quantum yield for sunlight is attributable to the high photon flux density (Table 1) used in its calculation. The proportion of light that induces isomerization is small compared to the light emitted between 400 and 500 nm (the upper limit of the actinometer). Therefore, if only the shorter wavelength radiation were used in the calculation, the quantum yield would be larger.

We have identified UV-A radiation as that most likely responsible for photoisomerization of ABA. It has been estimated to be about 3% of the total solar radiation (13) and to represent roughly 90% of the total UV light reaching the earth's surface (8). Thus, plants growing in sunlight are exposed to radiation which potentially could cause photoisomerization.

The presence of t-ABA in plant extracts is evidence for *in vivo* isomerization of ABA. Milborrow (19) found the t-ABA in rose leaves to be about 3% of the ABA present. Lesham *et al.* (15) reported t-ABA constituted up to 100% of the total ABA content of almond buds and noted a marked drop in the trans-isomer during bud break, suggesting that photoisomerization may have caused the drop as the new foliage became free of its bud scales. In contrast, t-ABA was not found in birch (14), apple (23), or *Rumex* (16). Studies indicate that the trans-isomer is conjugated much faster than the cis-isomer (19,23), which could account for the absence of t-ABA.

Although t-ABA was found both in free and conjugated forms in Douglas-fir seedlings (Table 4), no significant conversions were observed as a result of the radiation treatments. Lindoo *et al.* (16) found no increase in t-ABA in *Rumex* leaves after exposure to UV-B radiation, and proposed that the radiation was being attenuated by leaf constituents. This is plausible because many compounds found internally and externally in plants absorb radiation below 400 nm. Carotenoids and flavones have been shown to absorb strongly in the UV region, as have cuticular waxes (13).

Another explanation for the lack of response to the radiation treatments is that metabolism of ABA-related compounds is rapid enough to maintain nearly equal levels of ABA and t-ABA and their conjugates. In another study conducted in our laboratory, 2-trans-dihydrophaseic acid was found to parallel change in ABA. Dihydrophaseic acid has been shown to be a metabolite of ABA (20,27). This suggests that with high levels of ABA, more isomerization occurs, but the resulting

trans-isomer is rapidly catabolized to trans-dihydrophaseic acid.

Leaf compartmentalization of ABA has been proposed by several researchers (1,11,28) and may provide another explanation for the observed lack of treatment effect *in vivo*. Assuming that the small amount of t-ABA present in the free form represented the true PSS concentration in solution in the leaf, there would then have been 55/45 x (t-ABA concentration) ABA in solution. The additional ABA found in the plant extract could have been membrane-bound, or in some other way compartmentalized or sequestered, such that it could not photoisomerize. Because the small amounds of ABA and t-ABA in solution were already at equilibrium, further radiation treatments would not have changed the concentrations of the two isomers. The rate of t-ABA metabolism and conjugation would control the amount detected in plant extracts, and the varying rates would explain the absence reported in some species and the varying concentration found in others.

The photoisomerization of ABA *in vitro* has been demonstrated to occur rapidly, tha actual rate being dependent on light quality. Though we found no evidence for *in vivo* photoisomerization in Douglas-fir seedlings, the presence to t-ABA in both the free and conjugated form suggests that isomerization occurs.

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TABLE 1. The Photon Flux Density for Light Sources in the Photoisomerization Study Quantified for Wavelengths Below 500 mm.

Source	Photon Flux Density
	$\mu E/cm^2$-min
Sunlight	1.077
254-nm Lamp	0.226
350-nm Lamp	0.450
Fluorescent Lamp (reaction chamber)	0.294
Fluorescent (growth room)	0.004

TABLE 2. The Photoisomerization Half-Times of the Geometric Isomers and Their Methyl Esters for Several Radiation Sources

Source	Initial Compound	Half-time	
		Cis-isomer	Trans-isomer
		- - - min - - -	
350-nm Lamp	ABA	2.76	2.71
	t-ABA	1.24	1.61
	Me-ABA	4.53	4.72
	Me-t-ABA	3.45	3.07
Fluorescent Lamp	ABA	8.92	7.89
	t-ABA	6.14	6.31
	Me-ABA	10.29	10.60
	Me-t-ABA	6.77	6.61
Sunlight	ABA	2.99	3.47

TABLE 3. The Quantum Yield of ABA Photoisomerization for Three Light Sources

Source	Molecules/Quantum
350-nm Lamp	2.3×10^{-3}
Fluorescent Lamp	1.6×10^{-3}
Sunlight	8.2×10^{-4}

TABLE 4. The *In Vivo* Photoisomerization Kinetics of Douglas-fir Seedlings Irradiated with Either 350 nm or Fluorescent Light.

The values for ABA, t-ABA and their conjugates represent the mean of three studies and six samples. The means and the standard deviations, in parentheses, are expressed as a percentage of the total.

Source	Time	Free		Conjugate		Total
		ABA	t-ABA	ABA	t-ABA	
	min	- - - - % of total - - - - - -				ng/g dw
Fluorescent Lamp						
Control	10	11.4 (2.3)	3.3 (3.7)	63.4 (11.1)	21.8 (6.0)	6082 (2159)
Treatment		11.1 (3.3)	4.0 (2.5)	66.7 (12.0)	18.2 (10.8)	7361 (7349)
Control	25	12.4 (10.8)	3.9 (3.8)	65.6 (20.8)	18.1 (11.1)	5529 (918)
Treatment		18.4 (13.0)	9.9 (8.7)	60.7 (25.7)	11.0 (4.9)	5309 (1744)
Control	40	7.7 (3.0)	2.2 (0.9)	71.2 (12.8)	18.9 (10.7)	5517 (2271)
Treatment		9.9 (5.1)	6.3 (5.1)	61.4 (18.9)	22.3 (10.2)	5875 (3880)
Control	60	9.5 (6.7)	4.7 (2.1)	72.0 (6.0)	13.9 (3.2)	5347 (1277)
Treatment		13.0 (5.2)	6.2 (4.4)	69.9 (10.8)	11.0 (1.6)	5279 (1945)

TABLE 4. Continued

350-nm Lamp						
Control[a]	5	9.4	2.2	64.7	23.8	7555
Treatment[a]		7.3	10.6	62.5	19.5	8076
Control[b]	10	14.7 (1.3)	3.1 (1.3)	68.1 (6.7)	14.2 (4.2)	4391 (1635)
Treatment[b]		15.2 (6.6)	9.7 (0.7)	66.8 (18.5)	10.8 (7.6)	3801 (2143)
Control[a]	15	11.3	6.3	62.6	19.9	6270
Treatment[a]		27.9	24.2	37.8	10.1	10058
Control	25	9.5 (6.3)	5.2 (4.1)	58.4 (17.3)	26.9 (22.2)	8404 (6794)
Treatment		16.6 (9.4)	6.7 (5.8)	52.5 (18.6)	24.2 (5.9)	10068 (9480)
Control	40	9.8 (10.1)	5.3 (0.3)	68.1 (13.0)	16.8 (10.6)	3902 (1699)
Treatment		13.1 (9.7)	14.0 (16.7)	54.5 (25.0)	18.4 (8.3)	5066 (3619)
Control[b]	60	8.8 (7.9)	2.8 (1.0)	77.6 (15.9)	10.9 (7.0)	3284 (1780)
Treatment[b]		7.5 (5.4)	2.7 (1.0)	74.7 (20.2)	15.2 (13.8)	3466 (1381)

[a]Data represent one experiment.

[b]Means based on two experiments.

FIGURE CAPTIONS

Figure 1. The spectra of molar absorptivity of ABA and t-ABA in two solvents.

Figure 2. The spectra of trans/cis molar absorptivity for the free acid and the methyl ester of ABA.

Figure 3. The photolysis and isomerization kinetics of ABA in 254-nm light.

Figure 4. The photoisomerization kinetics of ABA and t-ABA in 350-nm light (a) initial isomer ABA, (b) initial isomer t-ABA.

Figure 1

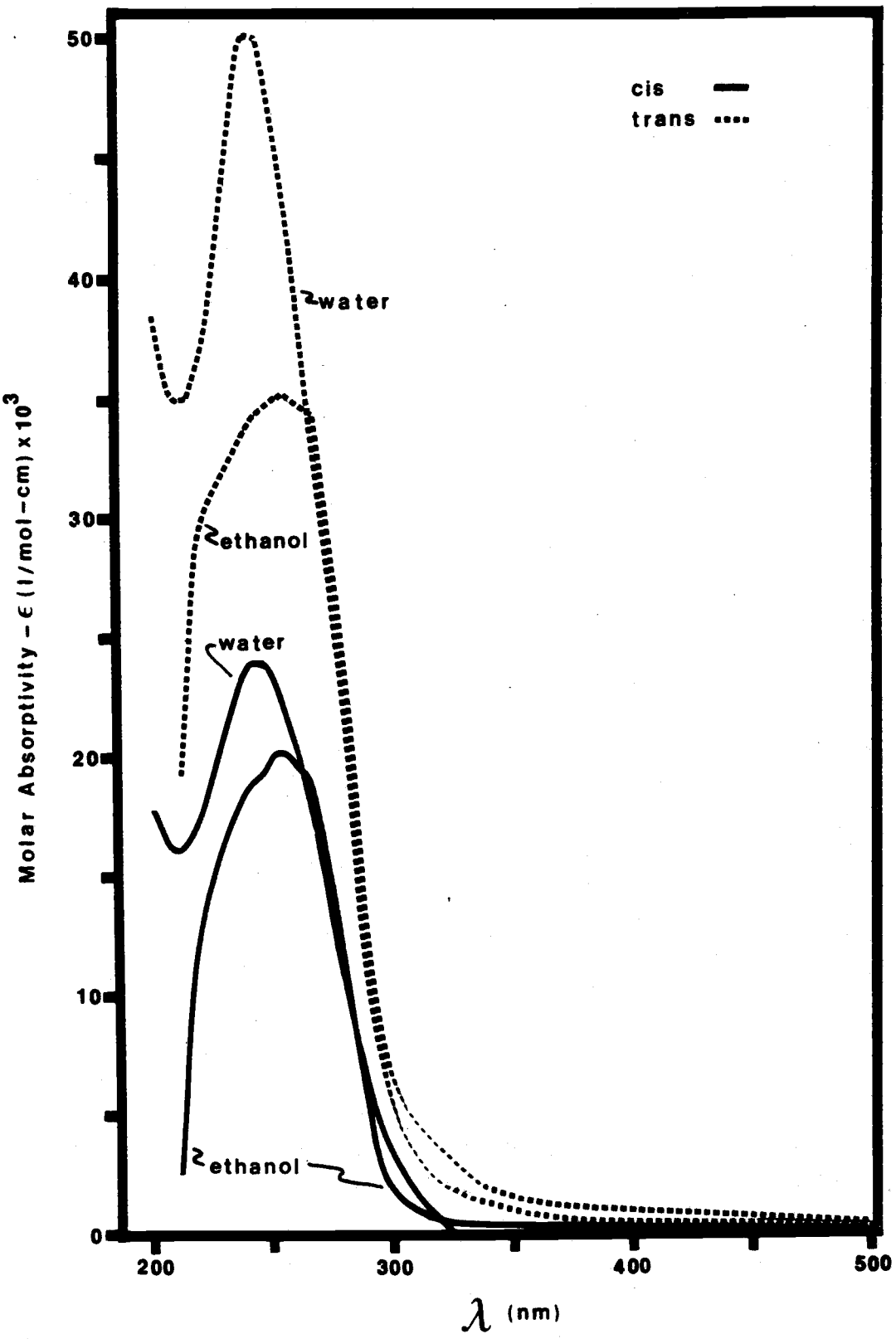

cis
trans
water
ethanol
water
ethanol
Molar Absorptivity - ε (l/mol-cm) x 10^3
50
40
30
20
10
0
200
300
400
500
λ (nm)

Figure 2

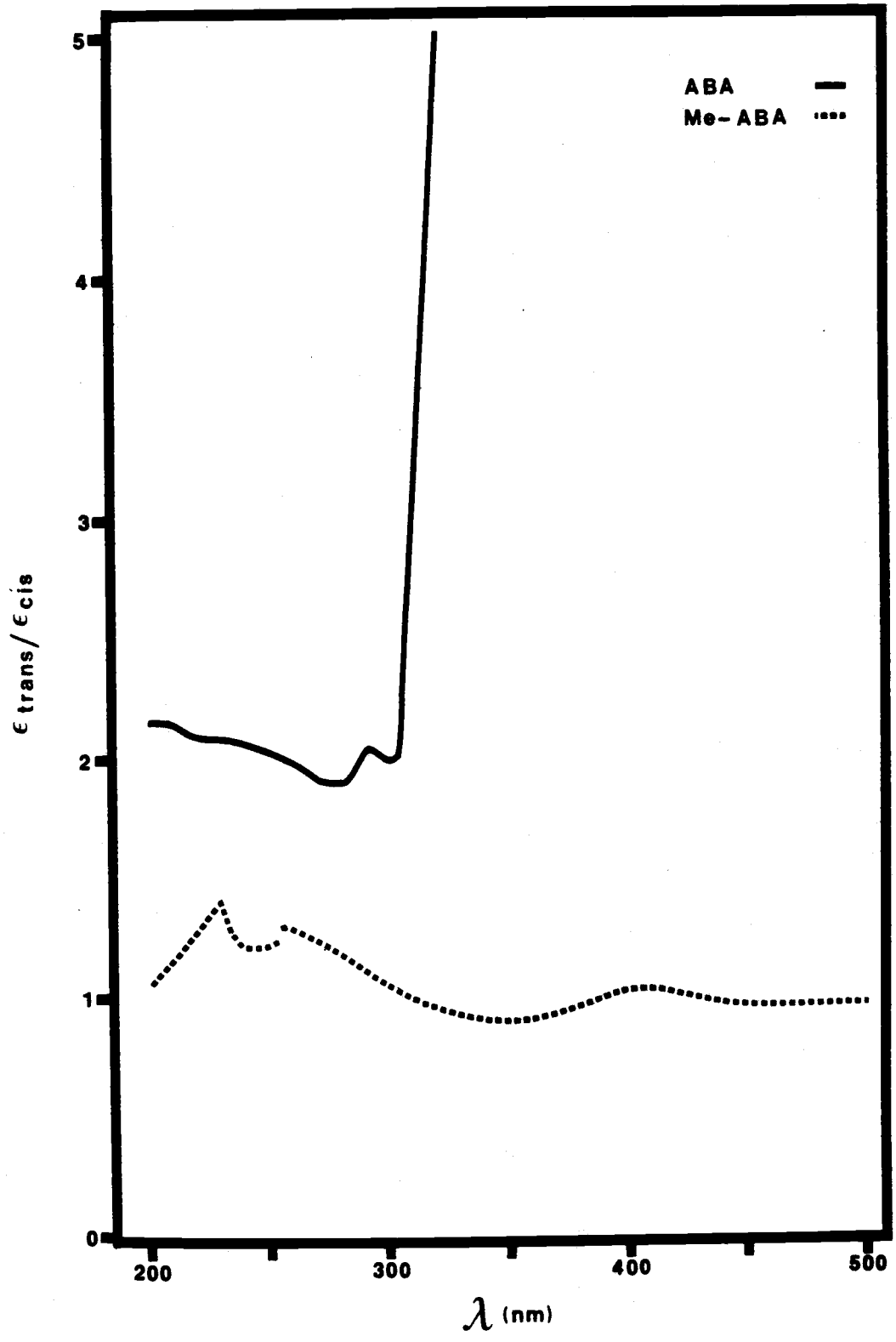


Figure 3

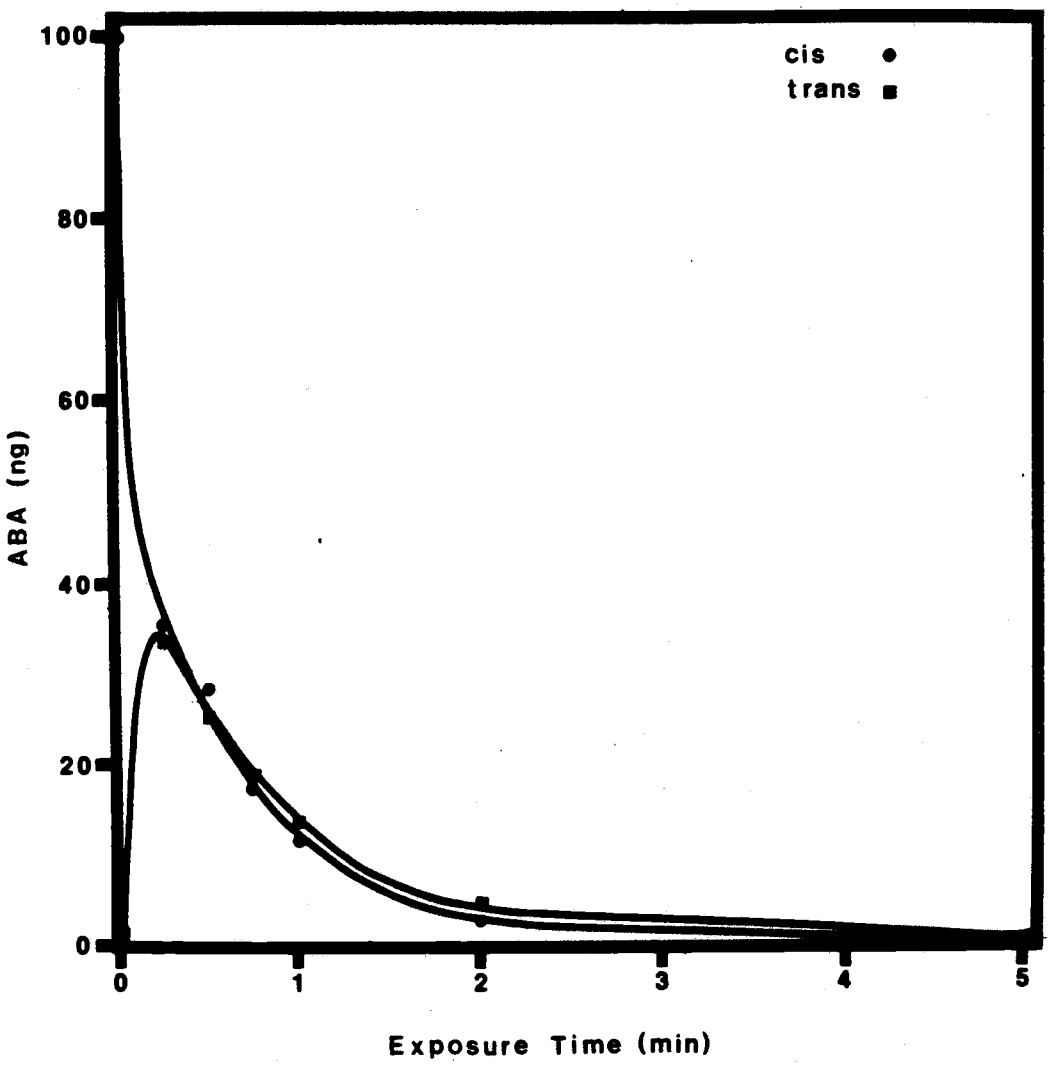
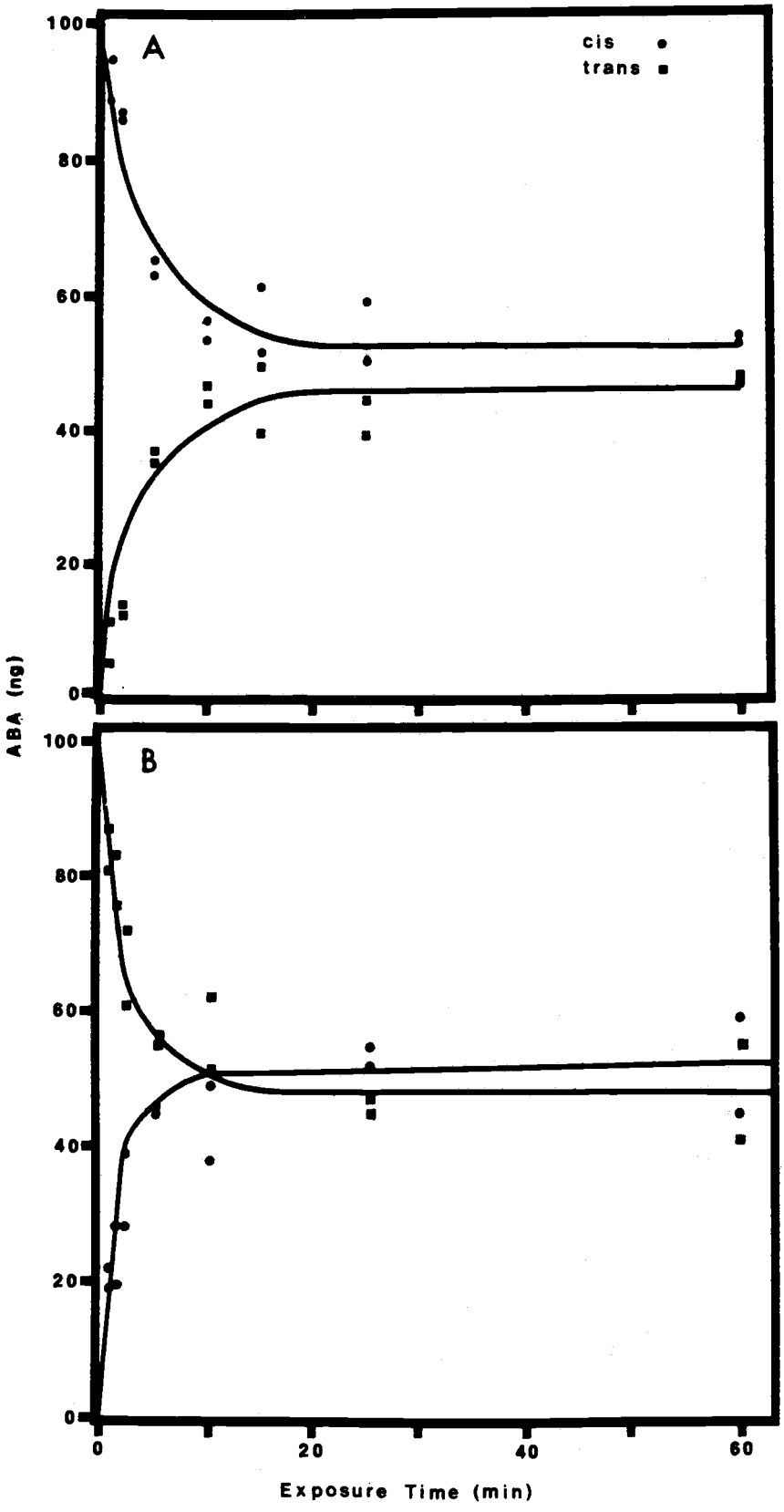


Figure 4



CHAPTER 5

The Effect of Abscisic Acid Metabolism in Douglas-fir During Water Stress on Stomatal Conductance[1,2]

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Abbreviation: ABA, abscisic acid; PA, phaseic acid; DPA, dihydrophaseic acid, epi-DPA, epi-dihydrophaseic acid; t-ABA, 2-trans-abscisic acid; g_ℓ, branch conductance; ψ_x, xylem water potential.

ABSTRACT

The changes in abscisic acid and its metabolites were followed through two drought cycles in *Pseudotsuga menziesii* seedlings to determine the metabolic pathway of the hormone and its effect on branch (stomatal) conductance.

Leaf conductance showed the typical water potential threshold, decreasing abruptly at -2.0 MPa. This corresponded to the simultaneous increase in abscisic acid level, from 500 to 850 ng g^{-1}. The relationship between abscisic acid and water potential was not definitive, though the general trend was an increase in the hormone as stress intensified until the water potential was -5.0 MPa, at which a sharp decline in concentration was evident. The results suggest the function of abscisic acid is to close stomates at the onset of water stress, but not to maintain closure throughout the duration of the stress. Physical limitations attributed to lack of foliar water appear to keep the stomates closed. No adjustment to stress was observed in any of the above relationships, but stress during the second cycle progessed at a slower rate.

Metabolism of abscisic acid was found to differ from reports of previous studies. A linear relationship between abscisic acid and its conjugate strongly implicated the importance of the interconversion of the two compounds for storage and supply of the free acid. This may have been due to the age of the tissue and preconditioning to water stress. Phaseic acid and epi-dihydrophaseic acid showed the greatest variation during the study suggesting that these were the primary

metabolites. Changes in trans-dihydrophaseic acid paralleled abscisic acid providing evidence for the occurrence of concentration-dependent photoisomerization, either of abscisic acid or one of its metabolites (phaseic or dihydrophaseic acid).

Stomatal regulation is complex and involves several controlling factors including light intensity (45), intercellular carbon dioxide concentration (35) and atmospheric humidity (23). These factors appear to be jointly controlling stomates during favorable plant water status (6). However, during water stress, leaf abscisic acid level increases, closing the stomates and overriding the other controlling factors (29,39).

The rise in ABA has been associated with a water potential (1,3, 4,5,24,32,43) and turgor potential (33) threshold. In some species, though, a gradual ABA increase occurred as water stress developed, exhibiting no threshold (9). In either case, stomates respond rapidly to enhanced ABA concentrations, closing within a few minutes to exogenous applications (8,20). Relief from water stress results in a drop in ABA concentration, attaining prestress levels within a few hours (3,16) or days (2,11). The stomates, however, take several days to achieve full turgor and complete opening (2,11,19).

The mechanism of stomatal control by ABA is not well understood, but evidence suggests that the site of ABA action is the guard cell plasmalemma. ABA causes the extrusion of K^+ and a drop in turgor potential (27).

The origin of the ABA associated with water stress has not been identified. *De novo* synthesis has been proposed by Milborrow (28,31)

and others (39,41,42), based on studies using (^{14}C)-ABA or one of its precursors. The reconversion of conjugated ABA, i.e. the saponifiable ester, during the initial onset of water stress has been speculated to account for the rapid increase in ABA, but supportive data are lacking (11,28,29,30,31,34). Walton *et al.* (40) took the view that synthesis and catabolism become elevated at the onset of stress as does transport to the active site. More recently, disruption of sink-source relationships and translocation from leaves was found to increase foliar ABA levels and close stomates (38).

The metabolism of ABA has been demonstrated in several species to follow a similar path. Phaseic acid is the first stable metabolite (14,29) which reduces the dihydrophaseic acid. Zeevaart and Milborrow (44) found an abundance of the epimer of DPA and suggested that the pathway splits at this point. The metabolism of t-ABA has not received much attention and as a result less is known about it. Both ABA and t-ABA are found in plants in a conjugated form, once believed to be the glucose ester (39). The function of the conjugates are not clearly understood; they may represent a rapid inactivation product (though t-ABA is not very biologically active) or they may be the first step in catabolism (29,39).

The study of ABA metabolism has been conducted on rapidly stressed (a few hours), very young tissue (several weeks old) with radioisotopes introduced through the cut petiole of detached leaves. These techniques create an extremely artifical experimental environment which may alter the process under investigation. Metabolism of radioisotopes applied in such a manner has been suggested to be different from the

metabolism of the endogenous compounds (29). Exogenous application not only could cause an imbalance of synthesis and metabolism due to higher concentrations, but there is no guarantee that the applied compounds ever reach the site of endogenous synthesis and metabolism (29).

The objective of this study was to determine the endogenous metabolic pattern of ABA in older, intact plants during naturally-induced water stress as it related to stomatal regulation. The role of *in vivo* photoisomerization in the metabolism of ABA was also investigated by including high intensity light treatments.

MATERIALS AND METHODS

Three-year-old *Pseudotsuga menziesii* (Mirb.) Franco seedlings, individually potted in soil, were maintained in an environment of 20 C, 60% RH under fluorescent lights (200 uE $cm^{-2}min^{-1}$) at a 16 h photoperiod. Control seedlings were watered throughout the study whereas water was withheld from the treatment seedlings and they were allowed to dry naturally. After the first stress cycle, the treatment seedlings were watered and branch (stomatal) conductance were permitted to attain control levels. Water was again withheld for the second stress cycle. Leaf conductance was measured with a steady-state porometer (Interface Instruments, Corvallis, Oregon), and xylem water potential (ψ_x) was determined with a pressure chamber.

Four treatment seedlings, four branches per seedling, were sampled at each stress level and each light treatment. Two branches were sampled before and two after the light treatments and were analyzed for ABA and its metabolites. The light treatments consisted of 30 min

exposure in a Raynet Photochemical reaction chamber (The Southern N.E. Ultraviolet Co., Middleton, Connecticutt) equipped with either 350-nm or Sylvania F8T5/cw lamps. Photon flux densities estimated with a Potassium Ferrioxalate actinometer (15) were: 350 nm - 0.45 uE cm^{-2} min^{-1}; fluorescent - 0.29 uE $cm^{-2}min^{-1}$; growth room - 0.004 uE cm^{-2} min^{-1}. Before irradiation, g_ℓ and ψ_x were sampled on each seedling, and g_ℓ was remeasured on the same branch following the light treatment. Four control seedlings, 2 branches per seedling, were sampled along with the treatment seedlings.

The needles (about 100) were stripped off of each branch, placed into a preweighed vial, and it was plunged into liquid N_2. They were ground into a fine powder with a mortar and pestle, returned to the vial, lyophilized, and weighed. The needles were extracted twice with 75 ml acetone at 5 C in the dark for 24 h. The acetone was filtered, 10^4 dpm of D,L-(2-^{14}C)-ABA (specific activity of 11.1 mCi $mmol^{-1}$, Amersham Corp., Arlington Heights, Illinois) and 50 ml NH_4OAc buffer (pH 8.0) were added, and the acetone was removed *in vacuo*. The pH of the buffered solution was readjusted to 8.0, and the solution partitioned against toluene (3 x 50 ml). The aqueous fraction was slurried with PVP for 10 min, filtered and the pH of the filtrate decreased to 2.5. Phase partition against $MeCl_2$ (3 x 50 ml) followed. Both fractions were retained this time. The aqueous fraction containing the conjugates was adjusted to pH 10.8 and saponified for 1 h at 60C., After dropping the pH to 2.5 and adding the ^{14}C standard, the phase partition with $MeCl_2$ was repeated as before, but only the organic phase was saved.

Both the free and conjugate fraction were further purified by removing the $MeCl_2$ *in vacuo*, solubilizing the residual in 10 ml NH_4OAc buffer (pH 3.0) and loading it onto a preparatory column (40 mm by 5 mm ID stainless steel, packed with octadecyl coated, solid core pellecular beads). The column was washed with d,d-H_2O, the sample was eluted with 40% (v/v) EtOH:H_2O, and taken to dryness *in vacuo*.

The samples were methylated with diazomethane, and then separated and quantified with gas-liquid chromatography. A Hewlett-Packard, Model 5750, chromatograph with a ^{63}Ni ECD was fitted with a glass column (1.16 m x 3 mm ID) packed with 2% Epon Resin 1001, 80/100 mesh (Analabs, Inc., N. Haven, Connecticutt). The carrier gas (Ar:Me - 90:10) was delivered through the column at 22 ml min^{-1}; the detector was purged at a rate of 75 ml min^{-1}. Temperature conditions were: column, 205 C; injection port, 210 C; detector, 290 C.

All of the compounds were identified by their retention times determined on XE-60 and Epon Resin 1001 columns. The metabolite standards were extracted from *Phaseolis vulgaris* seeds and identified by their published retention times (44). ABA and the conjugates of ABA and t-ABA were positively identified in needle extracts with a Finnigan Mass Spectrometer. No other compounds were found to be conjugated in Douglas-fir (39).

The compounds were quantified by digitizing the peak area, and calculating concentration in terms of ABA-equivalents since pure standards of the other metabolites were not available. The concentrations were expressed on a dry weight basis and corrected for extraction

losses (which averaged 20%) by scintillation counting of the ^{14}C standard.

The data were statistically analyzed with analysis of variance and regression analysis between selected variables. There was large inherent variability between branches and among seedlings as was reported in bean (40). To reduce variability, the data were converted to percentages based on the total amount of all detected compounds in a branch. This transformation significantly increased the homogeneity within the seedlings which suggests that such relative concentrations among metabolites may be more meaningful, physiologically (Table 1).

Statistical analysis of the data indicated that the light treatments had no significant effect on the concentration of ABA, t-ABA or any of the other metabolites. The pre- and post-treatment data were pooled for further analysis and discussion.

RESULTS

The relative concentration of ABA and its metabolites in the control seedlings were consistent throughout the study (Table 1), but variations in the sum of the compounds were evident. The high concentration of conjugated ABA (4600 ng g^{-1}) is notable as is the presence of a considerable amount of conjugated t-ABA (500 ng g^{-1}). The majority of the metabolites (PA, DPA and t-ABA) exhibited low concentrations (100 ng g^{-1}) whereas ABA, epi-DPA and t-DPA showed higher concentrations (500 to 600 ng g^{-1}).

The changes in the compounds through the two stress cycles were considerable and revealing (Fig. 1). ABA concentration increased as

water stress developed and g_ℓ dropped. However, as stress intensified, ABA concentration actually decreased at day 9 (Fig. 1A and D) and g_ℓ stabilized. After watering, ABA dropped to control level, and ψ_x and g_ℓ recovered at day 14. The second stress cycle showed plant adaptation to stress with a more gradual increase in ABA as ψ_x and g_ℓ decreased.

Further data analysis showed a drop in g_ℓ between -1.5 and -2.0 MPa (Fig. 2), indicative of a ψ_x threshold. This drop in g_ℓ corresponded to an ABA increase of between 5 and 10% above the control seedlings (Fig. 3). There was no evidence, however, of a shift in these relationships during the second stress, despite the appearance of adaptation in Figure 1.

The relationship between ABA and ψ_x was not definitive, but a general trend was evident as delimited by the two lines (Fig. 4). The point of the g_ℓ drop, i.e. -2.0 MPA (Fig. 2) and increases of 5 to 10% ABA (Fig. 3), does lie within these limits. In this figure, ABA concentration exhibited a decrease at high water stress as was evident in the first water stress cycle (Fig. 1A).

The reciprocal relationship between ABA and its conjugate (Fig. 1A, 1B and 5) strongly implicates interconversion between the two compounds as a dominant ABA supply process at the onset of water stress, and its rapid sequestration when stress is relieved. In figure 5, much of the increase above 0% ABA was due to a concommitant decrease in the conjugate as indicated by a slope that approaches -1. However, below 0% ABA, large changes in the conjugate are accompanied by small ABA variations which suggests the conjugate is being

metabolized. This relationship (Fig. 5), like the others, does not show any stress adaptation. No interconversion between t-ABA and its conjugate was apparent.

Phaseic acid shows a slight decrease at the onset of stress, but it recovered to control level and remained virtually unchanged (Fig. 1C). This drop and the subsequent increase indicate an initial metabolic disruption (partially responsible for the ABA increase), and then a re-adjustment to maintain the nearly constant PA concentration.

The next step in the catabolic pathway bifurcates to either DPA or epi-DPA (44). In Douglas-fir seedlings, ABA catabolism favors epi-DPA (Fig. 1C). DPA did not change significantly throughout the study and was not included in Figure 1. In constrast, epi-DPA lagged behind the initial PA decrease and then gradually increased to a maximum at day 24. A dramatic decrease occurred at day 29.

Trans-DPA levels paralleled changes in ABA (Fig. 1A and C) providing evidence for *in vivo* photoisomerization and a concentration dependency. However, this isomerization must proceed at a slow rate since the 30 min light treatments had no effect. An examination of the changes in the trans compounds (t-ABA, its conjugate and t-DPA) indicates the rapid catabolism of t-ABA and the minor importance of conjugated t-ABA, though it may be an intermediate in the catabolic pathway.

Analysis of variance between sample days was statistically significant ($P \leq 0.05$) for all of the compounds with the exception of DPA.

The statistics from the regression analyses are indicated in the descriptions of the figures.

DISCUSSION

The decrease in g_ℓ as water stress increased (Fig. 2) exhibited a similar threshold response as reported for Douglas-fir and other species (1,3,4,5,22,32,43). The ψ_x range at the threshold was broader (-1.5 to -2.0 MPa) and more negative than previously published values. Diffusive resistance of Douglas-fir seedlings have been observed to rapidly increase at -1.0 to -1.2 MPa (5,32). The abruptness of this threshold may be partly due to the use of resistance rather than conductance as in our study ($g_\ell = R_\ell^{-1}$). Cotton has been found to lack a discrete ψ_x threshold, and instead exhibits a gradual decrease in g_ℓ with ψ_x (9). Repeated water stress and leaf aging caused a shift in the ψ_x threshold to more negative values (1), implicating prior physiological condition in a plants response to stress. However, Davies (10) found that prestressed plants showed more rapid and complete stomatal closure. The seedlings in our study were grown, for the most part, outside which provide an opportunity for repeated mild water stress and subsequent adaptation during the 3 years prior to the study.

The decrease in g_ℓ in the threshold range was from 0.06 to 0.04 cm s^{-1} (Fig. 2), corresponding to an ABA increase of only 5% (Fig. 3) or a change from the control concentration of 500 to about 850 ng g^{-1}. This is an extremely low increase (1.7X) compared to other species which have shown up to a 50-fold increase in ABA (43). Douglas-fir

seedlings have previously exhibited increases of 3-fold (32) and 20-fold (5) in response to water stress. These discrepancies can be attributed to preconditioning as discussed earlier. Repeated water stress enhances stomatal sensitivity to ABA (1,10,12,37). In addition, older leaves tend to have higher residual ABA levels (18,37) and do not synthesize large amounts of ABA in response to water stress (1,4,18,36).

The poor ABA- ψ_x relationship (Fig. 4) suggests that ψ_x is only loosely related to the mechanism that stimulates ABA synthesis or increase. A better parameter may be the water potential or turgor potential (33) of the mesophyll cells surrounding the stomatal complex (25). The evident drop in ABA levels at high stress (Fig. 1 and 4) has been observed in a number of species, including Douglas-fir (5,32), cotton (9), tomato (37), *Tradescantia* and *Mentha* (12). This decrease suggests that the function of ABA is only involved with the initial closure of stomates at the onset of stress and not to maintain closure through the duration of the stress. It is apparent under intensifying stress that the lack of cellular water alone would physically reduce guard cell turgor and stomatal aperture.

Stress adaptation by osmotic adjustment (Fig. 2 and 4) or ABA stomatal sensitivity (Fig. 3) did not occur as a result of the two consecutive stress cyles, but water stress and the increase in ABA did develop more slowly during the second cycle (Fig. 1A and D). A shift in water potential relationships, attributed to osmotic adjustment (1,10,17), typically occurs with subsequent water stress. The seedlings in our study may have not been capable of adapting in

this manner due to their preconditioning or genetic composition. A comparison of Douglas-fir ecotypes (32) showed stress adaptation, a shift to a more negative ψ_x threshold, in the xeric seedlings whereas the seedlings from a mesic site exhibited no adaptation, similar to our study's results. This genetic influence was probably manifested in the roots, affecting the supply of water to the foliage since there was no evidence for adaptation in the foliage. This would account for the slower development of water stress during the second cycle and the similarity of the ψ_x and g_ℓ values at days 5 and 24.

The pattern of ABA metabolism during the study was unique in comparison to other studies (Fig. 1). Conjugated ABA levels varied inversely with ABA providing support for the hypothesis of the reconversion of the ester to the acid. The conjugate has long been speculated to perform a storage function which could be readily hydrolyzed and rapidly increase ABA concentration at the beginning of water stress (7,16,28,29). However, only low concentrations of conjugated ABA have been found in most instances, between 0.1 and 0.3 of the free acid (11,28,29,31) with the exception of citrus fruit (10:1, conjugate:ABA) (12), and grape leaves (3:1) (26). We found that conjugated ABA constituted about 70% of the total analyzed compounds, or about 10 times the amount of the free acid. Exogenous application of ABA have documented its conjugation, *in vivo* (28,34) and increases in the conjugate following changes in ABA during water stress have also been reported (11,28,30), but the reversal of this process, conjugate to ABA, has never been reported.

The interconversion of ABA and its conjugate occurs in Douglas-fir (Fig. 5). Its presence can be attributed to the plant material used in our study. As noted before, the needles had a higher ABA content under non-stress conditions and only a small increase occurred at the onset of stress. This ABA increase appears to come almost exclusively from the conjugate (Fig. 1A, 1B and 5). Metabolic changes in the foliage (due to conditioning) could account for the persistence of the large reservoir of the conjugate and the apparent decline in the foliage's capability for *de novo* ABA synthesis. In contrast, younger, never-stressed leaves seem to possess a metabolic pattern which favors synthesis over conjugate reconversion. This pattern may reflect stomatal insensitivity to ABA and the need to have large increases in ABA to affect the stomates.

Phaseic acid, the first stable product in ABA catabolism, did not change concentration during the study with the exception of a small decrease and recovery in the first stress cycle (Fig. 1C). Although pea seedlings (11) exhibited a similar pattern, several studies have reported an increase in PA during and after water stress (21,24,26,40). This further supports the proposed metabolic shift in older, previously, stressed plants.

The direction of metabolism to DPA or epi-DPA depends on the tissue and technique (44). Zeevaart (44) and Walton (39) reported DPA to be the major product of PA reduction and present at higher concentrations then its epimer. Epi-DPA, however, was produced in considerable quantities in bean plants fed (^{14}C)-ABA (39). In Douglas-fir, epi-DPA is apparently the major metabolite by virtue of its

significant changes during the study and the initial lag it exhibited in following the drop in PA (Fig. 1C). The pattern of epi-DPA can be explained by water stress-induced alteration in enzyme activity. Initially, no alteration occurred, but at day 9, an increase in catabolism resulted in the drop in ABA, increase in PA, and decrease in epi-DPA. Upon watering, the breakdown of epi-DPA lessens while ABA catabolism continued. This pattern continued until stress intensified (day 28) causing an increase in the breakdown of epi-DPA as in the first cycle.

Little variation in t-ABA and its conjugate was observed in Douglas-fir (Fig. 1A and B), and as indicated before, the light treatments did not influence the levels of these two compounds. However, their presence in the needles does indicate the occurrence of *in vivo* photoisomerization (28). Further evidence of isomerization is the parallel changes in t-DPA and ABA (Fig. 1A and C), and the dependency on ABA concentration. The data also suggest rapid metabolism of t-ABA and its conjugate with a limiting step at t-DPA. The isomerization of PA and DPA cannot be discounted and could equally cause the observed pattern in t-DPA.

In this study we showed that older Douglas-fir seedlings responded to decreasing ψ_x by closing their stomates. This process was mediated by a small increase in endogenous ABA concentration, supplied primarily by the reconversion of its conjugate. Metabolism of ABA occurred rapidly from ABA to PA and epi-DPA. DPA was of minor importance in the metabolism. Photoisomerization of ABA occurred continuously, but slowly and depended on ABA concentration. Trans-ABA and its conjugate

were rapidly catabolized to t-DPA. Metabolism was different in the older tissue and appeared to be affected by previous physiological condition.

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TABLE 1. The Mean Percentage of Each Compound in the Control Seedlings for Each Sample Day of the Study.

Day	ABA	epi-DPA	PA	t-ABA	DPA	t-DPA	Conjugated ABA	Conjugated t-ABA	Sum of Compounds
	percent of sum								ng/g d.w.
0	7.1	4.0	2.1	1.6	1.3	6.4	70.8	6.9	5636
5	6.7	2.1	3.7	1.0	1.5	4.4	73.3	7.4	8423
9	8.0	12.2	2.1	1.1	2.5	3.6	63.4	7.4	10400
14	11.0	12.9	2.1	1.6	2.9	11.3	51.6	6.6	5526
24	7.2	5.9	0.7	1.0	2.1	8.9	63.6	10.5	7152
28	8.0	12.2	2.1	1.1	2.5	3.4	63.4	7.4	5013
mean	7.9	7.6	2.1	1.3	2.0	6.3	65.3	7.6	7025

FIGURE CAPTIONS

Figure 1. The changes from control in ABA, its metabolites, leaf conductance and xylem water poential throughout the study. A - ABA (▬); t-ABA (▮▮▮): B- conjugated ABA (▬); conjugated t-ABA (▮▮▮): C - PA (▬); epi-DPA (▮▮▮); t-DPA (▨): D - g_ℓ(▮▮▮); ψ_x (▬). Vertical bars in upper corner of graphs A through C are the LSD at 5% level of significance.

Figure 2. The relationship of g_ℓ to ψ_x throughout the study, differentiated by stress cycle: first cycle (●); second cycle (★). The regression equation for the line is: $g_\ell = 0.029 + 0.089\ (\psi_x)^{-1}$, $R^2 = 0.50$.

Figure 3. The effect of ABA on g_ℓ during the two stress cycles. The symbols are the same as in Fig. 2. The line was hand-fitted.

Figure 4. The influence of ψ_x on ABA level during the two stress cycles. The symbols are the same as in Fig. 2. The lines were hand-fitted to indicate limits.

Figure 5. The relationship between ABA and its conjugate during the study. The symbols are the same as in Fig. 2. The regression equation is: conjugated ABA = $_e(3.91 - 0.05\ (ABA + 7))$, $R^2 = 0.66$.

Figure 1

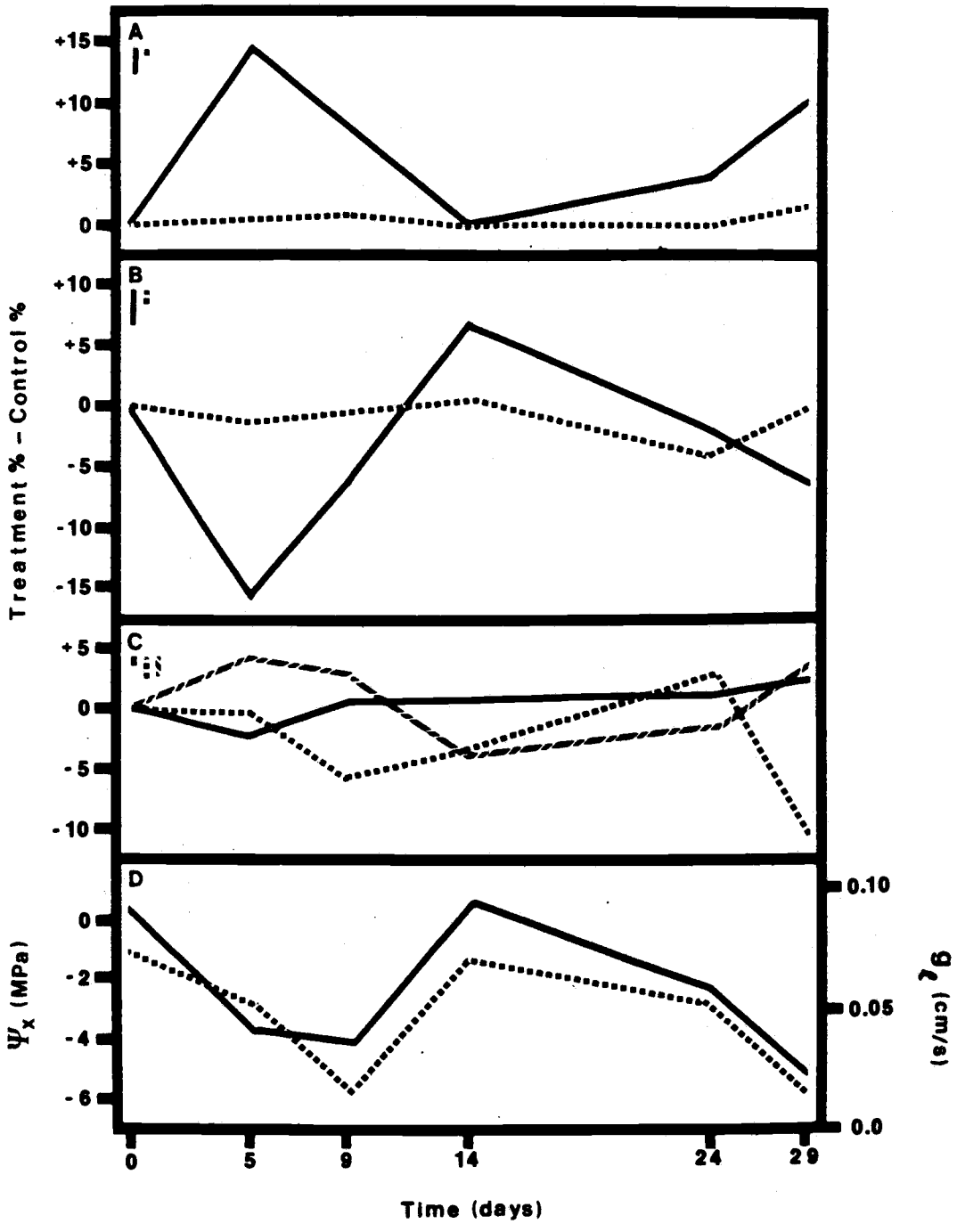

A
B
C
D
+15
+10
+5
0
-5
-10
-15
Treatment % - Control %
Ψ_x (MPa)
-2
-4
-6
0.10
0.05
0.0
g_c (cm/s)
0
5
9
14
24
29
Time (days)

Figure 2

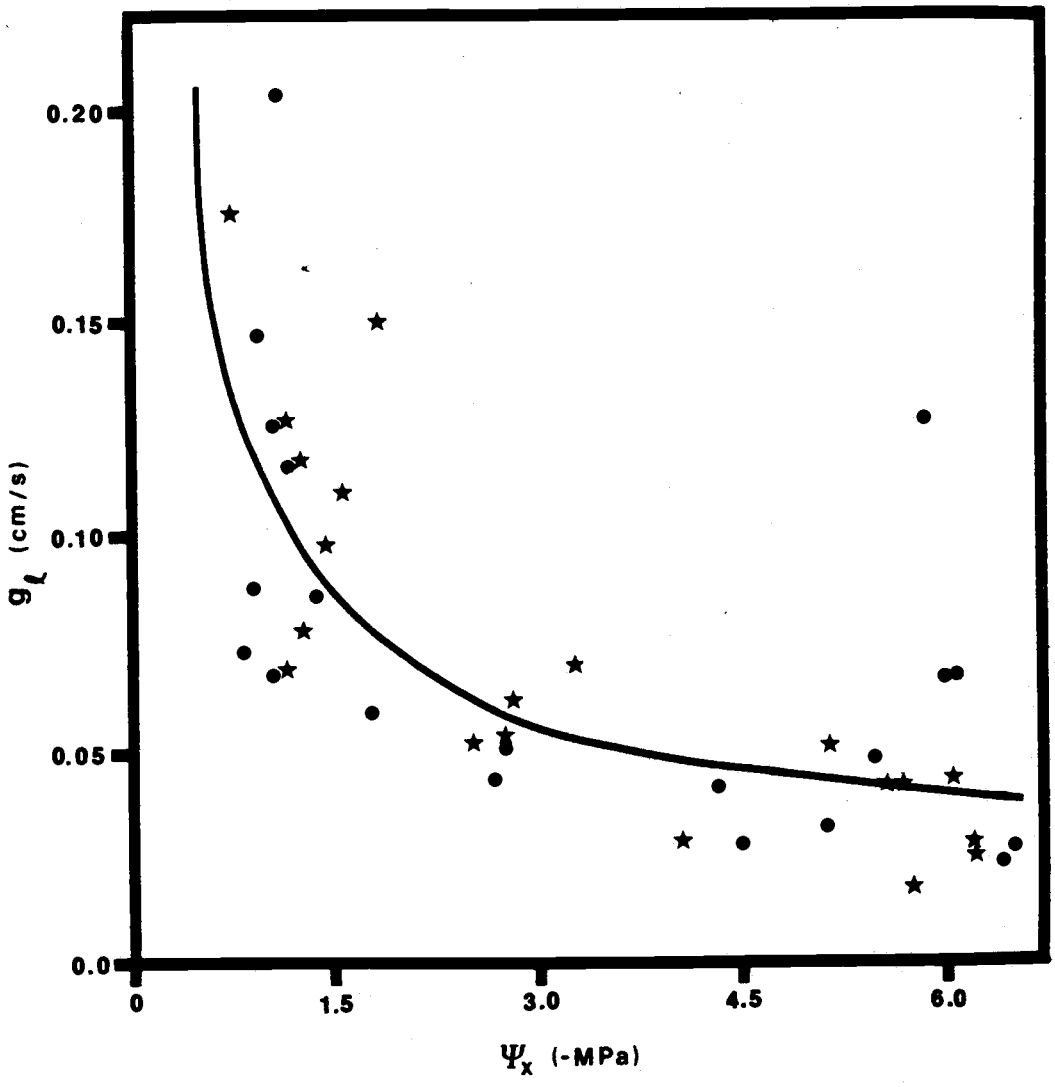


Figure 3

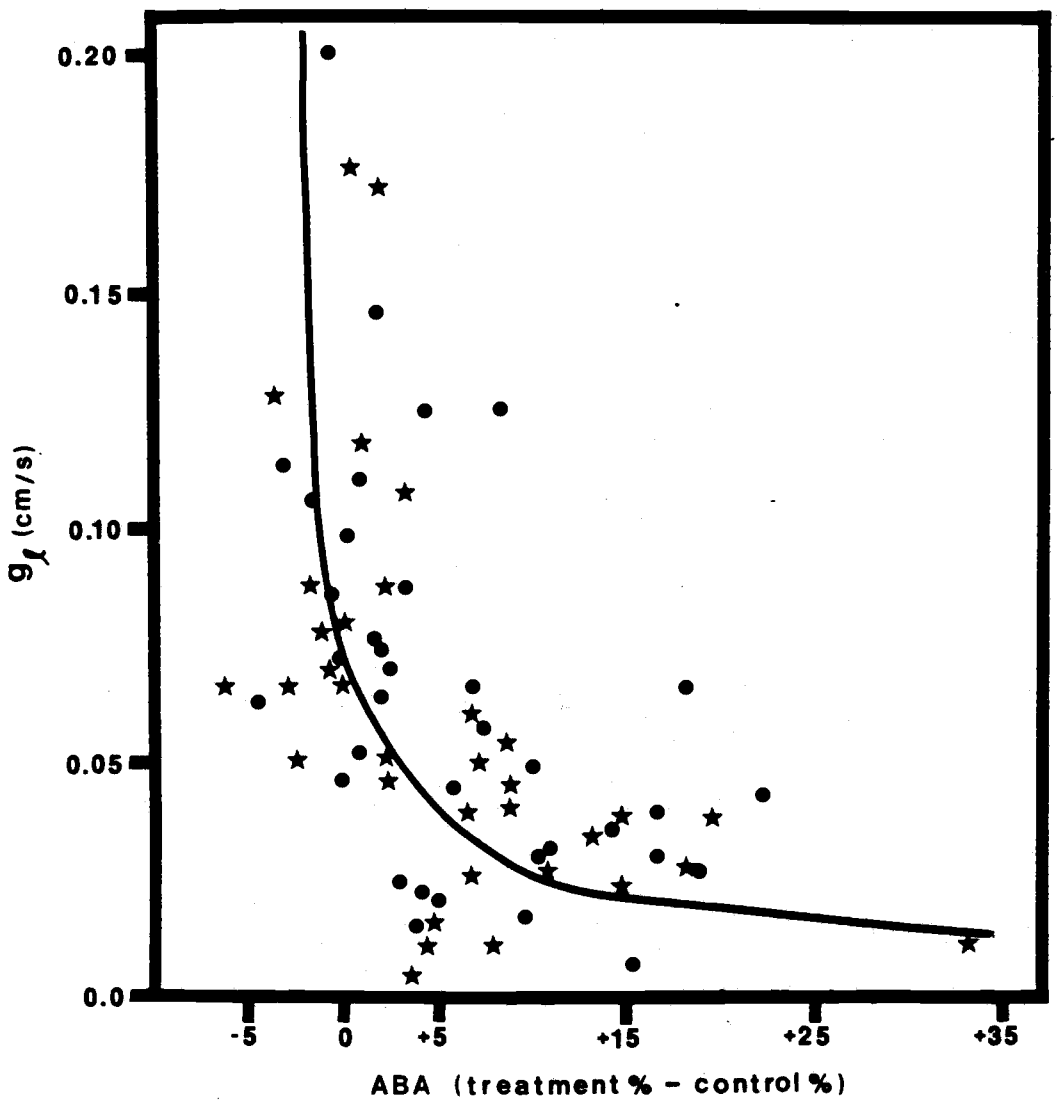


Figure 4

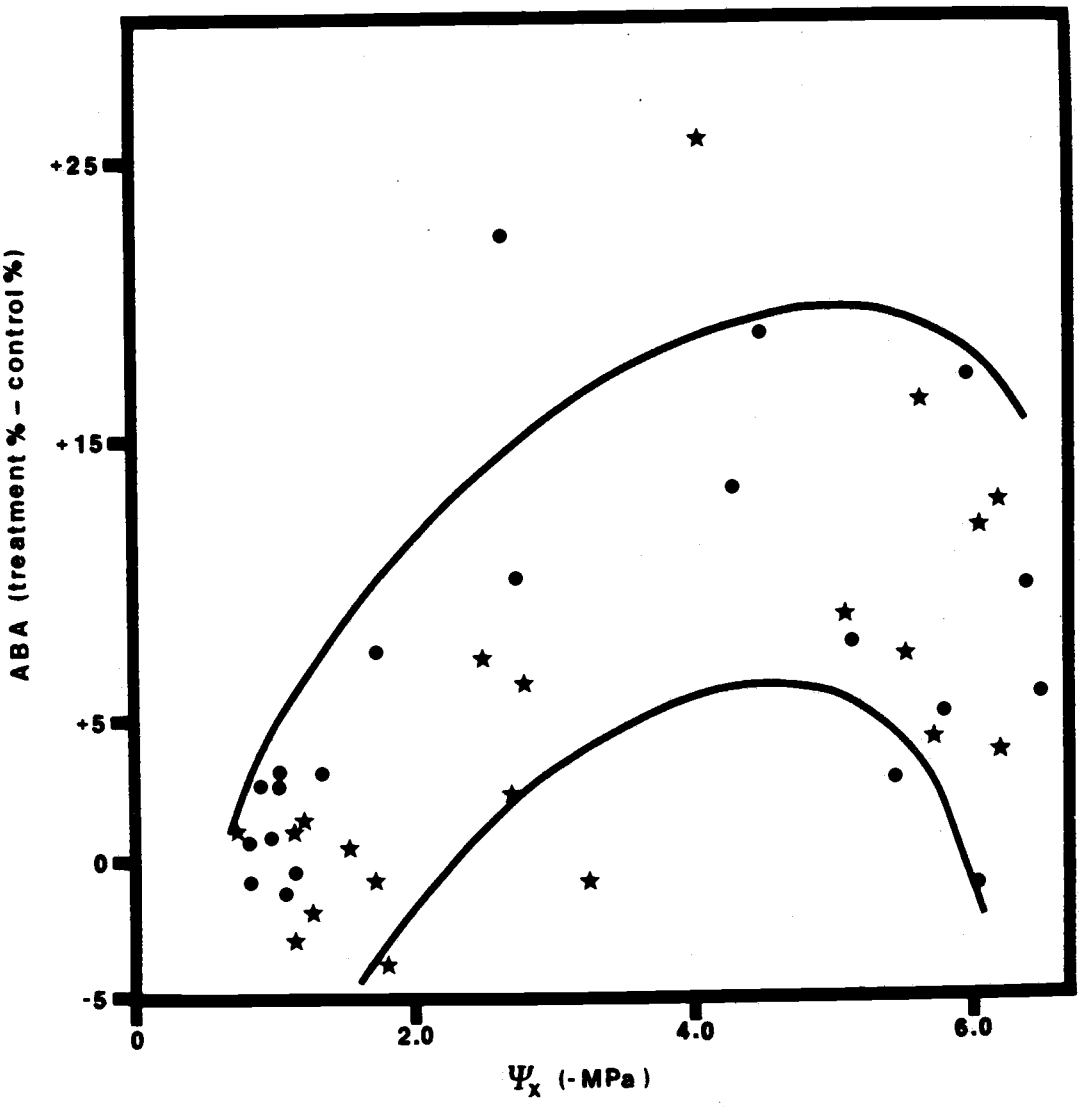
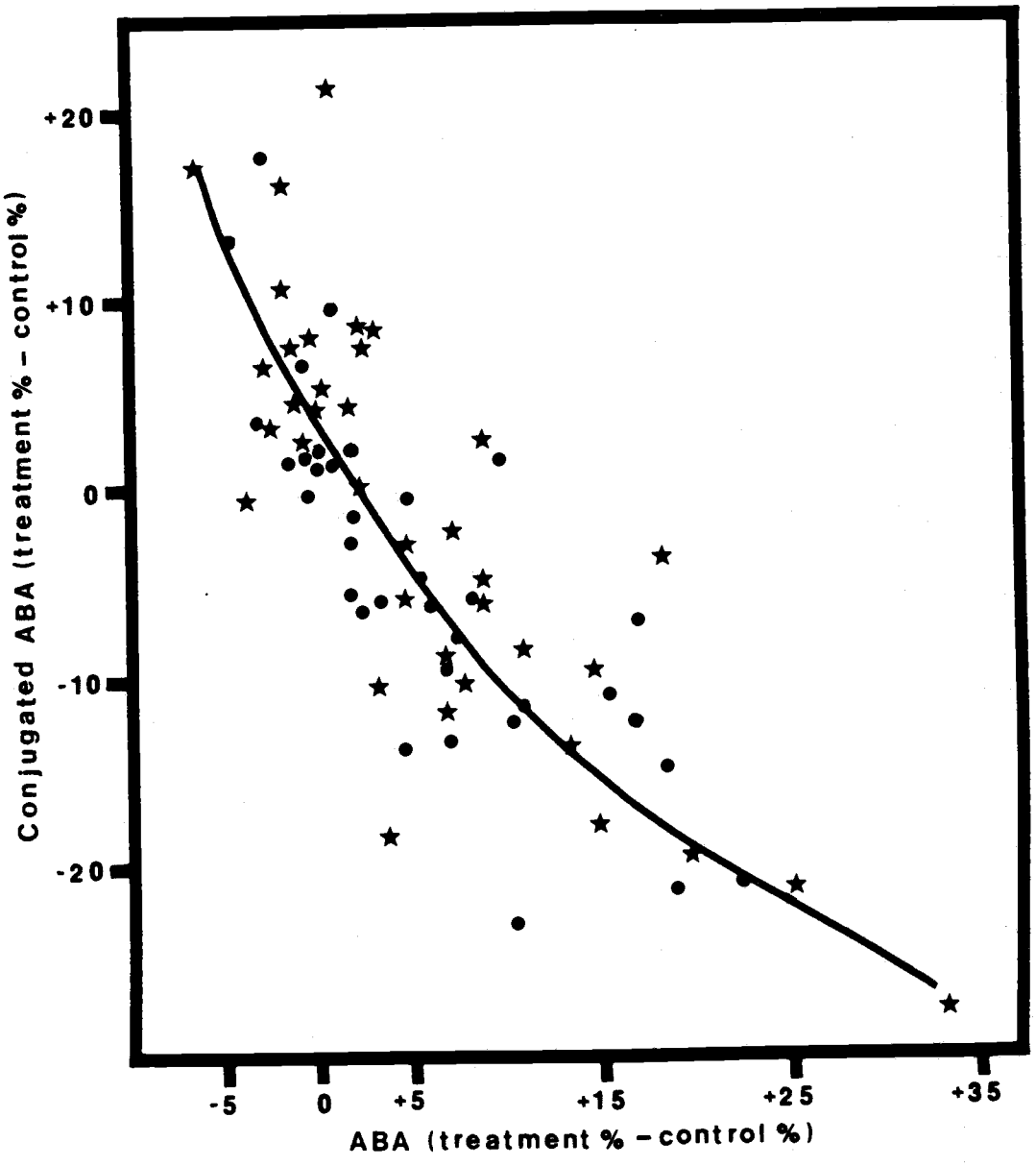


Figure 5


+20
+10
0
-10
-20
Conjugated ABA (treatment % - control %)
-5
0
+5
+15
+25
+35
ABA (treatment % - control %)

CHAPTER 6

Summary

The complexity of stomatal regulation was reiterated, and some of the influencing processes were elucidated and clarified by this research. When soil water was not limiting, stomates responded rapidly and dramatacially to changes in atmospheric humidity. Stomates responded so rapidly that even exposures of less than 30 seconds to low humidity affected the measurement of leaf and branch conductance. As water supply lessened and the seedlings became water stressed, the stomates initially were more sensitive to humidity, but this sensitivity decreased as the stress intensified. This change in sensitivity was attributed to increases in foliar abscisic acid levels. The ABA increase was due to the reconversion of its saponifiable conjugate, and effectively closed the stomates. However, ABA was involved only in the initial closure and did not appear to be required to maintain closure. Metabolism of ABA occurred by the normal pathway from phaseic acid to epi-dihydrophaseic acid and via photoisomerization of ABA to trans-dihydrophaseic acid.

Stomates respond directly to environmental factors such as light intensity and humidity when soil water is ample, but when water becomes limiting, physiological factors (abscisic acid and its metabolism) override the environmental influence and regulate the stomates, and hence control gas exchange.