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Uptake, transport and bioactivity of exogenously applied ABA and ABA analogues in white spruce and wheat seedlings

by

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A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming
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ABSTRACT

There are significant differences between conifers and herbaceous species in their stomatal sensitivity to exogenously applied ABA. Experiments on white spruce (*Picea glauca* (Moench) Voss) and wheat (*Triticum aestivum* L. cv Katepwa) seedlings, whose roots were sealed in an aeroponic misting chamber, confirmed that 200-fold higher concentrations (2×10^{-3} M) of exogenously applied (±)ABA were required to close stomata in spruce than in wheat (10^{-5} M). I tested the hypothesis that this difference in response between species was because: (i) stomata are inherently more sensitive to ABA in wheat than in spruce; (ii) in wheat, ABA is taken up more efficiently by roots and more ABA is subsequently delivered to the shoots and (iii) a combination of (i) and (ii). Tritiated ABA was applied to plants over approximately 10 hours and their water uptake (transpiration rate, E) measured continuously. ABA uptake efficiency (UE) was calculated as the ratio of the scintillation count of root and shoot tissue extract to the product of the activity of the misting solution and total water uptake. Transport efficiency (TE) was calculated as the ratio of the shoot to the total tissue scintillation count.

UE was almost twice as high in spruce (31.0 %) as in wheat (18.6 %). However, in spruce, virtually all of the ABA taken up remained in the roots (94.5 %). In contrast, in wheat, a much higher proportion of ABA taken up by the plant was delivered to the shoots (48.8 %). Thus TE was almost 9 times higher in wheat than spruce. Treatments such as increasing root temperature or the use of dimethyl sulphoxide as an organic solvent, brought about dramatic increases in UE in both species (in spruce, UE, in some cases, was almost 80%). However, in spruce this did not result in increased delivery of ABA to the shoots and TE declined. When the roots were excised from spruce seedlings, there was a 55-fold increase in the amount of ABA delivered to the shoots and a concomitant 20-fold increase in stomatal sensitivity to the application of ABA. Immunofluorescence labeling technique, used to localize ABA, showed that the cortical cells around the endodermis were the main site of exogenous ABA accumulation in spruce roots. In contrast, in wheat, the major portion of the exogenous ABA was found inside the vascular tissue in the roots. I conclude that in spruce, the roots provide a major barrier to the transport of ABA to the shoots. However, differences in TE between wheat and spruce, while very large, do not

fully account for differences in their stomatal response to exogenously applied ABA. Thus it is likely that wheat stomata are inherently more sensitive to ABA than those of spruce.

Experiments were also conducted on white spruce and wheat seedlings, to determine the uptake and transport from roots to shoots of (+)- and (–)-ABA enantiomers and their respective methyl ester derivatives. I tested the hypothesis that the higher biological activity, determined as their ability to affect gas exchange, of ABA enantiomers or specifically tailored analogues would be related to their being more efficiently incorporated into roots and subsequently transported to shoots. Tritiated ABA and MeABA enantiomers were applied, using an aeroponic root misting system, for 10 hours and seedling transpiration and photosynthesis rates monitored. Uptake efficiency (UE) and Transport efficiency (TE) were calculated as described earlier.

In both species, (+)-ABA was more biologically active than (–)-ABA. However, differences in TE between the ABA enantiomers were significant only in wheat with the natural enantiomer having twice as high a TE as (–)-ABA. In spruce, the UE of the methyl ester enantiomers (~87 %) was almost twice as high as that of the respective ABA enantiomers. However, virtually all of the MeABA taken up remained in the roots with less than 2 % reaching the shoots. Thus, despite its higher transport across root membranes, MeABA, at all concentrations tested, had a lower biological activity than ABA and there was no correspondence between root uptake and bioactivity. Adding an isopropyl ester to the C-1 carbon of ABA brought about an increased bioactivity only in spruce where (±)-iPrABA induced stomatal closure at a 10-fold lower concentration (10^{-4} M), than (±)-ABA. I conclude that a much larger proportion of exogenously applied ABA is sequestered in spruce roots than in wheat. Thus it is likely that, in the former species, any increased biological activity of ABA analogues depends on how effectively they are transported from the roots to receptor sites in the shoots.

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TABLE OF CONTENTS

Chapter 3

Uptake, transport and biological activity of exogenously applied ABA enantiomers and ABA analogues in white spruce (Picea glauca (Moench) Voss) and wheat (Triticum aestivum L. cv Katepwa) seedlings

LIST OF TABLES

LIST OF FIGURES

CHAPTER 2

Appendix B

LIST OF SYMBOLS AND ABBREVIATIONS

A	total projected leaf area (cm^2)
ABA	abscisic acid
ABA-GE	ß-D-glucopyranosyl abscisate
BHT	butylated hydroxy toluene
Ci	curie (radioactivity)
cpm	counts per minutes (of radioactive decay)
D	vapor pressure deficit (kPa)
DMSO	dimethyl sulphoxide
DW	dry weight (g)
E	transpiration rate (mmol m^{-2} s^{-1})
EDC	1-(3-dimethylaminopropyl)-3 ethyl carbodiimide
FITC	fluorescein-isothiocyanate
g_s	stomatal conductance to water vapor (mmol m^{-2} s^{-1})
g_{smax}	steady-state stomatal conductance measured over one hour, 1-2 h after the lights were switched on (mmol m^{-2} s^{-1})
I.D.	internal diameter (m)
IgG	Immunoglobulin G
iPrABA	isopropyl ester of abscisic acid
M	molar (moles L^{-1})
MeABA	methyl ester of abscisic acid
O.D.	outer diameter (m)
PBS	phosphate buffer saline
pH	$-\log(a_{H+})$
P_n	net photosynthesis rate (µmol m^{-2} s^{-1})
ppm	parts per million
Q	photosynthetic photon flux density (µmol $m^{-2}s^{-1}$)
TE	transport efficiency (%)
UE	uptake efficiency (%)

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DEDICATION

This thesis is dedicated to my father

CHAPTER 1

INTRODUCTION

Any factor that decreases plant growth and reproduction below the genotype's potential is defined as stress to the plant (Osmond et al., 1987). The internal and external signals that regulate plant growth are mediated, at least in part, by plant growth regulating substances, or phytohormones. Phytohormones are naturally occurring, organic substances which influence physiological processes at low concentrations in plants (Davies, 1988). Stressful environmental conditions are known to bring about many morphological and physiological changes in the plants including changes in plant hormones. Roots can influence hormone levels in the shoot either by exporting the hormone or hormone precursors or by acting as active sinks for phloem-mobile hormones produced in shoot tissue. On this basis, roots modify several kinds of hormonal messages by increasing their output (positive message), decreasing their output (negative message) or by becoming a less active sink for shoot sourced hormones (accumulative message) (Jackson, 1993). Abscisic acid (ABA) is one of the positive messages that increases substantially in many species following soil drying (Dodd et al., 1996).

ABSCISIC ACID

The plant growth hormone, abscisic acid (ABA), is a 15 carbon atom weak organic acid and possesses optical activity due to an asymmetric carbon atom at position C-1'. Therefore, there are two ABA enantiomers; (+)-ABA and (–)-ABA. Only the (+)-ABA enantiomer occurs naturally in plants. Commercially available synthetic ABA is a racemic 1:1 mixture of (+)- and (–)-ABA. Stress induced biosynthesis of abscisic acid (ABA) promotes characteristic developmental changes in plants. Examples of such changes are restricted shoot growth, reduction in leaf surface area, stimulation of root extension, lateral root growth and root hair development (Milborrow, 1978). These changes can help plants

cope with a range of environmental stresses. Reducing water loss, by promoting stomatal closure, is one of the actions of ABA in response to stress. Generally when a plant faces stress there is an increase in ABA concentration and action at the outer surface of the guard cell plasmalemma (Wilkinson and Davies, 1997). This is brought about by the following three mechanisms

i) ABA redistribution in leaves

ABA is the only known plant hormone which distributes across the cell compartment ideally according to the anion trap mechanism for weak acids. The undissociated lypophilic acid, ABAH, is the only permeant ABA species that passes through membranes by diffusion and is trapped in alkaline compartments (cytosol and chloroplast) as a completely non-permeant lypophobic anion ABA^-. Decreased leaf water potential inhibits leaf plasmalemma ATPase resulting in slower outward proton transport and consequently a more alkaline apoplast (Hartung et al., 1988). This results in diffusion of membrane permeable undissociated ABA into the alkaline apoplast and an increase in apoplastic ABA concentration. Symplastically isolated guard cell plasmalemma become more alkaline in water deficient cells hence there is movement of apoplastic ABA to its primary site of action (guard cell exterior) by diffusion (Correia and Pereira, 1994; Daeter and Hartung, 1995).

ii) ABA as a message from root system to the stomata

In leaves, stress can cause an increase in apoplastic ABA, but in roots the situation is less clear. Stress induced ABA biosynthesis in roots requires substantial soil water loss but ABA is released to the xylem even when soil water deficits are small. There must be some rapid and sensitive mechanism to redistribute ABA into xylem vessels in the absence of increased biosynthesis. Overall permeability of stele membranes to ABA has been found to be higher than that of cortical membranes. Using ^{31}P-NMR it was observed that there was an increase in pH gradient in cortical plasma membranes as stress acidifies the apoplast of

the cortex and alkalizes the cortical cytosol (Spickett et al., 1992). In addition, reduction in cytosolic pH of the stele leads to an increase in ABA released to the xylem (Hartung et al., 1998). Daeter et al. (1993) developed a model incorporating compartmental pH of unstressed and stressed root cells and the permeability coefficient of ABA in root membranes. According to this model, the pH gradient-dependent ABA redistribution under stress accounted for the two to three fold increase in xylem sap ABA. A further increase in xylem sap ABA originates from biosynthesis in roots. It has been shown by several research groups that dehydration of isolated root systems increases ABA biosynthesis (Zhang and Tardieu, 1996). Cornish and Zeevart (1985) found that ABA concentrations in roots of stressed *Xanthium strumarium* plants increased despite stem girdling to inhibit ABA export from shoots. The concentration of ABA in roots increased with decreasing soil water content and roots lower down the soil profile became progressively enriched with the hormone as the soil dried (Zhang and Davies, 1987). Since the increase in root ABA takes place in the absence of decreased leaf hydration, it is unlikely that roots received ABA transported from stressed leaves. Parry et al. (1992) confirmed that roots contain all the precursors necessary for ABA biosynthesis via the indirect violaxanthin pathway. Xylem vessels are in direct contact with the leaf apoplasm (Hartung, 1983), the only leaf compartment directly connected with the outer surface of guard cell plasmalemma. Thus transpiration flow deposits xylem ABA at its primary site of action.

iii) Change in sensitivity of stomata to ABA

In most plants, water deficits can cause ABA concentrations to increase by a factor of 10-30 (Wright, 1978) but in some species the increase in ABA is not so pronounced. Trejo and Davies (1991) found that the ABA concentration in the xylem sap of bean (*Phaseolus vulgaris*) increased only two fold, even when the seedlings were severely droughted. Smith and Dale (1988) also observed very low ABA increase in leaves of bean plants stressed by root cooling. In both these cases, however, the researchers observed a decline

in leaf stomatal conductance. It was suggested that perhaps guard cells of stressed plants are more sensitive to ABA than in unstressed conditions and that this accounted for the stomatal closure even though ABA concentrations were low. In field experiments with almond (Wartinger et al., 1990) and maize seedlings (Tardieu and Davies, 1992), a biphasic relationship was observed between xylem sap ABA and leaf conductance under changing environmental and leaf water status. Within a relatively narrow range of xylem sap ABA concentration, a small increase in ABA had a dramatic effect on leaf conductance (Tardieu, Zhang and Davies, 1992). This suggests that in many plants there is a highly sensitive stomatal response to ABA (Dunleavy et al., 1995; Hirasawa et al., 1995). Tardieu and Davies (1992) provide strong evidence that leaf water potential modifies stomatal sensitivity, with an increased sensitivity to ABA during afternoon hours when leaf water potentials are typically low. Model calculations by Hartung and Slovik (1991) also point to the fact that a small increase in xylem sap ABA is sufficient to cause a significant increase in ABA concentration at the primary site of action. These data suggest that stomatal responses to small increases in ABA, as observed in some plants, could be explained by leaf water potential dependent changes in guard cell sensitivity to ABA.

ABA AND PLANT GROWTH

It has long been known that reduced soil water can change the ratio of shoot to root in favor of the root system and that similar responses can be stimulated by exogenously applied ABA. Studies of root growth at reduced water potential show that tips of primary maize roots continue to grow under a reduced soil water potential, even when the growth and development of the shoot is already inhibited (Robertson et al., 1990). Treatment of the roots with the carotenoid synthesis inhibitor, fluridone, reduces the ABA concentration in the root tips and substantially reduces growth at a low water potential (Audus, 1983). Root growth was reduced when ABA synthesis was inhibited (Saab et al., 1990), recovered

when ABA levels were restored, and was again reduced when excessive amounts of synthetic ABA were applied (Sharp et al., 1994). Thus, at high concentrations, exogenous ABA acts as a root growth inhibitor rather than a promoter. For example, in maize and pepper seedlings, root elongation was limited at 0.1 mM ABA and was completely suppressed at 1 mM ABA, respectively (Leskovar and Cantliffe, 1992; Wightman et al., 1980). In the shoot, ABA causes a decline in cell elongation by reducing cell wall extensibility resulting in stunted growth (Munns and Cramer, 1996). Thus, ABA plays a direct role in both maintenance of primary root growth and inhibition of shoot growth at low water potentials (Frensch, 1997; Trewavas and Jones, 1991).

ABA AND PLANT STRESS RESPONSE

One of the first measurable responses of a plant to water stress is a decline in its stomatal conductance. Evidence linking stomatal aperture to the concentration of ABA in xylem sap is quite strong (Davies and Zhang, 1991). Loveys (1984) found that in well-watered field-grown plants, xylem sap ABA concentration increased during the first few hours of each photoperiod followed by a decrease in transpiration. Thus, an increase in xylem ABA is a potential cause rather than a consequence of stomatal closure. Since Loveys, various other researchers have shown a strong relationship between xylem sap ABA concentration and stomatal conductance in laboratory as well as in field-grown plants (Davies et al., 1986, 1994; Tardieu et al., 1992; Zhang and Davies, 1989, 1990a, 1990b). Possibly the most convincing evidence is from split-root experiments in which water was withheld from part of the root system. A decrease in stomatal conductance was observed even though the leaves were still well supplied with water from the rest of the root system, but stomatal conductance increased again when the dry roots were excised (Gowing et al., 1990; Khalil and Grace, 1993). The coupling between leaf conductance and ABA concentration in the xylem sap was far closer than between leaf conductance and bulk leaf ABA in droughted

and in irrigated maize plants (Tardieu et al., 1992). However, there is evidence that in some species, the antitranspirant activity of the xylem sap cannot be attributed to ABA. Munns and King (1988) found that ABA enriched xylem sap of droughted wheat was 100 times too dilute to close stomata in tests where synthetic ABA of the same concentration was administered to detached wheat leaves. The antitranspirant activity of the droughted wheat xylem sap remained unchanged even after the passage down an immunoaffinity column that removed ABA (Munns, 1990). Zhang and Davies (1991) repeated and extended Munns approach and found that xylem sap from unwatered maize plants reduced stomatal conductance in detached wheat leaves. In addition, the decrease in conductance was solely due to the xylem sap ABA content. The differences in the experimental conditions for the two studies are that Zhang and Davies (1991) used larger wheat leaves (16 cm) in their assay rather than short ones (8 cm) used by Munns (1990) and filtered the xylem sap to avoid particles (>0.2 μm) which may block water transport through detached leaves.

Various studies have demonstrated that the amount of ABA entering the leaf, as opposed to simply its concentration in the sap, influences the stomatal conductance. ABA concentration measured in xylem sap is subject to transpiration-linked variability. Data from some reports have to be examined carefully to ensure that the observed increase in ABA concentration is not simply a result of decrease in its dilution because a decline in stomatal conductance leads to decreased transpiration rates. Calculating delivery rates (concentration x sap flow rate) is a more reliable way of measuring ABA in the plant (Else et al., 1995; Jokhan et al., 1996). In experiments where delivery rates were examined, the results have indicated that when stomata close there is a marked increase in ABA input into leaves by xylem transport (Jackson et al., 1995; Schurr et al., 1992).

In spite of the compelling evidence that stomata are controlled by chemical messages originating from dehydrating roots, it is difficult to overlook the evidence, obtained over the years, of correlation between stomatal behavior and leaf water status. Kramer (1988) presented the argument that water deficiency, which almost invariably occurs in the shoots

before the roots, makes shoots the primary sensor of drought. Shoot turgor loss and wilting in hot sunny conditions, when roots were still in moist soil, has been reported by various researchers (Grime, 1989; Pierce and Raschke, 1980). In experiments with alder and Douglas-fir seedlings, it was demonstrated that increasing leaf turgor via root pressurization immediately opened stomata of plants in dry soil (Fuchs and Livingston, 1996). Similar response of stomata to soil pressurizing, observed by Saliendra et al. (1995) with birch seedlings, indicate a central role of leaf cells in sensing water stress.

To explain these contradictory findings, Tardieu and Davies (1993) reasoned that ABA synthesized in the leaf and sequestered in the chloroplast could have a role to play in the day to day regulation of stomatal behavior in response to leaf water status. Abscisic acid sequestered in the guard cells may be redistributed in the leaf in response to perturbations in the atmospheric environment and exert short term control over stomatal water loss and plant water balance. However, when stress is imposed or sensed, ABA moving in the transpiration stream from dehydrating roots controls stomatal conductance quite independently of any ABA sequestered elsewhere in the leaf (Tardieu et al., 1993).

Control of stomata conductance by leaf water status alone seems unlikely, as reported in some studies (Hartung and Davies, 1994; Gowing et al., 1993a, 1993b). The capacity of roots to produce ABA and the direct link between the site of production and the site of action provides a sensitive means of controlling leaf physiology as a function of soil water status (Tardieu and Simonneau, 1998). However, stomatal control via root message cannot be considered alone without accounting for leaf water status.

EXOGENOUS ABA AND FIELD USE PROSPECTS

The role of ABA in the control and maintenance of stomatal function is illustrated by studies on ABA deficient wilty mutants (Bradford, 1982). Raschke (1975) observed that water use efficiency in plants can be improved by external application of ABA. Findings of reduced ABA in xylem and foliage of well watered vines and its correalation with increased

stomatal conductance, carbon assimilation and growth, led Levoys (1991) to propose that ABA levels in the plants can be manipulated to increase crop production under conditions of low water availability. Plants transplanted from the high humidity conditions in propagation house to more stressful environments, such as in glasshouse or outdoors, have a tendency to wilt despite an abundant root-zone water supply. This phenomenon of transplant shock (inability to cope with increased evaporative demand) has been attributed to low ABA levels or poor response to endogenous ABA in seedlings. The response of these unhardened plants is similar to that of the ABA-deficient *flacca* mutant. Exogenous application of ABA restores the normal phenotype in *flacca* mutants (Neill et al., 1985; Quarrie, 1982; Tal et al., 1979). Levoys (1984) has shown that during hardening for outplanting, greenhouse grown vines show significantly increased levels of xylem ABA over unhardened clones. These facts suggest that there is a opportunity to increase WUE in the field and diminish transplantation shock by external application of ABA. Indeed, ABA has been successfully used as an antitranspirant (Hartung and Abou-Mandour, 1996). In *Pinus* seedlings, for example, exogenous ABA initially decreased transpiration rates for a few days. However, subsequently photosynthetic and transpiration rates were higher than pretreatment values, giving rise to increased water use efficiency (Davies and Kozlowski, 1975). In bell peppers, exogenous application of ABA improved the water status of the transplanted seedlings (Berkowitz and Rabin, 1988). Pospisilova (1996) proposed that exogenous ABA could be suitable for hardening tobacco plantlets *in vitro* to help their acclimation after transplantation to *ex vitro* conditions.

High cost, rapid metabolism and photodestruction limits the usefulness of the naturally occurring ABA; however, it is possible to synthesize and use analogues of ABA that posses high biological activity and improved stability in plants and environment. The biological activity of ABA depends on the presence of the carboxyl group, 2-cis and 4-trans pentadienoic side chain, 4'-ketone group and double bond in the cyclohexane ring (Walton, 1983). These limitations reduce the chances of producing a bio-active and stable analogues;

however, a few synthetic ABA analogues have been synthesized at low cost and are relatively stable in plant tissues (Walton, 1983). ABA analogues, which are able to resist enzymatic oxidation, are found to be more persistent and biologically active than natural ABA (Abrams et al., 1997; Todoraki et al., 1995). Thus, biologically stable analogues have proven to be beneficial as tools for investigating hormone action in plants as well as antitranspirants in agricultural use (Blake et al., 1990; Fuchs, 1998; Grossnickle et al., 1996).

THESIS OBJECTIVES

To date, most studies on the regulation of stomata by ABA have been focused on herbaceous agricultural species. Not many studies have been done using conifers, although the problem of drought stress affecting outplanted seedlings is significant (Livingston, 1988). There are differences between conifers and herbaceous species in their endogenous ABA levels and stomatal response to exogenously applied ABA. In conifers, exogenous ABA must be applied at very high concentrations in order to have any significant effect on plant water relations and gas exchange. This is in marked contrast to studies with herbaceous species where exogenously applied ABA brings about a significant reduction in transpiration at a much lower concentrations. This dissertation investigates the factors responsible for the differences in sensitivity between white spruce (*Picea glauca* (Moench) Voss) and wheat (*Triticum aestivum* L. cv Katepwa) to exogenous application of ABA. Furthermore, knowledge of the differences between the two species may be used to develop antitranspirant compounds that are markedly more biologically active than ABA at lower concentrations.

In chapter 1, differences in sensitivity between the two species to exogenously applied (±)ABA was investigated. I tested the hypothesis that the difference in response between species was due to higher ABA uptake and transport in wheat rather than an inherently greater stomatal sensitivity to ABA. Total exogenous tritiated ABA taken up by the

seedling, from an aeroponic misting system, over a period of 10 hours was quantified in terms of uptake efficiency which is the total concentration of compound taken up by the seedling relative to that applied. Firstly, the uptake and transport of ABA was quantified in the two species. Whether uptake is influenced by environmental conditions, such as temperature and humidity, or whether the use of organic solvent, such as dimethyl sulphoxide (DMSO) that increases the permeability of the membranes, could improve ABA uptake and distribution, was investigated. Furthermore, root bypass experiments were conducted to confirm if roots provide a major barrier to ABA transport in spruce. Finally, localization of exogenous ABA, in wheat and white spruce roots, was attempted using immunocytochemical procedure.

In chapter 2, the biological activity of the ABA enantiomers and ABA analogues was investigated, in relation to their uptake and transport in the two species. Comparisons were drawn between ABA enantiomers and their respective methyl ester derivatives. Additionally, the increased biological activity of an ABA analogue in spruce was analyzed, based on its ability to resist conjugation.

In appendix A, the relationship between the projected leaf area and leaf dry weight of wheat and white spruce is given. In appendix B, the responses of whole-plant transpiration rate to changes in vapor pressure deficits for spruce emblings and wheat seedlings are presented.

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CHAPTER 2

Uptake and transport of exogenously applied ABA in white spruce (*Picea glauca* (Moench) Voss) and wheat (*Triticum aestivum* L. cv Katepwa) seedlings

INTRODUCTION

Endogenous abscisic acid (ABA) is known to accumulate in tissues of plants subjected to water stress. It has been proposed that ABA is involved in the regulation of stomatal conductance and root and shoot growth (Audus, 1983; Davies et al., 1986; Saab et al., 1990). Numerous studies have shown that a plant's response to water stress can be mimicked by the exogenous application of ABA (see, for example, Trewavas and Jones, 1991) which reduces water loss by regulating stomata aperture (Johnson and Ferrell, 1983), and increases water uptake by the roots (Davies et al., 1982). Exogenous applications of ABA also provide a means to investigate the mechanisms by which ABA induces physiological and morphological changes in plants (Kuiper and Stall, 1987; Loveys, 1991).

Responses to exogenous application of ABA differ among species and cultivars (Blum and Sinmena, 1995; Sloger and Caldwell, 1970) and also depend on the environmental conditions and developmental status of the plant (Trewavas and Jones, 1991). There are reports that point to differences between conifers and herbaceous species in their stomatal sensitivity to exogenously applied ABA. For example, much lower concentrations of exogenously applied ABA are generally required to close stomata in herbaceous species. In detached wheat leaves, transpiration rates were almost halved after the application of 10^{-7} M ABA and were decreased by up to 80% after the application of 10^{-5} M ABA (Davies and Zhang, 1991). Stomatal opening in epidermal peels of *Vicia faba* L. leaves was completely inhibited by 10^{-9} M ABA (Dunleavy and Ladley, 1995). However, application of 10^{-5} M ABA, by means of an aerated root drench, did not reduce transpiration and photosynthesis

rates of lodgepole pine, Engelmann spruce, Douglas fir (Blake et al., 1990a) and black spruce seedlings (Blake et al., 1990b). Very high concentrations (10^{-3} M) of ABA were required to reduce stomatal conductance and photosynthesis rates in interior spruce seedlings (Grossnickle et al., 1996). These findings are consistent with reports that there are also differences in the concentrations of endogenous ABA between herbaceous and conifer species. Audus (1983), for example, reported an ABA content between 10–1000 ng ABA g^{-1} root dry weight in herbaceous species, whereas Roberts and Dumbroff (1986) reported values between 660–1492 ng ABA g^{-1} shoot dry weight in three conifer species.

The action of exogenously applied ABA is directly related to how effectively it is incorporated into plants. Uptake of ABA is strongly influenced by the external pH of the root solution, with maximum uptake at about pH 4.6 (Astle and Rubery, 1980). ABA transport across root membranes occurs mainly by diffusion (Hartung and Dierich, 1983) except at the apical region of roots where ABA carriers have been detected in a range of plant species (Astle and Rubery, 1983; Chen and Wang, 1992) and cell suspension cultures (Bianco-Colomas et al., 1991; Perras et al., 1994).

Leaves of well-watered plants contain enough ABA to bring about stomatal closure (Raschke, 1975), yet their stomata remain open. The physicochemical basis for ABA distribution between leaf compartments provides an explanation for the phenomenon. According to the anion trap concept, within-leaf ABA is accumulated preferentially in alkaline compartments such as the chloroplast stroma, and is thus effectively isolated from guard cells (Hartung and Slovik, 1991). ABA concentration is also regulated in the epidermal apoplast by various metabolic reactions (Trejo et al., 1993). Inactivation of ABA in plant tissue occurs by its oxidation to phaesic acid and dihydrophaesic acid (Zeevart et al., 1990). Apart from acidic metabolites, ABA is also metabolized to a conjugated form, ß-D-glucopyranosyl abscisate (ABA-GE), in plant tissue (Milborrow, 1970). The conjugation process is reported to be irreversible (Milborrow, 1978) and the conjugate

ABA-GE is sequestered in the cell vacuole (Bray and Zeevart, 1985; Lehmann and Glund, 1986). However, a reduction in leaf water potential can result in rapid ABA-regulated stomatal closure (Hartung et al., 1998). This could be because stomatal sensitivity to ABA increases as the leaf water potential decreases (Tardieu et al., 1992). Alterated mesophyll ABA metabolism in water stressed leaves (Zeevart and Creelman, 1988) or the release of ABA sequestered in the symplast can also lead to an increase in the ABA concentration in the apoplast of guard cells (Trejo and Davies, 1991; Trejo et al., 1995). Wilkinson and Davies (1997) have shown that there are differences in the compartmentation of ABA between well-watered and droughted plants. They suggest that ABA sequestration in well-watered plants is mediated by ABA uptake carriers, which when rendered inactive at pH 7.0 in droughted plants, favors the accumulation of apoplastic ABA. Thus, stomata close in response to water stress signal before any observable increase in bulk leaf ABA (Daeter and Hartung, 1995). However, these are short-term responses and prolonged stomatal closure requires an additional supply of ABA that is either synthesized in the leaves or supplied by the roots.

Several studies provide evidence for a central role of root-sourced ABA in water stressed plants (Davies and Zhang, 1991; Khalil and Grace, 1993). Soil drying stimulates ABA accumulation in plant roots (Zhang and Davies, 1989) including a 2 to 3-fold increase resulting from ABA redistribution in water stressed roots (Daeter et al., 1993). In addition to increasing ABA biosynthesis in response to stress, plant roots can regulate ABA concentration by slowing ABA catabolism (Liang et al., 1997; Walton et al., 1976) and by increasing the import of ABA from the shoots (Zhong et al., 1996).

To date, most research on the application of ABA has focused on the compound's mode of action and physiological effects rather than on its uptake. There is a growing interest in the use of ABA as an anti-transpirant and a need to quantify and maximize its uptake into plant roots and shoots. This study describes work undertaken to quantify and resolve differences between conifers and herbaceous species in their stomatal sensitivity to

exogenously applied ABA. The initial objective was to confirm that such differences in sensitivity do exist when ABA is applied under identical conditions. A second objective was to test the hypothesis that differences in sensitivity between species would be related to differences in the uptake of ABA by roots and its subsequent delivery to shoots. I hypothesized that in conifers, roots constitute a major barrier to ABA so that if root uptake was increased by increasing root membrane permeability (by either raising the root temperature or using organic solvents), or removing the roots altogether, then lower concentrations of ABA would be required to induce stomatal closure. A final objective was to test the hypothesis that ABA uptake is also related to the transpiration rate (E) or specifically to the flux of water through the roots. Thus, raising E by manipulating the aerial environment around the plant, would increase uptake. Experiments were conducted on wheat (*Triticum aestivum* L. cv Katepwa) and white spruce (*Picea glauca* (Moench) Voss), one of the most widely distributed forest species across North America and a very important commercial species. In British Columbia alone, 90 million white spruce seedlings are planted annually.

MATERIALS AND METHODS

Plant Material

Unless otherwise stated, all experiments were carried out on one year-old white spruce emblings and 10 day-old wheat seedlings. The spruce emblings, provided by B.C. Research Inc., Vancouver, B.C., Canada, were raised from somatic embryos (genotype U144) in solid agarose media and transplanted to styrofoam planting blocks containing a peat-vermiculite planting mixture. Six weeks before the start of experiments, emblings were removed from the styrofoam blocks and their roots washed thoroughly. The emblings were then transplanted into PVC cylinders (0.15 m I.D., 0.18 m high) filled with fine

sand. The sand was held in place by a nylon mesh (335 μm opening, 46% porosity) at the bottom of each cylinder. A half strength Hoagland's solution was applied to emblings every week. Wheat seedlings were germinated in test tubes (0.015 m I.D., 0.12 m long) filled with vermiculite. Prior to the experiments, the wheat seedlings and white spruce emblings were maintained in a growth chamber at a temperature of 20 °C and a daytime (14 h) photosynthetic photon flux density (Q) of 500 μmol $m^{-2}s^{-1}$.

Gas Exchange

Whole-seedling transpiration and net photosynthesis (P_n) rates were measured continuously using a computer controlled cuvette system (Livingston et al., 1994). In this closed system, seedlings are enclosed in a polycarbonate chamber (0.14 m I.D., 0.2 m high) with removable top and bottom plates. Vapor pressure is controlled by circulating chamber air through a $CaSO_4$ desiccant column supported on a digital balance (with a measurement resolution of 1 mg). Whole-plant transpiration is calculated as the rate of increase in desiccant mass with time. Stomatal conductance to water vapor (g_s) is calculated as E/(A x D) where A is the total projected leaf area and D is the vapor pressure deficit in the cuvette. Estimates of A were based on a linear relationship established between the leaf dry weight (DW) and leaf area (Appendix A). The leaf area was determined using a LI-3100 leaf area meter (Li-Cor Inc., Lincoln, NE, USA) after the leaves were dried for 36 hours using a vacuum freeze dryer. Because of the very high rate of air circulation in the chamber (approximately 0.025 m^3 s^{-1}) and very small differences in temperature between the leaves and air (< 0.1 °C), the boundary layer resistance was assumed to be zero (Livingston et al., 1994). Net photosynthesis rates are measured by integrating the output of a mass flow controller that injects CO_2 into the chamber to compensate for that assimilated by the plant. During darkness, respiratory CO_2 is scrubbed from the cuvette by pumping air from the chamber through a soda lime column. Light is provided by a high pressure sodium lamp using the light control system described by Livingston (1994).

During experiments, the cuvette was held at a temperature of 20 ± 0.05 °C, a CO_2 concentration of 350 ± 2.0 μmol mol^{-1} and Q of 1000 ± 5.0 μmol m^{-2} s^{-1} (measured at the top of the chamber). The vapor pressure deficit in the cuvette was maintained at 1.02 ± 0.02 kPa unless otherwise stated. Since D did not vary during any given experiment, E followed the course of g_s. Because of their very small size, eight wheat seedlings (with a combined leaf area of approximately 50 cm^2) were placed in the cuvette for the determination of P_n and g_s. Only one spruce embling was placed in the chamber.

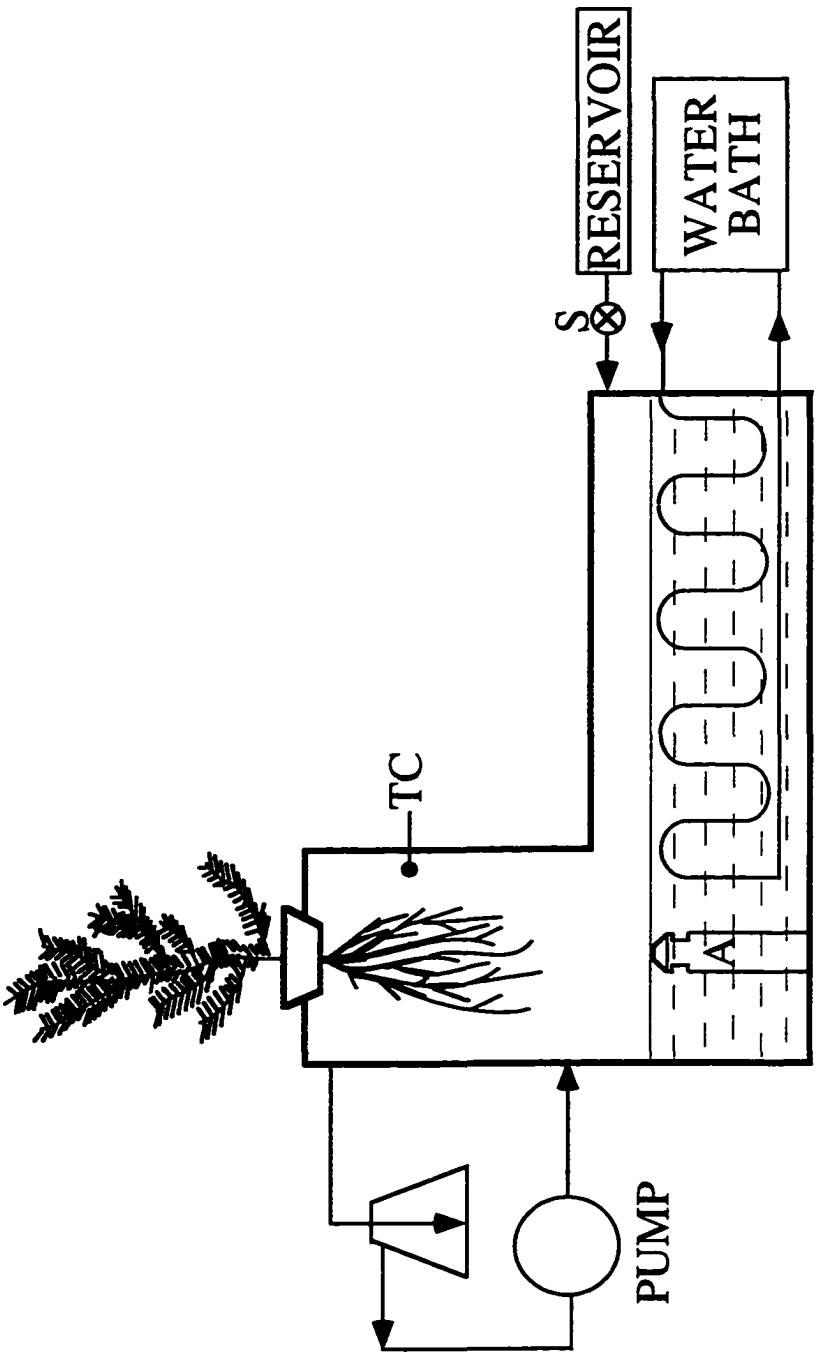
Measurements were carried out to determine typical P_n and g_s for well-watered wheat seedlings and white spruce emblings. Measurements were conducted over two days and repeated at least three times for both species.

ABA Delivery System

All determinations of ABA uptake were made on seedlings whose roots were enclosed in an aeroponic misting chamber. The chamber is a modified ultrasonic humidifier (Bionaire 201, Biotech Electronics, Montreal, Canada) (Fig. 2.1). Roots are held in a plexiglas cylinder (0.07 m I.D., 0.18 m high) and the solution is delivered to the roots in form of a fine mist using a piezoelectric sonic agitator. The temperature inside the misting enclosure is measured using a fine wire thermocouple and maintained at a given temperature (± 1 °C) by circulating water from a water bath through a coil of copper pipe (0.0065 m O.D.) in the reservoir of the misting chamber (volume 350 mL). Unless otherwise stated, root temperatures were held at 25 °C. An aeroponic delivery system was used because it removed the confounding influence of soil and soil microflora on uptake, it allowed the rapid introduction (and removal) of ABA into the misting solution, and minimized the amount of ABA solution required (500 mL per experiment).

Approximately 36 hours before ABA was applied, seedlings were removed from

Figure 2.1. Schematic diagram of the aeroponic misting chamber (not to scale). The mist in the root chamber is generated by an ultrasonic agitator (A). The ABA solution enters the misting system from the reservoir through a solenoid valve, S (SV-102, Omega, Stamford, CT, USA) activated by a float switch (inside the agitator). The mist in the root chamber, is circulated by a pump at a flow rate of 7.0 dm^3 min^{-1} (Dyna-pump, VWR Canlabs, Quebec, Canada). The root chamber temperature is measured using a thermocouple (TC).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their growing medium and their roots washed with tap water to remove soil debris. The seedlings were placed in the cuvette with their roots sealed in the aeroponic misting chamber. Seedlings were deemed to have successfully acclimatized to aeroponic misting if both g_s and P_n were not significantly lower than that measured when seedlings were grown in soil.

Determination of ABA Uptake

A solution of radiolabeled ABA, made on the day of the incorporation experiment, was applied to seedling roots using the misting chamber. Aliquots of labeled [^{3}H]-ABA stock, prepared in methanol, were added to 500 mL of distilled water to provide a radioactive concentration of 0.005 μCi mL^{-1} for (±)-[^{3}H]ABA. All glassware was silanized with 5% v/v dimethyl dichlorosilane (Sigma, St. Louis, MO, USA) in toluene prior to use. Typically, seedlings were fed with ABA for 10 hours until they had adsorbed at least 5 to 10 mL of labeled solution. Solution uptake was assumed to equal the cumulative transpiration over this period. Seedlings were then harvested and their roots washed with distilled water to remove any external labeled ABA. Roots and shoots were separated and immediately frozen in liquid nitrogen. Frozen tissue was dried in darkness for 36 hours using a vacuum freeze dryer. Lyophilized root and shoot tissues were weighed and ground to a fine powder in a mortar. Weighed amounts of powdered tissue were placed in 30 mL plastic centrifugation tubes and extracted in 20 mL of 80% (v/v) methanol/water containing 0.5% (v/v) acetic acid and 0.01% (w/v) butylated hydroxy toluene using a reciprocating wrist shaker. Tissue was stirred for two hours in darkness and then centrifuged at 8,000 g for 15 minutes. The pellet was re-extracted twice with 15 mL of methanol/acetic acid (+BHT) for 2 hours. The extracts were combined, filtered and reduced to aqueous phase by evaporating the organic solvents under reduced pressure in a rotary evaporator at 50 °C. The aqueous phase was then acidified to pH 2.5 with glacial acetic acid and partitioned with 10 mL hexane. The organic phase was discarded and the aqueous phase adjusted to 10%

(v/v) methanol and loaded on to Supelclean^{TM} LC-18 SPE silica cartridge (SUPELCO, St. Louis, USA). The cartridge (3 mL bed volume) was washed with 10% methanol (2 bed volumes) and the bound ABA was eluted with 6 bed volumes of 60% (v/v) methanol/water + 0.05%(v/v) acetic acid. The eluted fraction was collected and evaporated under reduced pressure in a rotary evaporator at 50 °C. The triplicate aliquot of sample was counted for [^{3}H] by liquid phase scintillation counting using Scinti-Versa II scintillation cocktail (Fisher Scientific, New Jersey, USA) and a scintillation counter (model LS6000 IC, Beckman Instruments Inc., California, USA).

Uptake efficiency (UE) was calculated as the ratio of the scintillation count of the tissue extract to the total count of the solution taken up by the plant (i.e., E x the activity of the misting solution) and expressed as percentage. Transport efficiency (TE), also expressed as percentage, was calculated as the ratio of the shoot count to the count of the combined root and shoot tissues. Values were adjusted to account for the relative proportion of root and shoot dry matter.

In separate preliminary experiments, the extraction protocol was carried out using a known radioactive concentration of (±)-[^{3}H]ABA. The percentage loss during extraction was 2.2 ± 0.9% (±SD). Uptake efficiency values, calculated for (±)-[^{3}H]ABA, include a correction for this small purification loss.

In wheat, the leaves comprised approximately 95% of the total shoot mass; however, in spruce, the needles constituted a much smaller fraction of the total shoot mass. In separate experiments, it was confirmed that there was no difference in transport efficiency determined for whole shoots (branches and needles) and needles alone. Thereafter, transport efficiency was determined for whole spruce shoots.

Roots Excision

Spruce emblings were transferred from their growing medium to the cuvette with their roots enclosed in a hydroponic chamber. The hydroponic chamber, made of plexiglas, is

0.1 m in length, 0.065 m in width and 0.1 m in height. The embling roots were continuously aerated by bubbling compressed air through the root solution. Following transfer to the hydroponic system, the emblings were monitored for at least 36 hours to ensure that both g_s and P_n were not significantly lower than that measured when the seedling roots were in soil. On the third day, after the lights were switched on and the emblings had reached steady-state g_s and P_n (after approximately 2 hours), they were removed from the cuvette their roots cut under water with a sharp scalpel and transferred back into the cuvette with the cut end of the stem submerged in aerated water. During this procedure, care was taken to ensure that the cut end of the stem was always submerged. Following root excision, the embling was left undisturbed until g_s and P_n reached the pre-excision values (approximately 1 hour). Exogenous (±)ABA was then applied (concentrations ranging from 0 to 10^{-11} M) and P_n and g_s measured over the next 10 hours.

Immunolocalization of Abscisic Acid

(i) Preparation of tissue

Seedling roots were exposed to exogenous (±)ABA for 10 hours in the aeroponic chamber. Seedlings were then harvested and the roots were cut into 1.0 cm sections and rapidly immersed in a freshly prepared 2% aqueous 1-(3-dimethylaminopropyl)-3 ethyl carbodiimide (EDC) solution (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA). Penetration of the EDC solution into the tissue was improved by vacuum pumping for 30 minutes. Postfixation was performed overnight in 4% paraformaldehyde + 1% glutaraldehyde mixture in 0.15 M phosphate buffer, pH 7.2 (Sotta et al., 1985). Following five rinses in phosphate buffer, root samples were dehydrated in a graded series of ethanol. After the 95% ethanol rinse, they were embedded into JB-4 resin (Polyscience, Inc., Warrington, PA, USA). Sections (5 µm) were cut with glass knives using a JB-4 microtome (Sorvall, Connecticut, USA) and affixed in pairs on glass slides precoated with

gelatin (0.5%) and dried overnight at 50 °C (Knox, 1982).

(ii) Immunocytochemical procedure

Monoclonal antibody solution was prepared by mixing 0.5 mg monoclonal antibody to (+)ABA (Idetek, San Bruno, USA) in 5 mL phosphate buffer saline (PBS, pH 7.2) containing 0.1% (v/v) Triton X-100 to prevent non-specific binding of antibody to the tissue. A drop (50 µl) of antibody preparation was placed over each section and incubated for 1 hour at room temperature. After incubation the sections were washed three times with PBS containing 0.1% Triton X-100. The specimens were then incubated in 50 µL of (1:500 dilution) fluorescein-isothiocyanate-conjugated goat anti-mouse IgG (Cedarlane Labs Ltd, Ontario, Canada) for 1 hour at room temperature in darkness. Following three rinses in PBS containing 0.1% Triton X-100, the sections were air dried and examined by epifluorescence microscopy with a Leitz (vario-orthomat) microscope using an I2 filter block.

EXPERIMENTS CONDUCTED

(1) Sensitivity assay

Experiments were conducted to determine the response of wheat (n=3) and white spruce (n=4) to varying concentrations of exogenous ABA. Racemic (±)ABA (Sigma, St. Louis, MO, USA) stock (1 M) was prepared in methanol. Solutions of ABA (ranging from 10^{-3} to 10^{-11} M) were prepared by adding aliquots of 1 M (±)ABA stock solution to 500 mL distilled water. The roots of the seedlings were exposed to the solution for at least 10 hours using the aeroponic misting system. During this period, P_n and g_s were measured continuously.

(2) Uptake and transport efficiency experiments

(i) Uptake of racemic ABA

Uptake and transport efficiency of tritiated racemic (±)-[^{3}H]ABA (Amersham, Arlington heights, IL, USA) was determined for both wheat (n=3) and white spruce (n=3). The concentration of (±)ABA in the radiolabeled solution used was only 2 x 10^{-11} M. In separate experiments on spruce emblings (n=3), radiolabeled (±)-[^{3}H]ABA solution was spiked with non-labeled (±)ABA to give a concentration of 10^{-3} M (±)ABA. These experiments were performed to determine whether uptake efficiency was a function of the concentration of the applied solution and, in particular, to determine whether a reduction in E, due to partial stomatal closure, would result in reduced uptake efficiency.

(ii) Effect of temperature and DMSO on uptake of racemic ABA

The effect of root temperature on uptake and transport efficiency was determined for spruce (at 17, 25, and 33 °C, n=3 for each root temperature) and wheat (25 and 33 °C, n=3). Uptake efficiency was also determined following the addition of 0.5% (v/v) DMSO (ACP Chemicals Inc., Quebec, Canada) to the misting solution for both species (spruce: 25 and 33 °C, n=3; wheat: 25 °C, n=3).

(iii) Effect of plant age on uptake of racemic ABA

Uptake and transport efficiency of tritiated (±)-[^{3}H]ABA was determined for 1 year and 2 year-old white spruce emblings (n=3 for each age class).

(iv) Effect of vapor pressure deficit on uptake of racemic ABA

The effect of varying vapor pressure deficit on (±)-[^{3}H]ABA uptake and transport efficiency was determined for spruce (D=1.0 and 2.0 kPa, n=3) and wheat (D=1.0 and 0.5 kPa, n=3). Vapor pressure deficit was adjusted so that the transpiration rates (on a per unit leaf area basis) of the two species were approximately equal. These experiments were

performed to test the hypothesis that sensitivity between the two species was related to flux of water through the roots or to the rate of delivery of ABA to receptor sites.

(3) Root bypass experiments

(i) Sensitivity assay

Experiments were conducted to determine the response of spruce (n=3) to varying concentrations of exogenous (±)ABA following root excision. Once g_s and P_n had reached pre-treatment values, an appropriate volume of the 1 M (±)ABA stock solution was dispensed into the hydroponic chamber using a syringe to achieve the desired concentration of ABA (from 10^{-4} to 10^{-7} M). The emblings were exposed to the ABA solution for 10 hours and g_s and P_n were measured continuously.

(ii) Uptake experiment

Following root excision, labeled [^{3}H]-ABA was added to the aerated solution in the hydroponic chamber to provide a radioactive concentration of 0.005 μCi mL^{-1}. After 10 hours of exposure to tritiated racemic (±)-[^{3}H]ABA, shoots were harvested for the determination of ABA uptake (n=3).

(4) Immunolocalization of ABA

For the immunolocalization experiments, spruce roots were exposed to 10^{-5} M (±)-ABA and wheat roots were exposed to 10^{-7} M (±)-ABA. The exogenously applied ABA had no effect on the seedling g_s and P_n. The long lateral, hair covered roots (absorbing roots) of white spruce were used for fluorescence labeling. In case of wheat, fluorescence labeling was performed on the root sections that were cut 3.0 cm away from the root tip.

Different controls employed to test the validity of the technique were: i) omission of the incubation in 2% EDC and ii) substitution of the anti-ABA antibody with PBS.

RESULTS

Sensitivity to exogenously applied ABA

The responses of wheat and white spruce, to varying concentrations of exogenously applied ABA are summarized in Figure 2.2. In spruce, the application of 10^{-3} M ABA resulted in a 50% reduction in g_s. In wheat, the application of a 1000-fold lower concentration of ABA brought about a 19% reduction in g_s. Almost complete closure of stomata in wheat and spruce was brought about by applications of 10^{-5} and 2 x 10^{-3} M (±)ABA, respectively.

Uptake and transport efficiency

Table 2.1 gives the uptake and transport efficiency of (±)-[^{3}H]ABA for wheat and spruce. At 25 °C (and D = 1.0 kPa) uptake efficiency was almost twice as high in spruce (31.0 %) than in wheat (18.6 %). However, in spruce virtually all of the ABA taken up remained in the roots and very little was transported to the shoots (TE = 5.4 %). In contrast, TE in wheat was almost 50% so that the total amount of ABA delivered to the shoots was nearly 9 times higher in wheat than spruce. In both species, (±)-[^{3}H]ABA counts in the roots always exceeded those in the shoots, although in wheat these differences were not statistically significant.

Typical time courses of P_n and g_s for a white spruce seedling following the application of 10^{-11} M (±)-[^{3}H]ABA are shown in Figure 2.3a. At this low ABA concentration, P_n and g_s were not reduced. Conversely, there was significant stomatal closure in seedlings given 10^{-3} M (±)ABA (nonlabeled) spiked with radiolabeled 10^{-11} M (±)-[^{3}H]ABA (Fig. 2.3b). However, the uptake efficiency was not significantly different to that determined for seedlings supplied with much lower concentrations of ABA. This suggests that in spruce the uptake efficiency is independent of the concentration of ABA applied.

Increased root temperature, DMSO treatment or a combination of these treatments, all increased the uptake efficiency of (±)-[^{3}H]ABA in both wheat and spruce (Table 2.1). In wheat, an increase in root temperature from 25 to 33 °C greatly increased UE (to 47.3 %) but because there was a proportional greater increase in ABA retained in the roots than ABA transported to the shoots, TE declined. Again, there was a decline in TE following DMSO treatment but it was not statistically significant. In spruce, despite huge increases in UE following treatments, TE was always less than 8% (Table 2.1). For example, DMSO treatment at 33 °C resulted in an UE of almost 80% but virtually all of the ABA was retained in the roots. In both wheat and spruce, when root temperatures were increased and 0.5% DMSO applied, without ABA treatments, there was no significant effect on seedling gas exchange. In separate experiments, it was determined that relatively large concentrations (> 5%) of DMSO are required to bring about stomatal closure.

Uptake efficiency in 2 year-old spruce emblings (11.7 ± 1.4 %) was less than half of that of 1 year-old emblings (31.0 ± 2.5 %). However, since there were no differences in shoot count (~1 %) between the age classes, TE increased significantly in two year-old spruce emblings (13.9 ± 2.2 %).

In wheat, when D was decreased from 1.0 to 0.5 kPa, E declined by 27% (from 1.9 to 1.4 mmol m^{-2} s^{-1}; Appendix B). There was a concomitant decrease in TE (from 48.8 to 33.6 %) because proportionally less ABA was transported to the shoots at the lower transpiration rates. Uptake efficiency did not differ significantly between treatments. In spruce, increasing D (from 1.0 to 2.0 kPa) led to transpiration rates (1.6 mmol m^{-2} s^{-1}; Appendix B) that were very close to those measured in wheat at lower D. However, UE and TE did not change significantly. Thus for the same E, TE in spruce was almost 10 times less than that in wheat (Table 2.1).

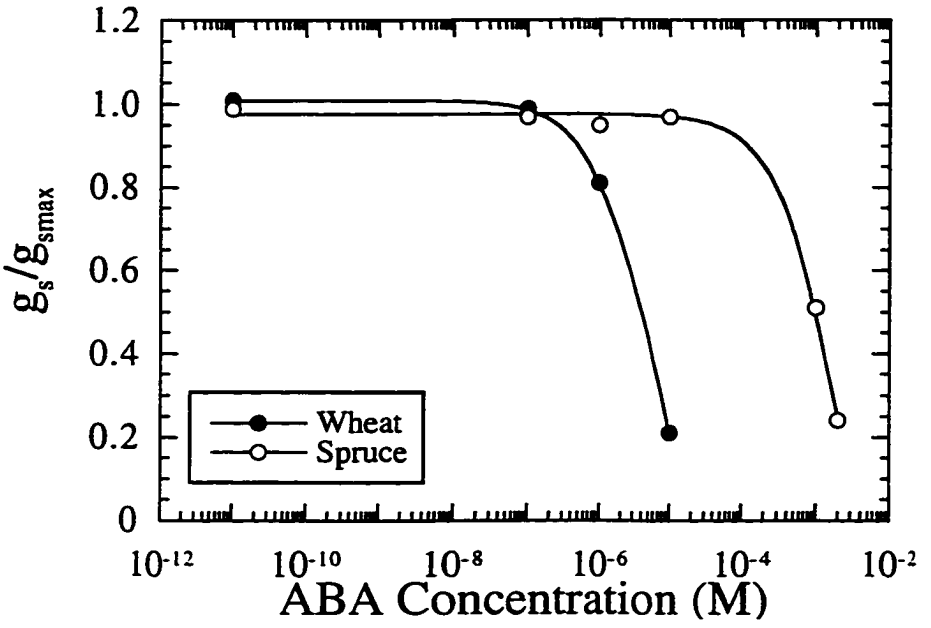


Figure 2.2. The relation between (±)ABA concentration and whole-plant stomatal conductance (g_s) normalized to the maximum stomatal conductance (g_{smax}) measured before the application of ABA. Each point is the average of 3 (wheat) or 4 plants (spruce). Values of g_s represent averages measured over 3 hours, at least 5 hours after the application of ABA. Standard deviations about the mean (not shown) were typically less than 0.05.

Table 2.1. Uptake, transport and distribution of 0.005 μCi mL^{-1} (±)-[^{3}H]ABA in roots and shoots of: (i) wheat seedlings with root temperature of 25 and 33 and 25 °C with 0.5% DMSO in the root solution, and 25 °C with vapor pressure deficit (D) of 0.5 kPa; (ii) white spruce emblings with root temperature of 17, 25, 33, 25 and 33 °C with 0.5% DMSO in the root solution, and 25 °C with D = 2.0 kPa. Unless otherwise stated, all treatments were conducted at D = 1.0 kPa. Each data point represents the mean (± SD) of three replicates. Mean values within each column followed by a different letter are significantly different at $\alpha = 0.05$ (Student-Newman-Keuls Test). Multiple comparisons were performed by species.

Species	Treatment	Uptake Efficiency (%)	Root Count (%)	Shoot Count (%)	Transport Efficiency (%)
Wheat					
	25 °C	18.6^a (±3.7)	9.6^a (±2.0)	9.0^b (±1.2)	48.8^b (±3.3)
	33 °C	47.3^c (±1.3)	31.7^c (±3.2)	15.6^c (±2.3)	33.1^a (±5.5)
	25 °C + DMSO	40.6^b (±4.2)	22.2^b (±2.1)	18.4^c (±2.4)	45.2^b (±2.3)
	25 °C + 0.5 kPa	16.6^a (±1.6)	11.1^a (±1.4)	5.5^a (±0.2)	33.6^a (±1.9)
Spruce					
	17 °C	24.2^a (±2.1)	22.4^a (±1.7)	1.8^a (±0.5)	7.1^b (±1.7)
	25 °C	31.0^a (±2.5)	29.4^a (±2.2)	1.6^a (±0.4)	5.4^b (±0.9)
	33 °C	57.5^c (±1.7)	55.9^c (±1.5)	1.6^a (±0.2)	2.9^a (±0.4)
	25 °C + DMSO	45.1^b (±3.1)	43.5^b (±2.9)	1.6^a (±0.4)	3.5^a (±0.6)
	33 °C + DMSO	79.4^d (±9.5)	77.7^d (±9.8)	1.7^a (±0.2)	2.2^a (±0.5)
	25 °C + 2.0 kPa	31.2^a (±1.2)	29.7^a (±1.1)	1.6^a (±0.4)	5.2^b (±1.3)

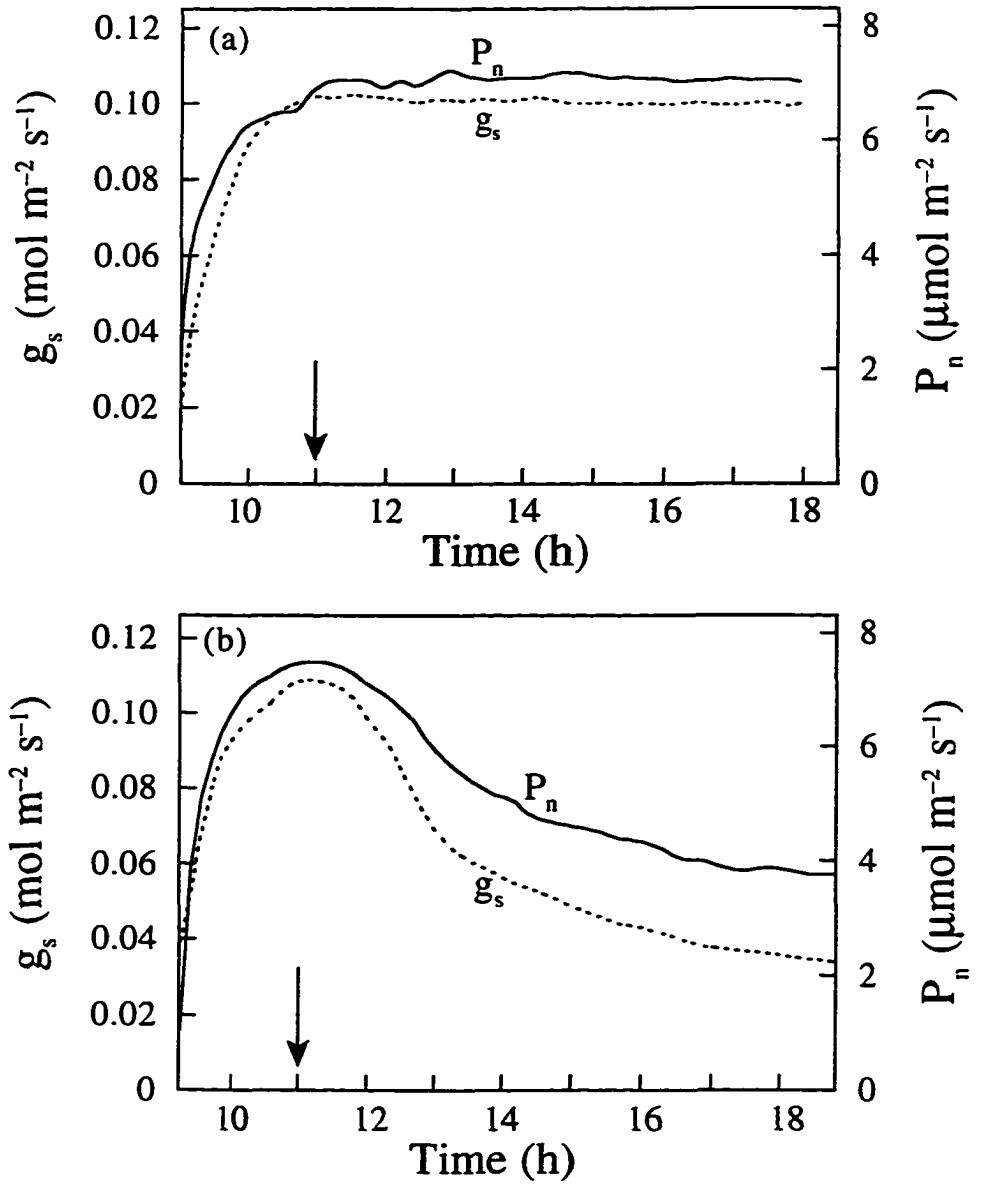


Figure 2.3. Net photosynthesis rate (P_n) and stomatal conductance (g_s) versus time for a one year-old white spruce embling. The lights were turned on at 09:00. In (a) and (b) the arrows represent the time of radiolabeled 10^{-11} M (±)-[^{3}H]ABA application, and 10^{-11} M (±)-[^{3}H]ABA + non-labeled 10^{-3} M (±)ABA application, respectively.

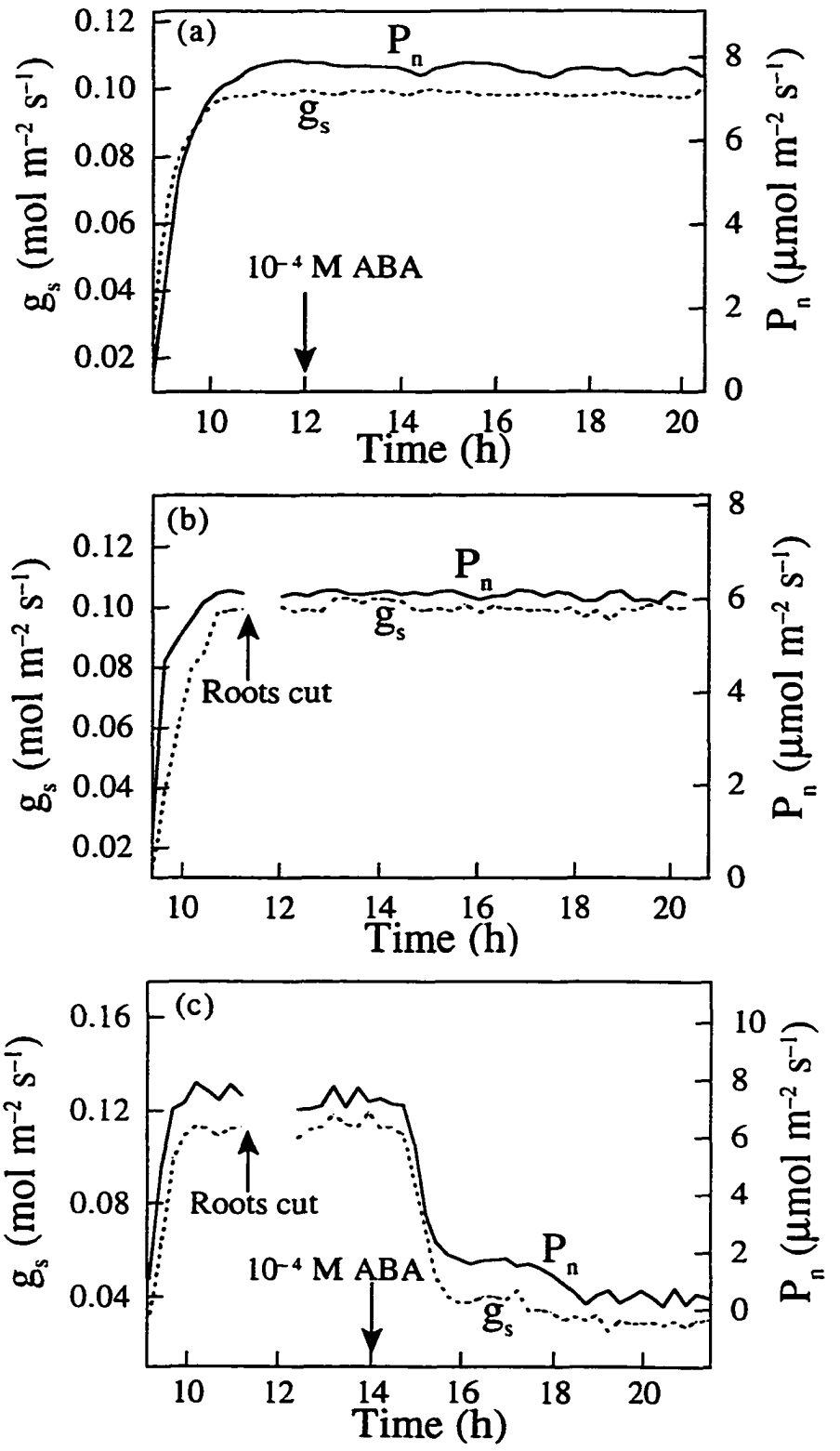
Root excision in spruce

Figure 2.4a shows a typical time course of P_n and g_s for a white spruce embling with its roots enclosed in the hydroponic system. The application of 10^{-4} M ABA did not significantly reduce P_n and g_s. There was no difference in response between emblings held in the aeroponic or hydroponic systems. A typical response to root excision in white spruce is given in Figure 2.4b. Following root excision, g_s and P_n returned to pre-treatment values within 1 hour and there was not a pronounced decline in g_s and P_n over the rest of the day. Figure 2.4c shows the response of a white spruce to 10^{-4} M (±)ABA, applied approximately 3 hours after the roots were excised. Within 2 hours of the ABA application, stomata were almost completely closed. The response of white spruce, with or without excised roots, to different concentrations of (±)ABA is summarized in Figure 2.5. Applications of 10^{-5} M (±)ABA following root excision, resulted in a 35% reduction in g_s. Applications of 10^{-4} M (±)ABA caused almost complete stomatal closure. Thus, excision of roots led to a 20-fold increase in sensitivity (2×10^{-3} to 10^{-4} M) to exogenous ABA in spruce. Root excision resulted in very large increase in the amount of (±)-[^{3}H]ABA transported to the shoots giving rise to a 55-fold increase in TE. The concentration of radiolabeled (±)-[^{3}H]ABA (2×10^{-11} M) used for these uptake experiments had no effect on g_s and P_n.

Immunolocalization of ABA in roots

Figure 2.6 shows the cross section of a white spruce (a) and wheat (b) root that were immunofluorescence labeled for ABA. There was a uniform fluorescence labeling in the inner cortical cells around the endodermis of spruce root section. However, in wheat, the fluorescence was localized inside the vascular tissue of the root section. No immunofluorescence was detected in sections of root samples that were not prefixed with 2% EDC or incubated in anti-ABA antibody.

Figure 2.4. Net photosynthesis rate (P_n) and stomatal conductance (g_s) versus time for a one year-old white spruce embling (a) before and (b) after root excision, and (c) after root excision and ABA application. The lights were turned on at 09:00. In (a), the roots were enclosed in a hydroponic chamber and the arrow indicates the time of 10^{-4} M (±)ABA application. In (b), the arrow represents the time of root excision. In (c), the arrows represent the time of root excision and application of 10^{-4} M (±)ABA. The gap in the data in (b) and (c) indicates the period when the seedling was removed from the chamber and its roots excised.


(a)
P_n
g_s
10^{-4} M ABA
g_s (mol m^{-2} s^{-1})
P_n (µmol m^{-2} s^{-1})
Time (h)
(b)
P_n
g_s
Roots cut
g_s (mol m^{-2} s^{-1})
P_n (µmol m^{-2} s^{-1})
Time (h)
(c)
Roots cut
P_n
10^{-4} M ABA
g_s
g_s (mol m^{-2} s^{-1})
P_n (µmol m^{-2} s^{-1})
Time (h)

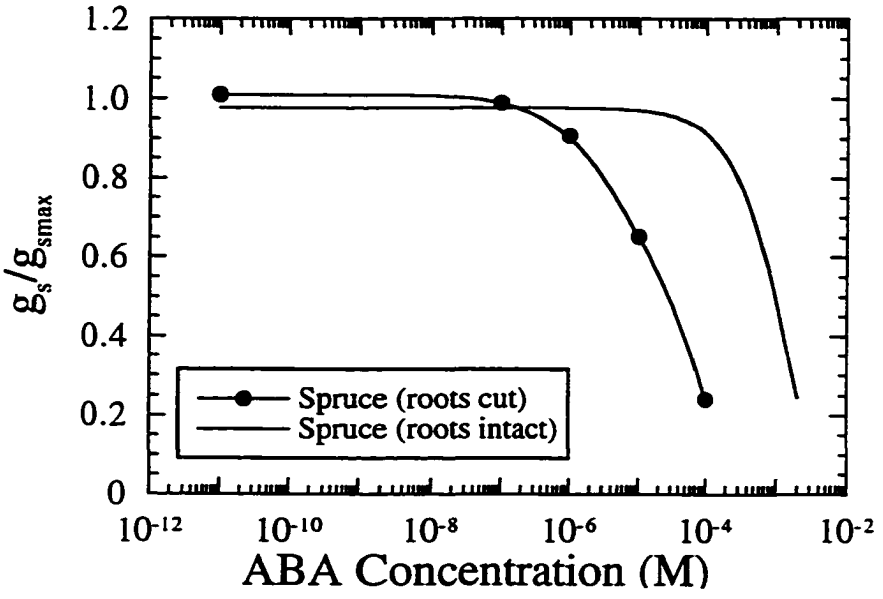
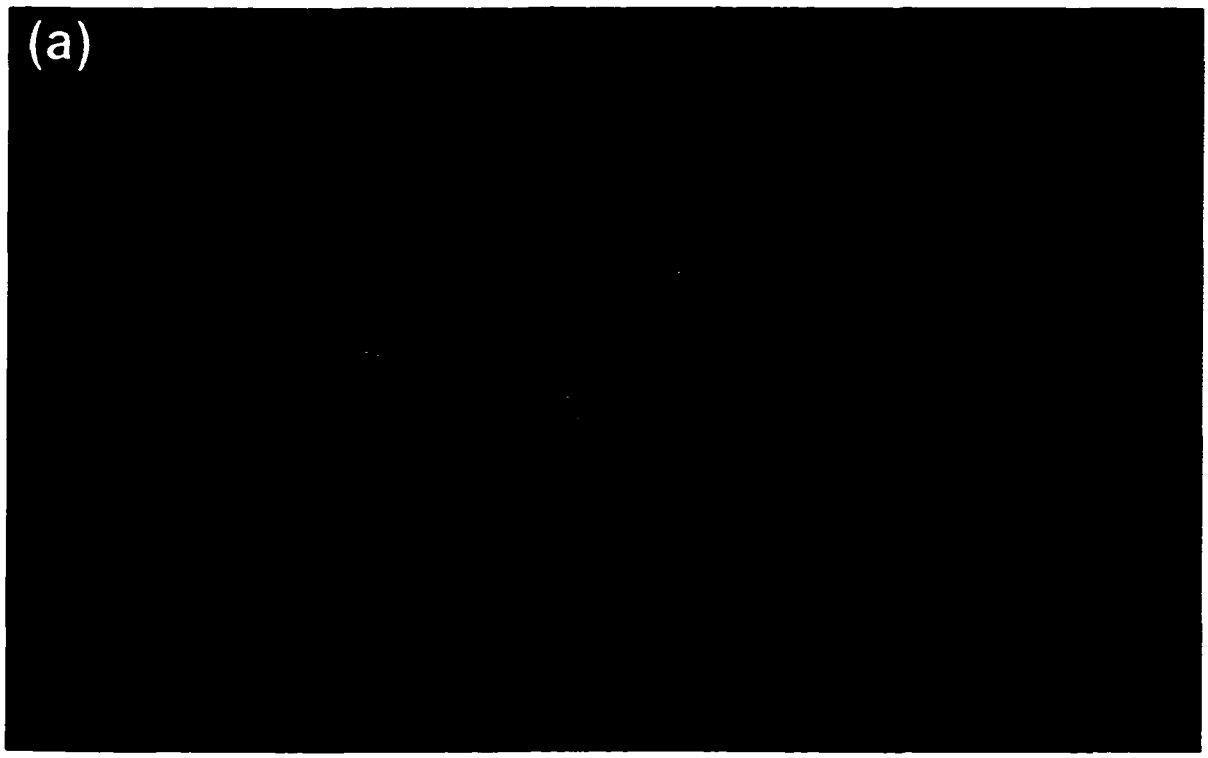
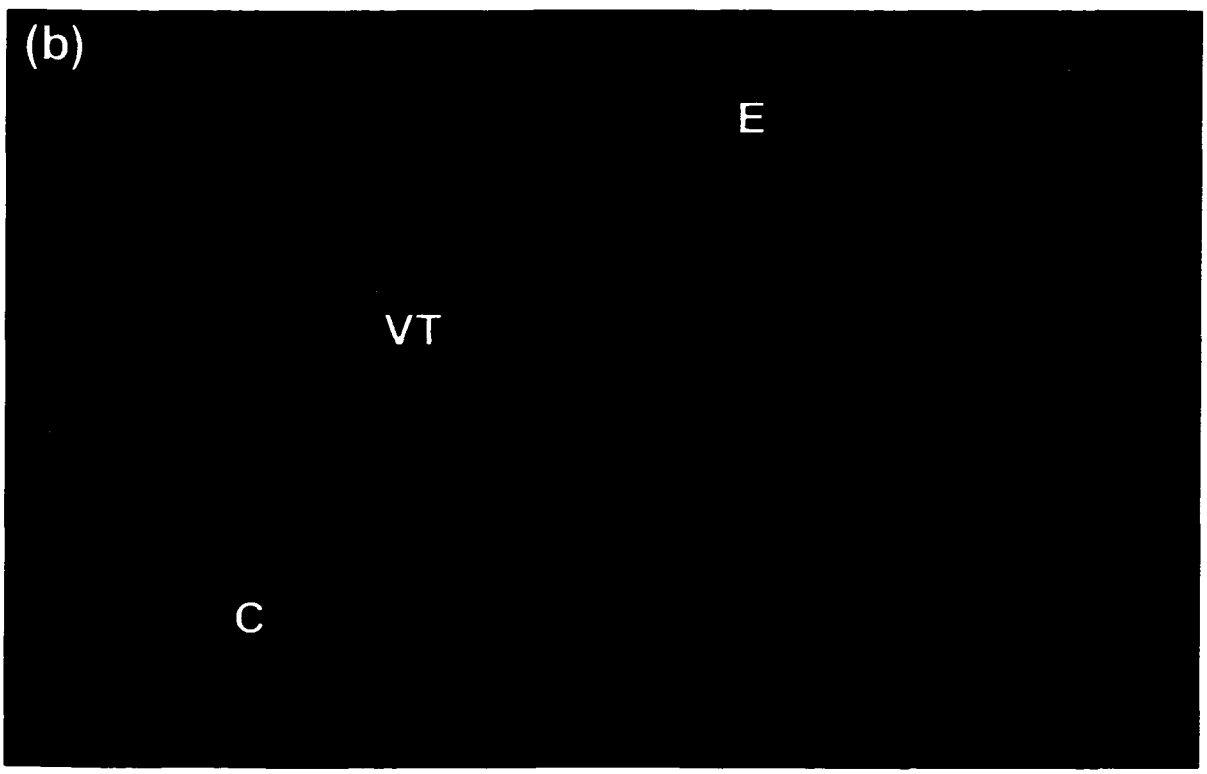


Figure 2.5. The relation between (±)ABA concentration and white spruce stomatal conductance (g_s) normalized to the maximum stomatal conductance (g_{smax}) measured before the application of ABA. Each point is the average of 4 (with roots intact) or 3 plants (with roots excised). Values of g_s represent averages measured over 3 hours, at least 5 hours after the application of ABA. Standard deviations about the mean (not shown) were typically less than 0.05.

Figure 2.6. Immunofluorescence localization of exogenous ABA in a JB-4 embedded white spruce (a) and wheat (b) roots. The root section were treated with (0.1 mg mL^{-1}) anti-ABA antibody followed by (1:500) FITC-labeled goat anti-mouse IgG. The sections were viewed by epifluorescence microscopy. Some weaker autofluorescence, mainly in the cortex (c) is distinguishable by its dull green color. The endodermis (E) and vascular tissue (VT) are also indicated.


(a)


(b)
E
VT
C

DISCUSSION

The results from this study show that exogenously applied ABA has to be applied at least at a 200 times higher concentration in spruce than in wheat to bring about the same degree of stomatal closure. Whilst there was up to 9-fold differences in transport efficiency between the species, this does not fully account for the differences in sensitivity to ABA application. This suggests that wheat stomata must be inherently more sensitive to ABA than spruce. This is consistent with reports that endogenous ABA concentrations in shoots are typically lower in wheat (146 ng ABA g^{-1} dry wt; Dallaire et al., 1994) than in white spruce (943 ng ABA g^{-1} dry wt; Roberts and Dumbroff, 1986). Thus, wheat might be more sensitive than spruce to low concentrations of exogenous ABA which it detects against a relatively low background level of endogenous ABA. However, in spruce only high concentrations of exogenous ABA bring about a pronounced decline in stomatal aperture. These results are also consistent with the finding that in drying soils, the stomatal conductance of woody species is relatively insensitive to chemical signals that might be generated in the roots (Fuchs and Livingston, 1996; Saliendra et al., 1995). This is in contrast to droughted herbaceous species where endogenous ABA produced in the roots has been implicated in bringing about stomatal closure (Gollan et al., 1986; Schurr et al., 1992). It is possible that in woody species, under well-watered conditions, high concentrations of endogenous ABA in the shoots are sequestered and therefore isolated from the guard cells (Hartung and Slovik, 1991). However, changes in leaf water status act as the stimulus for ABA redistribution in leaves causing rapid accumulation of ABA in the guard cells independently of any chemical signal from the roots. A similar argument is given by Schulze (1991) who argued that the long transport time required for chemical signals propagated from the roots to reach shoots in large trees suggest that in the short-term, stomata are more likely to be regulated by hydraulic influences.

Experiments carried out by Astle and Rubery (1980, 1983) using *Phaseolus coccineus* revealed saturable uptake carriers for ABA. Similar ABA carriers in woody species have

yet to be characterized. My results indicate that absorption of ABA into the spruce roots is not carrier mediated. Since high concentrations of unlabeled (±)ABA in the root system did not reduce the uptake of tritiated (±)-[^{3}H]ABA, it is likely that ABA transport across spruce roots occurs by diffusion. If ABA uptake was carrier mediated, the absorption rate of tritiated (±)-[^{3}H]ABA would have declined with increasing concentration of ABA because unlabeled (±)ABA would have competed for the carrier sites. Further, the root solution in my experiment was at pH 7.2 and carrier mediated uptake has been reported to be completely inactive at this pH (Perras et al., 1994).

The pronounced increase in uptake efficiency with increasing root temperature in both spruce and wheat might be related to concomitant increases in root hydraulic conductivity. Evidence for this in wheat (Carvajal et al., 1996) and cotton seedlings (Radin, 1990) showed that mineral nutrient deprivation is relieved by increasing the hydraulic conductance of the root cell membranes by raising root temperatures. These authors also drew attention to the correlation between the hydraulic conductance and the fluidity of the plasma membranes. The effectiveness of DMSO in greatly increasing uptake efficiency in both spruce and wheat might be related to its effects on the permeability of the phospholipid bilayer in root cells.

The significantly higher uptake efficiency in the younger, rather than the older spruce emblings, was likely related to the fact that the younger emblings had a greater proportion of white elongating roots, than brown roots. Although the root morphology of conifer seedlings is poorly described, some aspects have been investigated in white spruce (Johnson-Flanagan and Owens, 1985a, 1985b). There are changes in the morphology of spruce roots during the annual growth and dormant periods. The emergence of white root tips characterizes root growth and when elongation ceases, roots become brown as a result of two separate processes, suberization and metacutization. This could lead to significant changes in the absorption and translocation of exogenous ABA as the roots age.

The pronounced reduction in transport efficiency in wheat when D was halved and

transpiration reduced, strongly suggests that transport efficiency is dependent on the flux of ABA through the xylem to the leaves. A similar observation has been made for field grown maize plants where there were diurnal changes in the apparent stomatal sensitivity to xylem ABA concentration (Tardieu and Davies, 1992). During the day, ABA concentration in the xylem remained relatively constant but an increase in D resulted in increased leaf transpiration rates and thus in an increased delivery of ABA from the xylem stream to stomatal complexes. In contrast to wheat, in spruce, a 36% increase in E did not result in an increase in the transport of exogenous ABA to the shoots. Changes in D (and transpiration rate) did not have a significant effect on uptake efficiency in both species. This suggests that ABA transport across root membranes is not dependent on the transpiration rate.

In both species, more ABA was delivered to the roots than shoots. However, in all treatments, uptake efficiency was lower in wheat than in spruce. These results simply reflect the more rapid movement of exogenously applied ABA from roots to shoots in wheat seedlings. A similar trend in [2-^{14}C]-ABA distribution was reported in *Vicia faba* after exogenous application via roots (Bano and Hillman, 1989). Ten days after labeling, the major radioactive concentration was found in the roots (64%), followed by the shoots (29%) and the cotyledons (6%).

The results from my study suggest that the transport of exogenous ABA in spruce needles is always low and is virtually independent of root uptake. Very large increases in ABA uptake in the roots, whether brought about by increasing the root temperature or applying DMSO, did not translate into increased transport of ABA to the shoots. The possibility that exogenous ABA was metabolized very rapidly in the shoots can be ruled out since the labeling experiments were completed within 10 hours, a period much shorter than that required for significant metabolism (Lehmann and Schutte, 1984). Thus, my results strongly suggest that, in spruce, large amounts of ABA are sequestered in the roots. The root excision experiments support this conclusion.

Root excision did not bring about rapid stomatal closure in spruce. The effect of this treatment only became apparent after 12 hours, and in separate experiments were shown to increase over the next 3 days. Milligan and Dale (1988) presented similar results for *Phaseolus vulgaris*, where removal of 85% of the root system under water had no effect on seedling stomatal conductance or transpiration rate during the first photoperiod. Similarly, Smith and Dale (1988) reported no immediate rise in leaf ABA content following root excision in *Phaseolus vulgaris*. In contrast, an immediate response to root severing was observed in maize plants. Removal of 50% of the root system caused a 100-200% increase in ABA content of the remaining root system in 3 hours (Zhang, 1994).

My results indicate that over the short-term, root excision led to a higher sensitivity to ABA application in spruce because of the removal of a significant barrier to ABA uptake. Following root excision, delivery of ABA to the shoots increased by over 55-fold. This suggests that differences in transport of ABA from roots to shoots account for a major part of the differences in sensitivity to exogenous application of ABA between the two species. However, these results still indicate that wheat is inherently at least 10 times more sensitive to exogenously applied ABA than spruce. Certainly, the results of the root excision experiments demonstrate that exogenous ABA is sequestered in the spruce roots and the immunolocalization experiments confirm these observations.

The immunocytochemical techniques have been used successfully to provide useful information about hormone distribution within the plant tissue (Bertrand et al., 1992; Caruso et al., 1995). Immunolocalization of ABA using antibody requires that ABA molecule be irreversibly fixed in their original cellular position by EDC (Skene et al., 1987; Sossountzov et al., 1986). The control experiments confirm that the labeling is not an artifact arising from non-specific binding of ABA to tissue sections. From the analysis of the immunofluorescence labeled spruce root section, it is evident that ABA is almost exclusively localized in the inner cortical cells around the endodermis. In white spruce, the root endodermis is suberised and accumulates phenolic compounds (Johnson-Flanagan and

Owens, 1985a). Clarkson (1993) suggests that the presence of suberin lamellae in the endodermis increases resistance of the endodermal walls to the passage of solutes and this could account for the inability of ABA to cross the endodermis and reach the tracheids in spruce roots. However, in wheat the exogenous ABA is localized inside the root vascular tissue. These observations are in agreement with my results that much higher proportion of exogenous ABA is transported from roots to shoots in wheat than spruce. In addition, preliminary results of Hartung et al. (1998) suggest that, in some species like maize, root endodermis exhibits significant permeability to ABA.

My results provide compelling evidence that exogenously applied ABA is compartmented in spruce roots. Abscisic acid has a non-uniform distribution within a plant and since it only penetrates membranes freely when in a protonated form, its distribution within tissue will be highly dependent on pH gradients within that tissue (Hartung and Slovik, 1991). It is possible that in spruce roots, large amounts of ABA are sequestered as the anion (ABA^-) within a highly alkaline tissue fraction. In addition, exogenously applied ABA could be compartmented in spruce roots as ABA-GE. These possibilities could be investigated using the selective-ion monitoring (SIM) mode of combined gas chromatography-mass spectrometry (GC-MS).

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CHAPTER 3

Uptake, transport and biological activity of exogenously applied ABA enantiomers and ABA analogues in white spruce (*Picea glauca* (Moench) Voss) and wheat (*Triticum aestivum* L. cv Katepwa) seedlings

INTRODUCTION

In the previous chapter, I determined that a 200-fold higher concentration of exogenously applied (±)-ABA was required to close stomata in white spruce (*Picea glauca* (Moench) Voss) seedlings than in wheat (*Triticum aestivum* L. cv Katepwa). I concluded that this difference in response between species arose because of (i) differences in the uptake and subsequent transport of ABA within the plant and (ii) differences between species in their stomatal sensitivity to ABA. For example, in spruce, a very high proportion (95 %) of ABA taken up by the plant was sequestered in the roots and consequently less than 2 % of the applied ABA was transported to the shoots. In wheat, however, almost 9 times as much of the applied ABA reached the shoots.

In this study, I built on my previous work. The general objective was to determine whether ABA enantiomers and specifically tailored analogues would have higher biological activity (particularly in spruce) than racemic ABA. I hypothesized that if this was the case, then I would also observe a higher uptake and transport of these compounds in the two species. I had a particular interest in ABA analogues, as a cost effective alternative to the less stable, and in some cases, less biologically active naturally occurring form (Orton and Mansfield, 1974; Malloch and Fenton, 1979; Walton, 1983; Rademacher et al., 1989; Abrams and Milborrow, 1991; Todoroki et al., 1995; Rose et al., 1996; Abrams et al., 1997; Fuchs et al., 1998).

It is recognized that the uptake and action of ABA depend on its chemical configuration. For example, the cis-trans isomer of ABA penetrates more easily into soybean root tissue than the trans-trans isomer. This has been attributed to the former's hydrophobic character

(Markhart III, 1982). Both natural (+)- and unnatural (–)-ABA enantiomers are active in physiological assays but have different effects on physiological processes (Sondheimer et al., 1971) and gene induction (Walker-Simmons et al., 1992). For example (+)-ABA has a more pronounced effect than (–)-ABA on stomatal closure in intact plants (Cummins and Sondheimer, 1973), and much lower concentrations of (+)-ABA are required to close stomata in epidermal strips of *Commelina communis* (Milborrow, 1980) and *Vicia faba* (Hornberg and Weiler, 1984). Further, (+)-ABA is more effective than (–)-ABA at increasing freezing tolerance (Churchill et al., 1992; Wilen et al., 1996) and inhibiting seed germination (Gusta et al., 1992).

The action of each ABA enantiomer is likely directly related to how effectively it is incorporated into plants. ABA carriers in suspension-cultured barley cells have a strong preference for (+)-ABA over (–)-ABA (Perras et al., 1994). Windsor et al. (1994) reported that there is a stereoselective carrier for (+)-ABA in *Daucus* and that (–)-ABA transiently occupied the carrier receptor sites and inhibited the uptake of (+)-ABA. In contrast, Bianco-Colomas et al. (1991) reported that cells of the suspension culture of *Amaranthus tricolor* have saturable uptake carriers with no preference for either of the enantiomers.

There are also differences, between enantiomers, in metabolic pathways and rates of metabolism. The main route of (+)-ABA metabolism is the oxidative pathway via phaseic acid and dihydrophaseic acid, while (–)-ABA is preferentially conjugated to (–)-ABA-GE (Zeevart et al., 1990). Further, (+)-ABA is metabolized much faster than (–)-ABA. For example, in white spruce somatic embryo suspension cultures, there was quantitative metabolism of (+)-ABA to phaseic acid but (–)-ABA remained unmetabolised for up to 8 days (Dunstan et al., 1992). In maize cell culture, more than 90 % of (+)-ABA was converted to phaseic acid within 24 hours whereas only about 50 % of the added (–)-ABA was metabolized after four days (Balsevich et al., 1994).

Esterification of the carboxyl group of ABA produces an active analogue which has increased antitranspirant activity (Jones and Mansfield, 1971). It is likely that the increased activity of esters is due to their higher rate of transport across biological membranes, and their slower metabolism or hydrolysis to abscisic acid, the active species. Black spruce seedlings treated with a methyl ester analogue and then drought stressed 7 days after treatment, had water use efficiencies up to 75 % higher than non-treated control seedlings (Blake et al., 1990). In interior spruce seedlings, exogenous application of a methyl ester analogue reduced stomatal conductance for seven days but only reduced photosynthesis for one day and had no adverse affects on root growth (Grossnickle et al., 1996).

In this study, I quantify the uptake and subsequent transport of ABA enantiomers and analogues in white spruce emblings and wheat seedlings. Specifically, I test the hypotheses that; (i) the uptake and transport of (+)-ABA, and therefore the biological activity (assessed in terms of its effects on gas exchange), would exceed those of the unnatural enantiomer; (ii) the methyl ester derivative of ABA (MeABA) would be a better antitranspirant than ABA; (iii) the isopropyl ester derivative of ABA (iPrABA) would have biological activity at lower concentrations than the natural ABA and the methyl ester derivative of ABA and (iv) the bioactivity of exogenously applied ABA analogues would be related to the degree of sequestration in the roots, as observed in my previous study with (±)-ABA (Chapter-2).

In this study, I used ester derivatives of ABA because, the esters being more lipophilic than ABA should be easily transported across root membranes. In addition, the carbon-oxygen linkage in the C-1 terminal moiety is conserved, which is shown to be an essential requirement for higher bioactivity (Orton and Mansfield, 1974; McWha et al., 1973). The isopropyl ester derivative was used because, the O-C_3H_7 bond in isopropyl ester is sterically more hindered than the O-H or O-CH_3 bond in ABA or MeABA respectively, thus iPrABA should be less susceptible to conjugation and hence be more stable in plant tissue.

MATERIALS AND METHODS

Plant Material

Unless otherwise stated, all experiments were carried out on one year-old white spruce emblings (*Picea glauca* (Moench) Voss) and 10 day-old wheat (*Triticum aestivum* L. cv Katepwa) seedlings as described in detail in Chapter-2. The spruce emblings, provided by B.C. Research Inc. Vancouver, B.C. Canada, were raised from somatic embryos and transplanted to styrofoam planting blocks. Six weeks before the start of experiments the emblings were transplanted into PVC cylinders filled with fine sand. Wheat seedlings were germinated in test tubes filled with vermiculite. Prior to experiments, both wheat seedlings and white spruce emblings were maintained in a growth chamber at a temperature of 20 °C and with a daytime (14 h) photosynthetic photon flux density (Q) of 500 μmol m^{-2} s^{-1}.

Gas Exchange

Whole seedling transpiration (E) and net photosynthesis (P_n) rates were measured continuously using the computer controlled cuvette system described by Livingston et al. (1994). Stomatal conductance to water vapor (g_s) was calculated as E/1.6(A x D) where A is the total projected leaf area, and D is the vapor pressure deficit in the cuvette. Light was provided by a high pressure sodium lamp using the light control system described by Livingston (1994).

During experiments, the cuvette was held at a temperature of 20 (± 0.05) °C, with a vapor pressure deficit of 1.02 ± 0.02 kPa, a CO_2 concentration of 350 ± 2.0 μmol mol^{-1} and Q of 1000 ± 5.0 μmol m^{-2} s^{-1} (measured at the top of the chamber). Since D was not varied during experiments, E followed the course of g_s. Because of their small size, eight wheat seedlings (with a leaf area of approximately 50 cm^2) were placed in the cuvette for the determination of P_n and g_s.

Delivery System

All determinations of ABA uptake and transport were made on seedlings (or emblings) whose roots were enclosed in an aeroponic misting chamber held at 25 °C (Chapter-2). These seedlings were removed from their growing medium not less than 36 hours earlier. Solutions were delivered to the roots (previously washed with tap water to remove soil debris) in the form of a fine mist using a piezoelectric sonic agitator. Experiments were undertaken on seedlings that were deemed to have successfully acclimatized to the aeroponic misting system, i.e. if both g_s and P_n were not significantly lower than that measured when seedlings were grown in soil.

Determination of Uptake and Transport

Aliquots of optically pure tritiated ABA and ABA analogues, prepared in methanol, were added to 500 mL of distilled water to provide a radioactive concentration of 0.01 μCi mL^{-1} for (+)-[^{3}H]ABA, (–)-[^{3}H]ABA, (+)-[^{3}H]MeABA and (–)-[^{3}H]MeABA.

Typically, seedlings were fed with ABA compounds for 10 hours until they had adsorbed at least 5 to 10 mL of labeled solution. The amount of solution taken up was calculated as the cumulative whole plant transpiration over this period. Seedling's roots and shoots were then separated and freeze dried, and lyophilized tissue weighed and ground to a fine powder. The subsequent extraction procedures are described fully in Chapter-2.

Triplicate aliquots of extracts were counted for [^{3}H] by liquid phase scintillation counting (model LS6000 IC, Beckman Instruments Inc., California, USA) using a Scinti-Versa II scintillation cocktail (Fisher Scientific, New Jersey, USA). Uptake efficiency (UE) was calculated as the ratio of the scintillation count of the (root and shoot) tissue extract to the total count of the solution taken up by the plant (i.e., E x the activity of the misting solution) and expressed as a percentage. Transport efficiency (TE, %) was calculated as the ratio of the shoot scintillation count to the count of the combined root and

shoot tissues. Values of UE and TE were adjusted to account for the relative proportions of root and shoot dry matter.

In separate experiments, the extraction protocol was carried out using a known radioactive concentration of ABA and ABA analogues and the percentage loss during extraction determined (Table 3.1). Values of UE and TE, include corrections for these small purification losses (1.8 to 7.7 %).

In wheat, the leaves comprised approximately 95 % of the total shoot mass, however, in spruce the needles constituted a much smaller fraction of the total shoot mass. In previous work with (±)-ABA (Chapter-2) I confirmed that there were no differences in values of UE or TE determined for whole shoots (branches and needles) and needles alone. In spruce, therefore, needles were not separated and UE and TE were determined for whole shoots.

EXPERIMENTS CONDUCTED

(i) Sensitivity assay

The response of wheat and spruce (both n=3) to the exogenous application of varying concentrations (10^{-11} to 10^{-3} M) of the (±)-MeABA (Sigma, St. Louis, MO, USA) and isopropyl ester [(±)-iPrABA] analogues was determined. In both cases, seedlings' roots were exposed to the analogues for 10 hours and P_n and g_s measured continuously.

(ii) Uptake and transport efficiency

The uptake and transport efficiency of tritiated natural (+)-[^{3}H]ABA and unnatural (–)-[^{3}H]ABA enantiomers and their respective methyl ester derivatives [(+)-[^{3}H]MeABA and (–)-[^{3}H]MeABA] (Balsevich et al., 1994) was determined for wheat and one and two year-old (ABA enantiomers only) spruce (in all cases n = 3).

Table 3.1. Percentage loss of optically pure ABA and ABA analogues during extraction. Each number is the mean of three replicates. Standard deviations are given in parentheses.

Compound	Loss during extraction (%)
(+)-ABA	1.9 (±1.3)
(–)-ABA	1.8 (±0.9)
(+)-MeABA	3.8 (±1.9)
(–)-MeABA	7.7 (±0.2)

RESULTS

In spruce, applications of 10^{-4} and 10^{-5} M (±)-iPrABA brought about 35% and 12% reductions in g_s, respectively (Fig. 3.1 and 3.2a). However, the same concentrations of (±)-MeABA, (and (±)-ABA, Chapter-2) had no effect on seedling gas exchange (Fig. 3.2a). Even at 10^{-3} M, (±)-MeABA had only a small effect on g_s.

In wheat, while both (±)-MeABA and (±)-iPrABA closed stomata at lower concentrations than in spruce (Fig. 3.2b), both compounds were slightly but not significantly less effective than (±)-ABA at inducing stomatal closure.

Table 3.2 summarizes the uptake and transport of optically pure ABA and MeABA enantiomers for both species. In one year-old spruce, the UE of the ABA enantiomers was approximately half of that of their methyl ester derivatives, but because there was a proportionally greater amount of methyl ester analogue retained in the roots, TE of the methyl ester analogues was almost half of that of the ABA enantiomers. Thus, even though the UE of both [(+) and (–)] methyl esters was almost 90 %, TE was less than 2%. There were no significant differences in UE between the optically pure ABA enantiomers or between their methyl ester derivatives.

The uptake efficiency of both ABA enantiomers was more than twice as high in the one year-old, than in the two year-old spruce. However, a much higher proportion of ABA was retained in the roots of the younger emblings, so that TE for this age class was actually lower than that for the older emblings. This was likely related to the fact that the younger emblings had a greater proportion of white elongating roots than brown roots. Johnson-Flanagan and Owens (1985a, 1985b) draw attention to differences in the morphology of different aged roots in white spruce. Such differences could have profound effects on absorption and translocation of exogenous ABA.

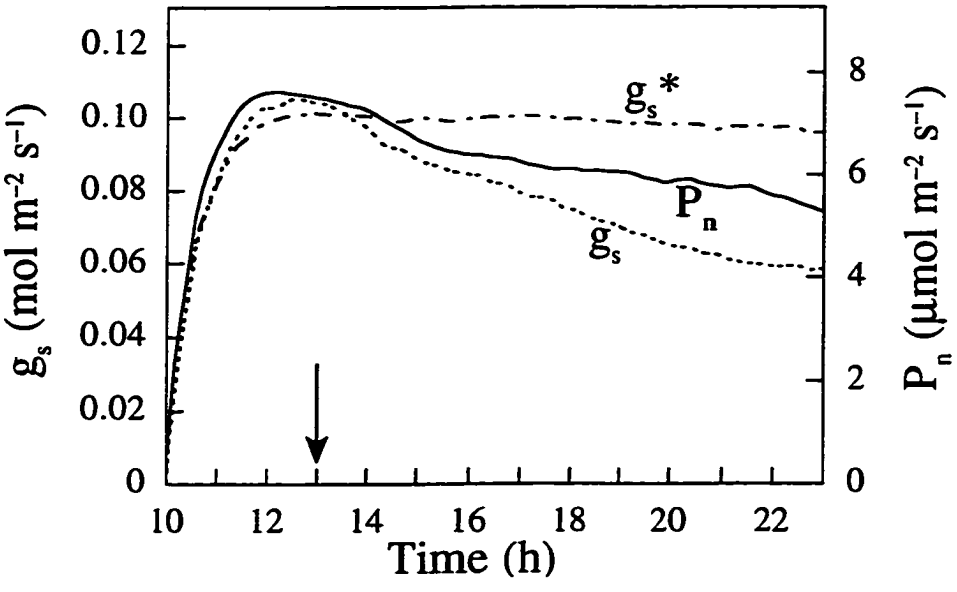

Figure 3.1. Net photosynthesis rate (P_n) and stomatal conductance (g_s) versus time for a one year-old white spruce embling. The lights were turned on at 10.00. The arrow represents the time of application of 10^{-4} M racemic isopropyl ester derivative of ABA. For comparison, g_s* represents g_s after the application of 10^{-4} M (±)-ABA (from Chapter-2).

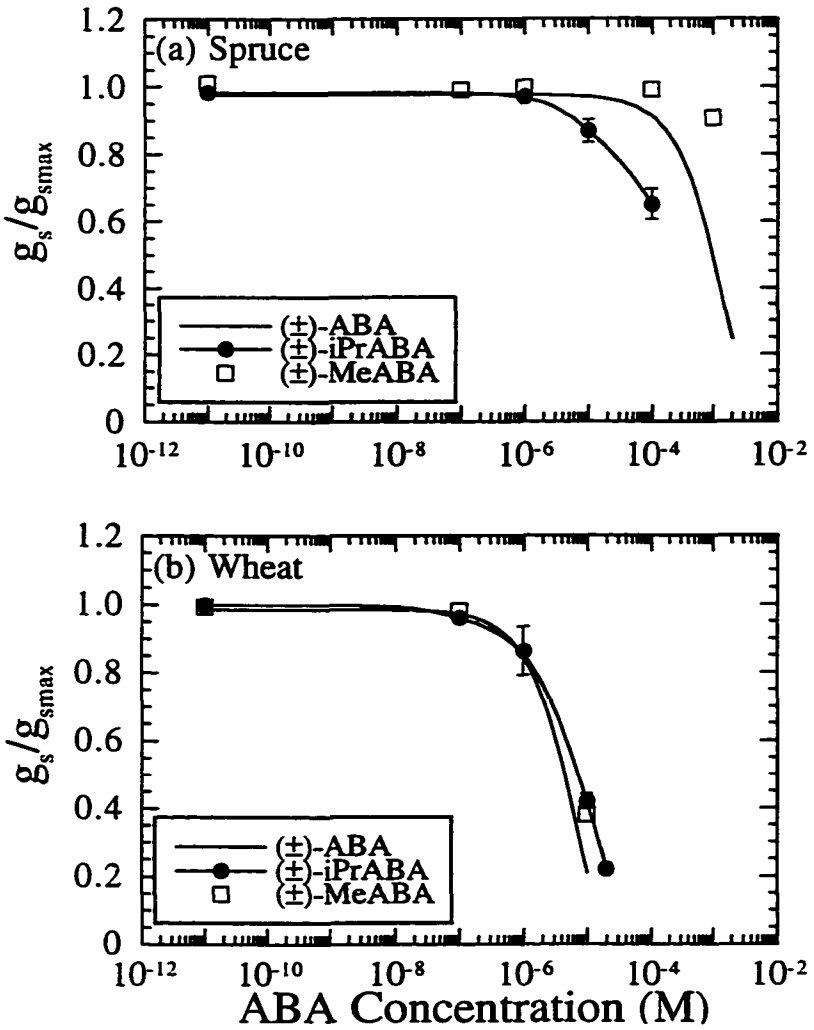

Figure 3.2. The relation between the concentration of exogenously applied methyl (MeABA) and isopropyl ester derivative of ABA (iPrABA) and stomatal conductance (g_s) normalized to the maximum stomatal conductance (g_{smax}) measured before the application of ABA analogues for (a) white spruce and (b) wheat seedlings. Each data point represents the average of 3 replicates. Values of g_s represent averages (±SD) measured over 3 hours, at least 5 hours after the application of ABA analogues. Data for (±)-ABA are also shown (from Chapter-2).

Table 3.2. Uptake and transport of 0.01 μCi mL^{-1} optically pure [^{3}H]ABA and [^{3}H]MeABA in roots and shoots of 1 year- and 2 year-old spruce emblings and wheat seedlings. Each number is the mean of three replicates. Standard deviations are given in parentheses. Mean values within each column followed by a different letter are significantly different at $\alpha = 0.05$ (Student-Newman-Keuls Test). Multiple comparisons were performed by species.

Species	Compound	Uptake Efficiency (%)	Root count (%)	Shoot count (%)	Transport Efficiency (%)
Spruce					
1 year-old					
	(+)-ABA	45.9^c (±2.9)	44.5^c (±3.2)	1.4^a (±0.3)	3.0^b (±1.9)
	(–)-ABA	41.2^c (±2.8)	39.4^c (±2.3)	1.8^a (±0.5)	4.4^b (±1.1)
	(+)-MeABA	87.3^d (±8.0)	85.9^d (±8.5)	1.4^a (±0.7)	1.7^a (±0.9)
	(–)-MeABA	88.1^d (±4.8)	86.9^d (±4.8)	1.2^a (±0.2)	1.4^a (±0.2)
2 year-old					
	(+)-ABA	9.4^a (±1.2)	8.1^a (±1.3)	1.3^a (±0.2)	13.3^d (±3.5)
	(–)-ABA	17.7^b (±0.5)	16.3^b (±0.6)	1.5^a (±0.2)	8.2^c (±1.4)
Wheat					
	(+)-ABA	32.8^b (±1.5)	21.1^b (±1.0)	11.7^b (±1.1)	35.5^b (±1.0)
	(–)-ABA	29.0^b (±0.6)	22.6^b (±0.3)	6.4^a (±0.5)	22.2^a (±1.3)
	(+)-MeABA	20.6^a (±2.3)	13.6^a (±1.6)	7.0^a (±0.8)	34.2^b (±0.7)
	(–)-MeABA	38.5^c (±1.7)	28.6^c (±0.8)	9.9^b (±1.8)	25.0^a (±1.1)

In wheat, differences in uptake and transport efficiency between the ABA enantiomers and the methyl ester derivatives were far less pronounced than in spruce (Table 3.2). The ABA enantiomers had similar UE, but TE of the (+)-ABA enantiomer was significantly higher than that of the (–)-ABA enantiomer. There were also significant differences in uptake and transport efficiency between the two MeABA enantiomers.

DISCUSSION

A primary objective of this study was to determine whether the biological activity of ABA enantiomers and analogues would be closely related to their respective uptake by roots and subsequent transport to the shoots. My results suggest that in wheat, the higher biological activity of the natural ABA enantiomer (than (–)-ABA) is, in part, due to it being more efficiently transported to the shoots than the unnatural form. While the uptake of the two enantiomers was not significantly different, almost twice as much (+)-ABA was transported from the roots to shoots as (–)-ABA. This is consistent with the observation made by Rose et al. (1996), that (+)-ABA is a more effective antitranspirant (in wheat) than (–)-ABA.

In spruce, similar amounts of the two ABA enantiomers were delivered to the shoots, yet Fuchs (1998) and Fuchs et al. (1998) reported that spruce stomata are markedly more sensitive to exogenous application of (+)-ABA than (–)-ABA. Thus, the differences in bioactivity between the two enantiomers may have reflected differences in their rate of metabolism or the presence of two different binding sites for ABA in the guard cells (Walton, 1983).

Windsor et al. (1994) reported that (–)-ABA is absorbed more slowly than (+)-ABA into the cells of carrot suspension culture even though the two enantiomers compete equally for the "docking site" on the saturable uptake carriers of these cells. In my study, I did not observe any such preferential uptake of (+)ABA over (–)ABA in either wheat or spruce.

In both species, MeABA was less effective in bringing about stomatal closure than ABA. However, the differences in activity were more pronounced in spruce than in wheat. The lower biological activity of MeABA could have been due to its slow hydrolysis to ABA. In contrast to the observations of Jones and Mansfield (1971) that esterification of the carboxyl group of ABA increases its bioactivity, a number of studies have demonstrated that acids are generally more biologically active than their similarly substituted esters (Churchill et al., 1992; Fuchs et al., 1998; Walker-Simmons et al., 1994). The activity of the esters has been attributed to the hydrolytic release of ABA (Walton and Sondheimer, 1972). However, Churchill et al. (1992) argued that hydrolysis is not a general requirement for ester activity. Van Der Meulen et al. (1993) found no trace of ABA, formed through hydrolysis, in barley aleurone protoplast incubated with ABA esters. Differences in the metabolism of ABA and MeABA have been reported in sunflower leaves (Loveys and Milborrow, 1984); however, in most cases the metabolic fate of ABA analogues in plant tissue is unknown.

In a previous study (Kaul et al., 1997), we found that in spruce, high concentrations (10^{-3} M) of exogenously applied, unlabeled (±)-ABA did not reduce the uptake of tritiated (±)-[^{3}H]ABA. This suggests that the absorption of ABA into roots is not carrier mediated but occurs by passive diffusion. Thus, the methyl ester derivative of ABA, being less polar than ABA, should have a higher rate of diffusion across root membranes. The very high uptake of the methyl ester enantiomers into the spruce roots confirms that these esters are relatively easily transported across root membranes. My results show, however, that high root uptake of a given ABA enanantiomer or analogue does not necessarily translate into a correspondingly high delivery of the compound to the shoots. Further, as in the case of ABA, there might not be any correspondence between root uptake efficiency and biological activity.

Unfortunately, it was prohibitively expensive to synthesize a tritiated isopropyl ester so that I could not quantify its uptake and transport. However, the fact that in spruce iPrABA

was significantly more biologically more active than (±)-ABA, suggests that it was more readily taken up by the roots and transported to the shoots. This is consistent with it being a much more lipophilic molecule than ABA and thus being able to cross root membranes more rapidly by diffusion. In addition, it is possible that the rate of hydrolysis of iPrABA is lower than that of ABA and MeABA, and hence a greater amount reaches the shoots.

In contrast to spruce, in wheat, the biological activity of iPrABA was slightly lower than that of ABA but similar to the methyl ester analogue. This was likely due to the presence of membrane bound ABA carriers in the roots. ABA carriers have been characterized in herbaceous species (Astle and Rubery, 1980) and are specific for the impermeant ABA anion. Since esters are unable to form a carboxylate anion, they can not be transported via ABA carriers (Perras et al, 1997).

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CHAPTER 4

SUMMARY AND CONCLUSIONS

The purpose of the first part of the study was to determine whether differences in sensitivity between white spruce and wheat to exogenously applied ABA might be related to differences in (i) ABA uptake and distribution and/or (ii) their inherent sensitivity to ABA. This was done by measuring the uptake, distribution and biological activity of the exogenously applied ABA in both species.

A 200-fold higher concentration of exogenous applied (±)-ABA was required to close stomata in spruce than in wheat, but differences between the species in transport efficiency of ABA, while significant (typically 9-fold) were not large enough to account for the differences in stomatal sensitivity. The results suggest, therefore, that wheat stomata is inherently more sensitive to ABA than spruce. In both species, uptake efficiency, which was always higher than transport efficiency, was dramatically increased by the use of 0.5% DMSO and by increasing root temperature. However, the additional ABA that was taken up was sequestered in the roots and not transported to the shoots. Thus, transport efficiency declined. Uptake efficiency was independent of transpiration rates in both species. However, it was only in wheat that lower transpiration rates resulted in lesser amounts of ABA being transported to the shoots. Roots are significant barriers to ABA uptake in spruce and their removal brings about a 55-fold increase in the delivery of ABA to shoots and almost a 20-fold increase in sensitivity to exogenously applied ABA. Immunofluorescence labeling suggests that a major portion of exogenously applied ABA is sequestered around the root endodermis in spruce. This is in contrast to wheat, where a major portion of the exogenous ABA was located inside the root vascular tissue. Thus, the difference in sensitivity between the two species is primarily due to sequestration of exogenous ABA in spruce roots, but in part, due to the higher inherent sensitivity of wheat stomata to ABA concentration than spruce.

In the second part of the study, the techniques used in the first study were used to quantify uptake, distribution and bioactivity of ABA enantiomers and ABA analogues in white spruce and wheat seedlings. The objectives were to compare the bioactivity of ABA and ABA analogues and to investigate a possible relationship between uptake (and subsequent transport) and bioactivity of these compounds in the seedlings. The results obtained have confirmed that white spruce is less sensitive than wheat to exogenous application of (+)- and (–)-ABA and their respective methyl derivatives. In all cases, the uptake of the compounds tested was greater in spruce than in wheat. This did not translate into higher biological activity because, in spruce, at least 95 % of that taken up was sequestered in the roots and only very small amounts (typically 10 - 20 fold less than in wheat) were transported to the shoots. In spruce, sequestration might serve as an inactivation mechanism to reduce the pool of physiologically active ABA. This study points to major differences in the total uptake, distribution and bioactivity of ABA enantiomers and analogues between species. However, at this stage, little is known about the metabolic fate of these compounds in plant tissues.

In spruce, the biological activity of the isopropyl ester derivative of ABA (iPrABA) was higher than that of racemic ABA and a range of other analogues tested in the laboratory (Fuchs et al., 1998) and the field (Grossnickle et al., 1996). Thus, iPrABA might prove to be an effective antitranspirant in reforestation programs.

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APPENDIX A

Estimates of total projected leaf area for wheat and white spruce

Estimates of the total projected leaf area of the seedlings, used in the experiments, were based on a linear relationship established between the leaf area (A) and leaf dry weight (DW) for white spruce in Figure A. 1 and wheat in Figure A. 2. The total projected leaf area of the seedlings was measured with a LI-3100 leaf area meter (Li-Cor Inc., Lincoln, NE, USA). Dry weight of the tissue was measured after leaves had been dried for 36 hours using a vacuum freeze dryer. Six seedlings of each species were used to establish the relationship between leaf area and leaf dry weight.

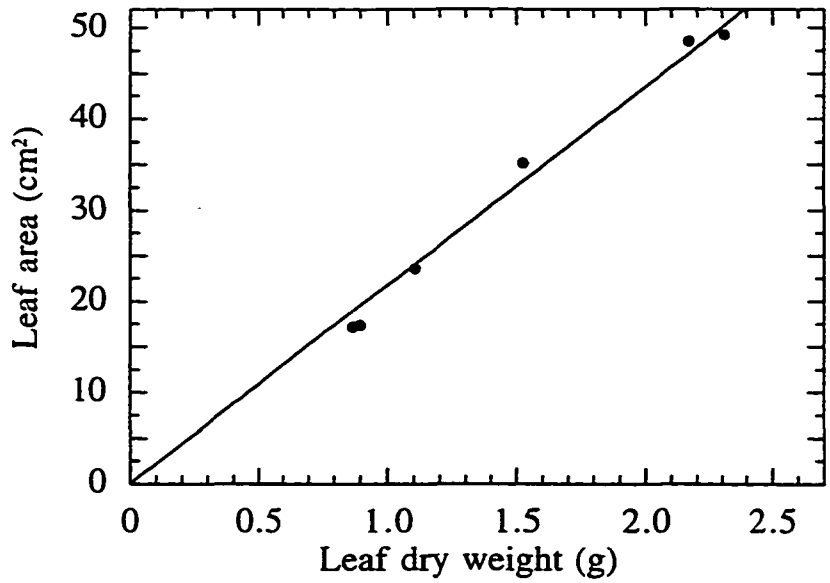

Figure A. 1 The relationship between the projected leaf area and leaf dry weight of white spruce needles: A = 21.7 * DW (r^2 = 0.98). The intercept of the regression line was forced through zero. Each data point represents one seedling.

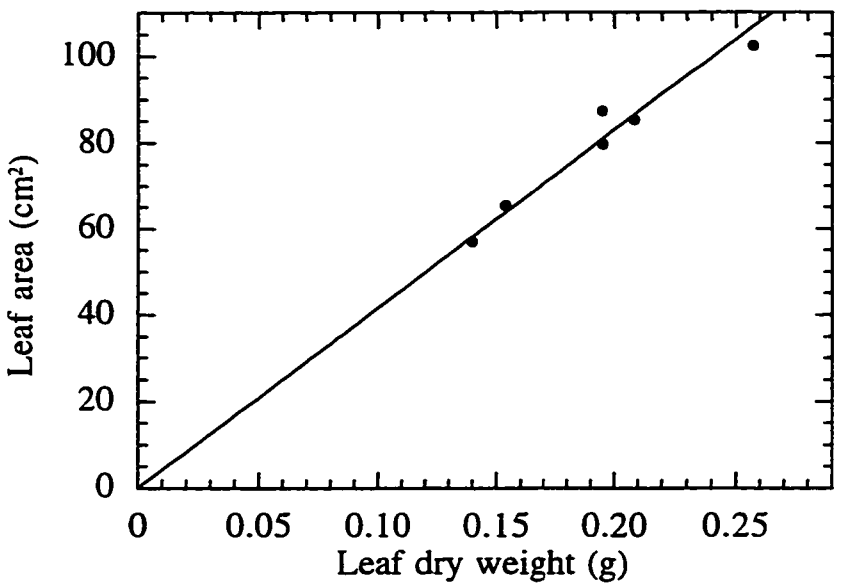

Figure A. 2 The relationship between the projected leaf area and leaf dry weight of wheat leaves: A = 414.3 * (DW) (r^2 = 0.96). The intercept of the regression line was forced through zero. Each data point represents one seedling.

APPENDIX B

Response of whole-plant transpiration rate to vapor pressure deficit

The stomatal humidity response curves for white spruce and wheat seedlings are presented in Figure B. 1. The responses of whole-plant transpiration rate (E) to changes in vapor pressure deficit (D) were determined by increasing D by 0.5 kPa every 2 h from 0.5 kPa to 2.0 kPa for white spruce and from 0.5 kPa to 1.5 kPa for wheat. The treatment was imposed 2 h after the lights were switched on, when stomatal conductance had reached steady state.

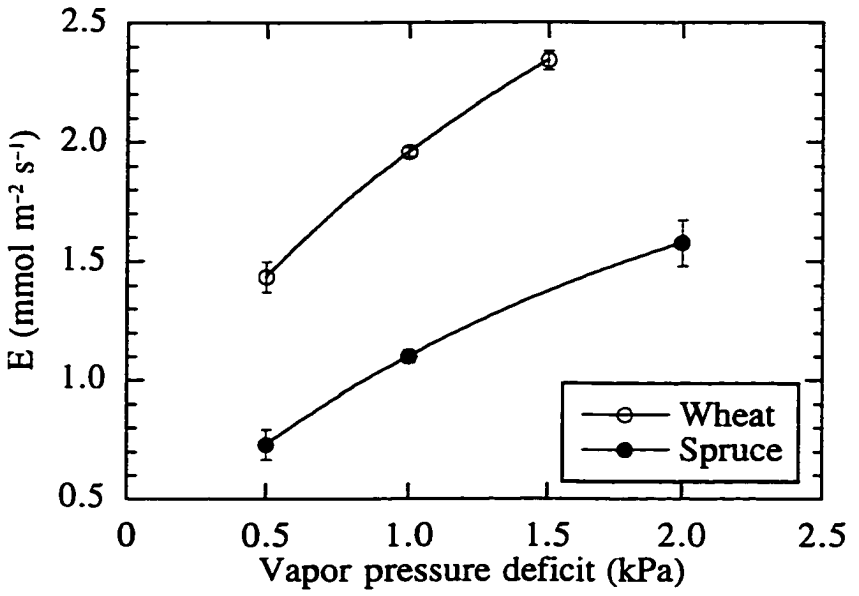

Figure B.1 Vapor pressure deficit (D) vs whole-plant transpiration rate (E) measured over the day. Each data point is the mean (±SD) of four seedlings. The photon flux density, air temperature and ambient CO_2 concentration were 1000 μmol m^{-2} s^{-1}, 25 °C and 350 μmol mol^{-1}, respectively.