The role of endogenous ABA and exogenously applied ABA and ABA analogs on the gas exchange of conifer seedlings

by

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ABSTRACT

Two main sets of experiments were conducted. The first set of experiments was conducted to determine the relative influence of root and leaf water status on stomatal conductance (g_c) in one-year-old Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) and 2-3 month old alder (*Alnus rubra* (Bong)) grown in drying soil. These results have been published and are presented in the appendix. The second set of experiments was conducted to; a) determine structure-function relationships of ABA analogs in white spruce (*Picea glauca* (Moench) Voss) and b) evaluate which analogs might assist conifer seedlings planted on reforestation sites maintain a favorable water balance and thereby improve seedling establishment.

The relative influence of root *vs* leaf water status on g_c was investigated by manipulating shoot water status independently of the roots by using a pressure chamber that enclosed the root system. Pressurizing the chamber increases the turgor of cells in the shoot but not in the roots. Seedling shoots were enclosed in a whole plant cuvette and transpiration and carbon assimilation rates measured continuously. In both species stomatal closure in response to soil drying was progressively reversed with increasing pressurization. Responses occurred within minutes of pressurization and almost immediately returned to pre-pressurization levels when the pressure was released. Even in wet soils, there was a significant increase in g_c with pressurization. In Douglas-fir, the stomatal response to pressurization was the same for seedlings grown in dry soils for up to 120 days as for those subjected to drought stress over 40 to 60 days. The stomatal conductance of both Douglas-fir and alder seedlings was less sensitive to root chamber

pressure at higher vapor pressure deficits (D) and stomatal closure in response to increasing D from 1.04 to 2.06 kPa was only partially reversed by pressurization. My results are in contrast to other studies on herbaceous species, even though I followed the same experimental approach. They suggest that it is not always appropriate to invoke a "feedforward" model of short-term stomatal response to soil drying, whereby chemical messengers from the roots bring about stomatal closure.

ABA analog structure-activity relationships were determined by testing an array of 19 different ABA analogs on 1-year -old clonal white spruce (*Picea glauca* (Moench) Voss) raised from somatic embryos. The contribution of specific structural features to analog activity was determined from the relative effect of aeroponically applied 10^{-3} M analog solutions on seedling gas exchange. Seedling transpiration and carbon assimilation rates were measured continuously during treatment by means of a whole plant cuvette system which enclosed the shoot. The analogs were racemic about the C-1′ chiral center and were derived from changes imposed on 6 regions of the ABA molecule. Changes were made in; the C-1 oxidation level (the acid functionality was changed to the ester, aldehyde and alcohol), the side chain bond order at C-4, C-5 (changed from a trans double bond to an acetylenic linkage), ring saturation (varied by replacing the C-2′, C-3′ ring double bond with a single bond or replacing the C-5′, C-6′ single bond with a double bond), the C-7′ methyl (fluorinated to form CHF_2), and at C-2 of the side chain (C-1 and C-2 were deleted and replaced with a ketone terminus. The activity of optically pure (+)-S-ABA and (-)-R-ABA were also determined. Activity was reduced by any change in the C-1 functionality and ring structure natural to ABA. The ring C-2′, C-3′ double bond was important but not essential to activity. The activity lost through changes in ring structure and C-1 functionally was, in many cases, almost fully restored by replacing the C-4, C-5 double bond with an acetylene bond. Therefore, acetylenic analogs were more active than their equivalents with a dienoic side chain overall. Fluorination of the C-7′ methyl caused

a relatively moderate reduction in analog activity. Truncation of C-1 and C-2 from the side chain reduced activity to near zero. Racemic ABA was as active as optically pure (+)-ABA but required more time to achieve its full effect. The unnatural, (-) ABA enantiomer was inactive. At least 5 of the analogs caused large reductions in spruce seedling transpiration rate and also improved seedling water use efficiency. This suggests that these analogs, if applied to seedlings prior to planting on reforestation sites, may reduce seedling water stress and increase seedling survival rate.

The reduction in transpiration rate in *Picea* in relation to the concentration of (+)-ABA applied to the roots was determined through a separate set of experiments. At least 10^{-3} M (±) ABA had to be applied to bring about significant stomatal closure. This contrasts with experiments in wheat (S. Kaul, University of Victoria, personal communication) in which application of 10^{-5} M (±) ABA closed stomata. [^{3}H]-ABA incorporation experiments performed on both wheat and spruce seedlings provided evidence that spruce is less responsive to exogenously applied ABA than wheat because ABA is absorbed into its shoot less efficiently and because spruce is inherently less sensitive to ABA.

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A	carbon assimilation rate (μmol m^{-2} s^{-1})
ABA	abscisic acid
AcOH	acetic acid
cpm	counts per minute (of radioactive decay)
C_a	ambient CO_2 concentration (μmol mol^{-1})
C_i	intercellular CO_2 concentration (μmol mol^{-1})
Ci	Curies (radioactivity)
D	water vapor pressure deficit
DW_l	leaf dry weight (g)
e_a	water vapor pressure (kPa)
E	transpiration rate (mol m^{-2} s^{-1})
EtOH	ethanol
g_c	stomatal conductance to carbon dioxide (mol m^{-2} s^{-1})
g_s	stomatal conductance to water vapor (mol m^{-2} s^{-1})
I.D.	internal diameter
J_{ABA}	flux of ABA (μmol m^{-2} s^{-1})
L	projected leaf area (m^2)
M	molar (moles l^{-1})
MeOH	methanol
NPK	nitrogen, phosphorus, potassium fertilizer
O.D.	outer diameter
P	leaf turgor (MPa)
pH	-log (a_{H+})
P_n	net photosynthesis rate (μmol m^{-2} s^{-1})
ppm	parts per million
Q	photosynthetic photon flux density (400-700 nm) (μmol m^{-2} s^{-1})
SPE	solid phase extraction
v/v	volume per volume (dimensionless)
WUE	water use efficiency (mol-$mmol^{-1}$)

θ_v	Volumetric soil water content
Ψ or ψ	water potential (MPa)
Ψ_l or ψ_l	leaf water potential (MPa)

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I came to Nigel Livingston's lab at an early phase when there were more ideas than equipment to go around. In time, as equipment arrived and the experiments began to get under way, an infectious energy and enthusiasm took hold. This made working in the lab a pleasure. There was very much a feeling of being involved and of doing something constructive with your time and I thank Nigel for the opportunity to be part of this. I also want to thank for him for his strong personal support which enabled me to see this work through. He is without a doubt one of the finest men I know.

I also had the further the advantage of exceptional lab mates (Steeve Pepin, Greg Filek, Robin Percy, Erin Linger, Wendy Bennink & Sun Zhongia) to whom I also owe a debt of gratitude. In particular I want to thank Steeve and Greg. I preyed heavily on Steeve's time over the years and found him not only exceptionally capable but also totally unstinting with his time. There is no one in the lab who has not been a recipient of Steeve's good will and help at one time or another. I want to thank Greg for showing me how to build and how to sail. I have always felt a little like Greg's apprentice. I'll never have his flair for invention and building, but as a result of working around him, I am less intimidated by tasks that previously boggled me.

Also, my thanks to those of my cohort who variously served as sage and not-so-sage council over the years; Mungo Marsden, Mary Powers, John Dower, Barry Milligan, Irene Hay, Donald Skinner, Derek Harrison, Mark Deeley, Niamh Kinsella and the crazies in biochemistry (which includes most of the biochemists). Finally, to Erica, for jump starting my write up, my deepest thanks.

DEDICATION

To my mother, Zita and my father, Theodore (1918-1994)

CHAPTER 1

Literature Review

1.1 Introduction

Plants are affected by many stresses including drought, flooding, salinity, chilling, high temperature, soil compaction and inadequate nutrition (Taiz & Zeiger 1991). While different forms of stress affect plant physiology through different processes, all have a number of common symptoms and consequences including reductions in gas exchange and carbon assimilation which contribute to reduced biomass and high mortality. Compared to other forms of stress, drought has the greatest impact on agricultural production and imposes the largest limitation on crop geographic range (Boyer 1982).

During photosynthesis, most terrestrial plants transpire large amounts of water to the atmosphere. Depletion of soil water reserves through transpiration has been seen as an unfortunate consequence of having to expose moist, water laden tissues to a relatively dry atmosphere to fix carbon (Kramer 1983). However, transpiration is not entirely a liability. It has been argued that transpiration improves plant performance by enhancing processes such as the absorption of soil minerals (Clements 1934) and the transport of nutrients and hormones within the plant (Kramer 1983). Evaporative cooling caused by transpiration also results in more favorable leaf temperatures which prevents heat injury (Gates, 1968) and simultaneously increases net photosynthesis.

The leaves of terrestrial plants are typically covered with a layer of hydrophobic epicuticular wax which reduces transpiration. Therefore, most water transpired through leaves (80 - 90 %) escapes through open stomatal pores. Since the size of the stomatal pore can be modulated and reduced to zero under certain conditions, stomata provide the

principal means of regulating water movement through the plant and curtailing losses to the atmosphere. The importance of water to production and of stomatal behavior in preserving water status has underscored efforts aimed at elucidating stomatal responses to drought.

1.2 Models of stomatal control and the controversy over whether roots or shoots are the primary sensors of drought.

Early models of stomatal control contended that changes in stomatal aperture were mediated hydraulically as a function of leaf water status (Nimah and Hanks 1973; Hillel *et al.* 1976). According to these hydraulic models, soil water depletion results in increased resistance to water flow in the soil and roots. In turn, shoot water supply is reduced, creating a shoot water deficit which manifests itself as a decrease in leaf turgor (P). Loss of turgor causes stomatal closure resulting in a reduction in transpiration rate (E) (Turner, 1974; Kramer 1983). Another tenet of the model is that shoots are the primary sensors of drought. This follows from the fact that the atmosphere is virtually always drier than the soil. As a result, shoot water deficits precede those that eventually develop in roots as the soil becomes progressively drier. Shoots then, experience drought first and induce a stomatal response before drought is even perceived in the roots (Kramer 1988). The view that shoots are the primary sensors of drought is supported by observations of shoot stress, apparent during mid-day wilt, that precedes onset of any perceivable root water deficits (Laker *et al.* 1987).

Evidence gathered from drought studies over the last 15 years indicates that stomatal conductance (g_s), a measure of diffusive gas resistance through the stomatal pore and a function of stomatal aperture, is not invariably determined by leaf water potential (Ψ_l) and in some instances may be more closely associated with root water status. Results from experiments on field grown plants which displayed an isohydric response to drought

suggested the possibility that root conditions might regulate stomatal behaviour. An isohydric response is characterized by stomatal closure during the initial phases of drought that results in reduced transpiration rates and the maintenance of Ψ_l at levels characteristic of a well watered plant. While isohydric behavior was reported decades ago (Berger-Landefelt 1936; Stocker 1956) the implications of these findings were not appreciated until recently. Bates and Hall (1981) hypothesized that stomatal closure in cowpea, despite high Ψ_l, was the result of some form of root-sourced signal. The possibility that root to shoot signaling mediates the response to drought was suggested earlier by Jones (1980) and Blake and Ferrell (1977).

The results of Bates and Hall (1981) were supported by Jones (1985) who noted g_s decreased although Ψ_l in droughted apple trees exceeded that in controls, even after 10 weeks of drought. Turner *et al.* (1985) reported that g_s changed consistently only with soil water status and not Ψ_l which was manipulated by different humidity regimes imposed on the shoot. Henson *et al.* (1989) found that g_s decreased in droughted lupins prior to any notable change in Ψ_l and suggested a local reduction in water potential in the epidermis might account for a corresponding decrease in g_s. They argued that standard measurements of Ψ_l using psychrometry only assess bulk Ψ_l , so that differences in potential between the epidermis and mesophyll would not be detected. Further, the epidermal potential can easily drop below bulk leaf levels if hydraulic contact with the underlying mesophyll is poor, a condition noted by Turner (1989) in some woody species. For example, Shackel and Brinkmann (1985) demonstrated that transpiration affected epidermal turgor in *Trandescantia* more than mesophyll or bulk leaf turgor. Whether such localized changes in the water potential of tissues within the leaf has a significant role in regulating stomata as soil water deficits increase remains unresolved.

During soil drying, reduced shoot growth rate often accompanies the decline in g_s (Davies and Sharp 1981; Jones 1983; Blackman and Davies 1985) suggesting a common

control mechanism for both shoot processes. Although it is linked to soil water status, the control mechanism is seen as non-hydraulic since reductions in shoot growth rate and g_s are effected independently of loss of shoot water status (Schulze 1986). Evidence that non-hydraulic root signals affect g_s and shoot growth has been gained through two types of root manipulation experiment; 1) split root experiments whereby roots of a single plant are divided vertically across an impermeable barrier and shoot water status is preserved by supplying water to roots on one side of the barrier (Blackman and Davies 1985; Zhang and Davies 1987; Khalil and Grace 1993) 2) root pressurization experiments whereby the root system is enclosed in a pressure chamber and shoot water status is sustained by applying a compensating pressure to the roots which counters the increase in soil suction as the soil dries (Gollan *et al.* 1986; Passioura 1987; Schurr *et al.* 1992). Both forms of root manipulation have shown that g_s decreases as roots experience drying soil conditions despite maintenance of leaf water status at non-stress levels.

Zhang and Davies (1987) found that increased concentrations of the phytohormone abscisic acid (ABA) in root tissue and in leaf epidermis coincided with soil drying and decreased g_s. They concluded that ABA of root origin is increased by soil drying and may induce stomatal closure and possibly act as a mediator of a drought response involving root to shoot communication. This view was supported by Schulze (1986), who stated that stomatal closure prior to wilting was the result of a root sourced hormone. However, Kramer (1988) rejected these interpretations and suggested the experimental conditions gave rise to misleading, non-representative results. He argued that although it is possible to create scenarios in which roots are stressed in advance of shoots, resulting in production of hormones which alter shoot responses (growth and g_s), these results do not reflect natural field conditions and cannot be extrapolated to the field. Nonetheless, in unwatered field grown maize, Zhang and Davies (1989) found an early drop in turgor and water potential of fine secondary roots in the upper soil horizon but no attendant change in leaf water status. Deeper roots maintained turgor throughout the experiment and continued to supply water to the shoot. Further, a progressive decrease in

g_s was noted two weeks prior to any noted decrease in Ψ_l. Thus in a plausible field situation; 1) portions of the root system nearer the surface exhibited symptoms of stress before shoots and deeper roots and 2) shoot growth and stomatal conductance were inhibited well in advance of any apparent decline in shoot water status. Passioura (1988) contends that in the field, some portion of a root system will be in dry soil and that Zhang and Davies' (1987) observations are a better portrayal of the normal circumstance of plants in the field than Kramer's (1988) whereby shoots wilt in the face of high evaporative demand even though roots are adequately supplied with water. According to Passioura (1988), the argument suggested by evidence from root manipulation experiments is not that shoot water status is irrelevant to shoot behaviour, rather, it is that in circumstances where part of the root system is water stressed, root sourced signals have the potential to override shoot effects on such important aspects of shoot physiology as growth and stomatal conductance.

An alternative to the view that stomatal behaviour is independent of Ψ_l is suggested by recent pressure chamber experiments on woody species. In contrast to the experiments on herbaceous species which show that g_s reduced by soil drying is only slightly increased by root pressurization (Gollan *et al.* 1986; Schurr *et al.* 1992), the equivalent experiments performed on woody species show that full or partial stomatal re-opening occurs within minutes of root pressurization (Saliendra *et al.* 1995; Fuchs and Livingston 1996). These experiments also show that stomatal closure occurs immediately after pressure inside the chamber is released. The difference in the response to pressurization by non-herbs versus herbs suggests that there may be a fundamental difference in the way that stomata are controlled in woody species compared to herbaceous species. Schulze (1991) has argued that the long transport time required for a chemical signal propagated from the roots to reach the shoots in large trees suggests that in the short term, stomata are more likely to be regulated by hydraulic influences.

However, there is some evidence that hydraulic signals may be the dominant influence in stomatal regulation in woody species in general, independant of size. For instance, Saliendra et al. (1995) cite some unpublished data (J.P. Comstock) on the small desert shrub, *Hymenoclea salsola*, in which stomata opened in response to soil pressurizing.

The results of Whitehead *et al.* (1996) also provide evidence for stomatal control by means of a rapidly propagated hydraulic signal. This study is noteable because the stomatal effects seen were not achieved by root pressurization but by hydraulic shock caused by varying the proportion of foliage under shade. Whitehead *et al.* (1996) found an almost immediate increase in g_c (stomatal conductance to CO2) and A (carbon assimilation rate) occurred in the upper foliage upon shading the lower foliage of a 7 year old tree of *Pinus radiata* (a woody coniferous species) The similarity of these findings to those obtained from root pressurization experiments counters possible arguments that the results achieved by root pressurization are a laboratory artifact.

The root pressurization experiments conducted by Saliendra et al. (1995) (referred to above) were performed on *Betula occidentalis*, an isohydric species. Their experiments demonstrated that bulk Ψ_l did not increase despite the influx of water into the shoot upon root pressurization presumably because, the increase in g_s and E that accompanied pressurization compensated for the additional H_2O supplied and averted such an increase. They concluded; 1) an increase in g_s can occur without a detectable change in bulk Ψ_l and therefore, 2) isohydric behaviour, which has been accepted as evidence for root versus shoot signalling, is not definitive proof of stomatal regulation by root influences.

1.3 Does abscisic acid mediate root to shoot communication ?

The arguments outlined above indicate that there is still controversy surrounding the extent of the role chemical messengers play in stomatal control during plant water deficits. The relative influence of different chemical messengers seen as potential contributors to stomatal control also is not entirely clear; but it is evident that in most cases abscisic acid (ABA) has the most significant role in root to shoot communication and in coordinating other aspects of the drought response (Zeevaart and Creelman 1988; Hartung and Davies 1992).

Abscisic acid was co-discovered in the 1960's by Wareing and Addicott. Besides its putative role in stomatal control, it has been implicated in leaf abscission (seasonal and flood induced), as well as seed and bud dormancy (see Addicott 1983). Chemically, it is a C-15 sesquiterpenoid derived from a mevalonate precursor via farnesyl pyrophosphate directly (Addicott 1983) , and indirectly via an alternate carotenoid based pathway which produces the immediate aldehyde precursor (Parry and Horgan 1991). The occurrence of ABA is pervasive; it is found in all higher plants surveyed to date (Zeevaart and Creelman 1988) as well as certain algae (Tietz and Kasprik 1986) and in several phytopathogenic fungi (Dörffling *et al.* 1984)

The role of ABA in stomatal regulation was suggested by exogenous applications of ABA which caused stomatal closure (Tucker and Mansfield 1971). Supporting evidence came from studies of mutant *flacca* strain tomato plants. *Flacca* plants are prone to early wilt during mild drought due to poor stomatal control. This is associated with low levels of endogenous ABA (Tal and Imber 1970). ABA applied exogenously caused the wild type stomatal response in mutants and proved that the stomata were capable of closing normally. The results indicated poor stomatal control resulted from inadequate

ABA production and not because of dysfunctional stomata. Studies have since revealed that *flacca* and *sitiens* mutants are deficient in the last synthetic step of ABA production (Taylor *et al.* 1988).

Mounting correlative evidence also indicates that ABA is active in stomatal regulation. Many laboratory studies have shown that increased ABA in the roots, xylem sap and epidermis coincides with soil drying and correlates negatively with stomatal conductance (Zhang and Davies 1987; Zhang and Davies 1989; Neales *et al.* 1989; Khalil and Grace 1993; Gowing *et al.* 1993). Similar determinations have been made in several large scale field studies involving maize (Wartinger *et al.* 1990; Tardieu *et al.* 1991; Tardieu *et al.* 1992b)

ABA synthesis was first detected in leaves (Wright and Hiron 1969). More recently, it has been determined that ABA can also be synthesized in roots. Walton *et al.* (1976) showed that excised root segments exposed to dry air had increased ABA content. Zhang and Davies (1987) found that ABA increased in root tips as turgor decreased. Cornish and Zeevaart (1985) experimented with whole plants instead of isolated root segments. They determined root ABA content increased when roots were water stressed by exposure to a stream of dry air. By steam girdling plant stems to prevent ABA transport from leaves to roots they proved that increased root ABA resulted from *de novo* ABA synthesis in roots.

The correlation between increases in root and shoot ABA content was not immediately accepted as proof of root to shoot communication since increases in leaf ABA might also be explained by local synthesis. Root to shoot signaling requires that ABA be transported from roots to shoots. Zhang and Davies (1987) suggested that ABA is moved in the xylem sap during transpiration. Additional experiments verified that ABA loaded into roots in the dark subsequently accumulated only in transpiring leaves.

ABA concentration did not increase in shaded, non-transpiring leaves. Hartung (1983) and Hornberg and Weiler (1984) determined that ABA produced in the roots and carried in the transpiration stream arrived directly at the ABA action site on the stomatal guard cell plasmalemma.

Zhang and Davies (1990) investigated whether increased xylem [ABA] could fully account for drought induced stomatal closure and found that increased xylem [ABA] brought about by exogenous application of ABA or soil drying resulted in similar reductions in g_s. Their results indicated that stomatal closure was mediated entirely through ABA. Similar observations were made by Tardieu *et al.* (1993) who manipulated xylem [ABA] by soil drying and feeding ABA through the stem instead of roots. Zhang and Davies speculated that xylem [ABA] acts as integrated signal provided by the entire root system and is a good indicator of soil water availability. Tardieu *et al.* (1992a) found a close relationship between pre-dawn water potential and xylem ABA concentrations in non-transpiring plants, corroborating the view that xylem ABA concentration is related to soil water availability.

1.4 Chemical vs hydraulic signals in regulation of shoot growth.

It is generally acknowledged that leaves must maintain a positive turgor to grow and that diminished turgor results in reduced growth (Bradford and Hsiao 1982; Kramer 1983). Observations that show reduced leaf growth with soil drying and without a drop in leaf turgor suggest that growth is inhibited by increased xylem ABA (Passioura 1988; Saab and Sharp 1989; Gowing *et al.* 1990; Zhang and Davies 1990)

Chazen and Neumann (1994) concluded that the primary event in leaf growth inhibition is a mechanical restriction caused by cell wall hardening that is initiated by a hydraulic signal from the roots. They found that this hardening process was evident within 2 minutes of imposing water deficit on maize seedlings by immersing roots in a non-permeating osmoticant, even when roots were freeze killed prior to exposure. Freeze killing roots eliminated the possibility of root hormonal or electrical signals. However, the authors suggested a role for non-hydraulic controls in preserving wall hardening and growth inhibition with persistence of water stress over a period of days. Nonami and Boyer (1993) also indicated that the primary event in growth inhibition might have a hydraulic basis. Earlier work indicated that a growth induced gradient in water potential from the xylem to enlarging tissues enables water uptake into growing cells (Westgate and Boyer, 1985). The water potential gradient apparently originates with cell enlargement that reduces turgor. Elevated turgor normally balances the influx of water in response to increased cell solute content. The reduction in cell water potential that results from turgor reduction results in an influx of water from the apoplast and gives rise to a negative tension which ultimately draws water from the xylem. Nonami and Boyer (1993) suggested that growth would be arrested within one minute if the water potential in the xylem dropped sufficiently to cause an inversion in the water potential gradient which is normally downward from epidermis to xylem. Such an inversion would immediately block the transfer of water from the xylem to enlarging tissue and stop growth. Thus, their results suggest that initially, growth might be inhibited solely as a result of arrested water supply preceding cell wall hardening. Marcelo and Matthews (1994) determined that in *Begonia* leaves, water deficit imposed by withholding water actually increased extensibility of cell walls so that growth could persist under reduced turgor, a result in

direct contrast with that of Chazen and Neumann (1994). The disparity might reflect a difference in responses between species or differences in methodology.

The growth inhibitory effect of ABA might result from its effect on cell walls. Van Volkenburgh and Davies (1983) reported that ABA decreased cell wall extensibility, possibly by inhibiting proton secretion across the plasmalemma into the apoplast (Chen and Kao, 1988) which reduces apoplast acidity and cell wall loosening reactions dependent on acid conditions (Van Volkenburgh and Davies 1983). Reduced cell wall loosening in turn prevents wall stretching and arrests leaf growth.

1.5 Effects of ABA on root growth.

In contrast to its inhibitory action in shoots, in roots, ABA acts to maintain growth and water uptake during soil water deficits. Tal and Nevo (1973) suggested that ABA might aid water uptake as the soil dries by increasing root hydraulic conductance. However, Markhart *et al.* (1979) subsequently demonstrated that exogenous ABA actually decreases root hydraulic conductance. Despite this effect, Fiscus (1981) found that ABA increased volume flow into roots at low flow rates by stimulating ion accumulation in the roots which increased the driving force for water uptake. Davies *et al.* (1982) made similar determinations at low flow rates but at higher rates found ABA did not promote further increases in flow. They suggested that high flow diluted ABA induced ion accumulations thereby dissipating the driving force responsible for flow increases at a low flow rate. Since ABA failed to increase flow into roots under conditions of high transpirational demand, when the need for water is arguably the most urgent, Davies *et al.* (1982) deduced this was unlikely to be the role of ABA in the root and

proposed instead that ABA acts principally to maintain root turgor and growth by stimulating osmotic adjustment. Continued root growth in turn, enables roots to penetrate dry soil and access lower, wetter soil horizons. Root growth is further promoted by ABA induced shoot inhibition which diverts photosynthate to the roots.

ABA also aids roots in soil penetration through its effects on root morphology. Changes resulting from ABA exposure include an increased root cortical diameter (Robertson *et al.* 1990) and an increased number of root hairs (Biddington and Dearman 1982). Root hairs provide anchorage which assists the root in penetrating drying soil (Russell 1977). In compacted soil with high mechanical impedance, roots display the same morphological changes induced by ABA (Hartung *et al.*1994). Masle and Passioura (1987) found that wet soil with high mechanical impendance restricted shoot growth more than root growth even though leaf potential was maintained. They suggested that root perception of soil resistance rather than water content might be the stimulus for root ABA synthesis. Tardieu *et al.* (1991, 1992b) also found that maize grown in the field in compacted soil with a high water content had increased xylem ABA concentration and reduced shoot conductance. The responses they observed were consistent with root water deficits resulting in ABA production. However, Tardieu *et al.* (1992a) found that soil compaction promoted root clumping and that even moderately clumped roots developed water potentials as much as 1.0 MPa below those in the relatively well watered surrounding soil. They concluded that high soil mechanical impedance does not stimulate root ABA synthesis directly but acts indirectly by promoting root clumping that creates root water deficits.

1.6 Is root to shoot signalling during drought mediated exclusively by ABA?

The ability of plants to synthesize ABA in response to water deficits and the suite of effects ABA has on shoot growth, stomatal conductance, and root turgor and growth has led to the view that ABA is the preeminent drought stress hormone (Hartung and Davies 1992). On the genetic level this view is supported by findings that show a strong correlation between the pattern of gene expression elicited by drought compared to that elicited by exogenously applied ABA (Bray 1988) suggesting that ABA mediates many of the effects of drought.

However, several studies have indicated that other compounds such as cytokinins play an active role in stomatal regulation during drought. Blackman and Davies (1983) determined that soil water deficits resulted in a root supply of cytokinins to shoots. They speculated that a continuous supply of cytokinins may be necessary to sustain maximal stomatal opening. Itai and Vaadia (1965) suggested that a nitrogen deficiency resulting from soil drying could bring about a decline in cytokinins in the xylem since nitrogen is required for cytokinin synthesis. Davies *et al.* (1987) pointed out that the relationship between cytokinin concentration and stomatal aperture is log-linear. He concluded the few percent decrease in cytokinin concentration noted during soil drying would not be sufficient to induce a decline in stomatal conductance consistent with experimental results. Gowing *et al.* (1990) performed a standard split root experiment whereby a single root system was separated into dry and wet soil compartments. They found that severing the roots growing in dry soil restored leaf growth and g_s. They reasoned that if reduced cytokinin supply from the roots caused stomatal closure, then root severance should have further reduced cytokinin supply, in which case, stomata would have remained closed. Therefore it is unlikely cytokinins modulate stomatal

behaviour , atleast during drought (Gowing *et al.* 1993). Masia *et al.* (1994) imposed a gradual drying regime on *Helianthus* roots but did not find consistent differences in root cytokinin concentrations between controls and treatment plants that accounted for stomatal behavior.

In water stressed wheat, Munns and King (1988) observed that the xylem [ABA] was two orders of magnitude lower than that of maize that had the same leaf conductance. They concluded the xylem [ABA] in wheat was inadequate to account for such low leaf conductance and speculated a novel, auxiliary antitranspirant was active. Their hypothesis was supported by their finding that immunocolumn removal of ABA from xylem sap did not fully eliminate its antitranspirant activity (Munns 1990) In contrast, a detached leaf transpiration assay of wheat leaves by Zhang and Davies (1990) showed that an artificial sap and xylem sap from a drought stressed maize plant with equal ABA contents reduced transpiration equally. They concluded that xylem [ABA] fully accounted for the antitranspirant activity that they observed.

1.7 The role of leaf ABA in stomatal function.

While it is well established that roots produce ABA in response to stress (Davies and Zhang 1991), leaf tissues also produce ABA (Pierce and Raschke 1980) and it is not certain that ABA from roots accounts for observed stomatal behaviour. Loveys (1977) demonstrated that the leaves of well watered plants contain significant amounts of ABA, yet maintain open stomata. ABA inactivity is explained by sequestration. Although ABA synthesis and degradation occurs in mesophyll cell cytoplasm (Hartung *et al.* 1980; Kaiser *et al.* 1985) ABA collects in the alkaline environment of the chloroplast stroma

(Cowan *et al.* 1982) and is effectively isolated from its action site, the apoplast and plasmalemma of the stomatal guard cell (Hartung 1983). ABA is trapped in alkaline compartments because alkaline conditions favor formation of the anion which cannot permeate membranes. Therefore, when permeant, uncharged ABA crosses a membrane, anion formation prevents back diffusion, resulting in increased ABA in alkaline regions and a corresponding depletion from acidic compartments such as the apoplast (Hartung *et al.* 1982).

Increased leaf water deficits brings about a release of chloroplastic ABA (Cowan *et al.* 1982) and simultaneously reduces the pH gradient between the cytosol and apoplast which becomes alkaline. As a result ABA concentrates in the apoplast adjacent to the active site in the plasmalemma and stomata close (Hartung *et al.* 1988). An increase in xylem sap pH resulting from soil dehydration may enhance ABA redistribution to the apoplast by; a) increasing apoplast alkalinity (Gollan *et al.* 1992) and b) increasing the tendency for root synthesized ABA to diffuse into the xylem and be conveyed to the shoot (Schurr *et al.* 1992). However, while redistribution of leaf ABA reserves may close stomata initially, maintained closure requires additional ABA to counter ABA removal from the apoplast by carrier uptake into the cytosol, followed by conjugation or catabolism which render it inactive (Hartung *et al.* 1990). The additional ABA might be supplied by the roots and would provide a reinforcing signal for stomatal closure. In cases where water deficits result in significant drops in leaf turgor, ABA synthesized in the leaf may be also be contributed. Pierce and Raschke (1980) demonstrated that leaf ABA concentration rose substantially when turgor approached zero suggesting that turgor loss stimulates ABA biosynthesis.

1.8 Stomata show varying sensitivity to ABA

Several studies have shown a poor correspondence between endogenous ABA levels and stomatal behavior (Beardsall and Cohen 1975; Burschka *et al.* 1983; Trejo and Davies 1991). In the latter study, *Phaseolus vulgaris* grown in a drying soil column displayed reduced g_s prior to a detectable drop in Ψ_l or increase in xylem [ABA]. Amongst other explanations (redistribution of leaf ABA, non-ABA anti-transpirants) the authors speculated that *Phaseolus* may be inherently sensitive to small changes in ABA concentration which it detects against a relatively high background level of ABA. This suggestion follows from assays that have shown that cultivars of *Phaseolus* maintain xylem and root ABA at concentrations ten times higher than those found in maize with an equivalent water status. Also, water deficits increase xylem [ABA] concentration only 2-5 fold in *Phaseolus* while in maize, 100 fold changes are typical. Therefore, it is conceivable that if species have a high sensitivity to small changes in ABA, stomatal closure may be initiated prior to a detectable increase in xylem [ABA] (Slovik and Hartung, 1992).

It is quite likely that different species vary in their natural sensitivity to ABA. Different stomatal sensitivities were evident in apple and cherry (Kim *et al.* 1984) Quarrie & Lister (1983) also noted that stomatal sensitivity to exogenously applied ABA varied widely even between different genotypes of wheat. Stomatal sensitivity also appears to vary within individual plants according to physiological conditions. In particular, Ψ_l appears to affect the responsiveness of stomata to xylem [ABA] (Tardieu and Davies 1992). For instance, Tardieu *et al.* (1992b) found that comparable xylem [ABA] produced lower g_s in plants with low leaf water potential. As a result they suggested that leaf water status may alter stomatal sensitivity to the chemical message.

Supporting evidence came from Schurr *et al.* (1992) who found that when Ψ_l was maintained by root pressurization in a pressure chamber, g_s decreased by only 20 % despite high xylem [ABA]. Also, Tardieu *et al.* (1993) determined xylem [ABA] had less influence on g_s when Ψ_l was maintained through feeding an ABA solution into the xylem stream despite high evaporative demand as

Recognition that Ψ_l could modulate a stomatal response to a chemical signal led Tardieu and Davies (1993) to propose a stomatal control model that integrates hydraulic and chemical influences. They formulated a plausible stomatal control system based on the following premises; 1) that the chemical message (xylem [ABA]) emanating from the roots varies directly with root water potential and reciprocally with the water flux through the roots 2) that g_s resulting from the message depends on Ψ_l such that;

$$g_s = g_{s\,min} + \alpha \exp\{[ABA]\beta\exp(\delta\Psi_l)\} \qquad [1]$$

where $g_{s\,min}$ is the minimum stomatal conductance, $(g_{s\,min} + \alpha)$ is the maximal stomatal conductance and β and δ are fitted parameters derived from a field data set and Ψ_l serves as a sensitivity factor to xylem [ABA]. Tests of the model on an independent field data set, accurately predicted diurnal stability of the xylem [ABA] signal. Subsequent simulations in which the effect of Ψ_l on ABA perception was discounted indicated that the role of chemical signals acting alone was marginal and lead to perilously low Ψ_l even in very dry soil. Conversely, an assumption of exclusively hydraulic control demonstrated reasonable regulation of Ψ_l, achieved via a feedback system involving changes in g_s. However, Tardieu and Davies (1993) pointed out that this control system is equally unlikely since comparable levels of Ψ_l are observed for different values of g_s. A viable feedback system would demand a stricter correspondence between these

parameters. The integrated model predicts that dynamic stomatal control is achieved through changes in leaf sensitivity to ABA since quick stomatal responses cannot be accounted for by changes in xylem [ABA] which is stable throughout the day. A typical response to a change in evaporative demand then, would be a change in Ψ_l that alters stomatal sensitivity to the current xylem [ABA].

Tardieu and Davies also postulated that a xylem [ABA] which increases slowly over days, but is relatively stable on a daily basis is suited to control of developmental responses to drought, such as changing root to shoot ratio. They reasoned that a steady message that is resistant to large fluctuations would provide more reliable information on soil water reserves and resistances to water transfer, and is therefore better suited to proper regulation of long-term morphological changes.

Recently, Trejo *et al.* (1993) found that the physiological basis of changes in leaf sensitivity to xylem [ABA] may reside with mesophyll ABA metabolism. This connection was suggested by experiments which showed that stomata of isolated leaf epidermis responded to much lower ABA concentrations than stomata of intact leaf pieces when each was floated on ABA solutions. Further, sensitivity to ABA increased in intact leaf segments if they were first incorporated with tetcyclacis, an inhibitor of ABA degradation to phaseic acid. Together these facts indicate that enhanced ABA sensitivity, at least in part, is a function of reduced ABA catabolism which leads to the accumulation of ABA in the mesophyll and subsequently in the epidermis. Activity of enzymes involved in mesophyll ABA metabolism is a function of leaf water status (see Zeevaart and Creelman 1988). Therefore, it follows from the preceding discussion, that sensitivity to xylem [ABA] depends on leaf potential which substantiates the integrated model of stomatal control deduced empirically by Tardieu and Davies (given above).

The mass of ABA delivered to the leaf varies as a function of the xylem [ABA] and the transpiration rate. Trejo *et al.* (1995) pointed out that transpiration rates are held constant in many experiments that examine the relationship between xylem [ABA] and stomatal behavior. Therefore, only the effects of increasing xylem [ABA] on stomatal behavior are considered. Typically, in the field, the vapor pressure deficit increases during the day resulting in increased leaf transpiration rates. Since, xylem [ABA] is relatively constant during the day (Tardieu and Davies 1992) the flux of ABA (J_{ABA}) into the leaf should rise with the increase in transpiration rates (Trejo *et al.* 1995). Lately, there has been interest in the relative influence of changes in J_{ABA} *vs* changes in xylem [ABA] on stomatal behavior and how an ABA signal from the roots may be "read" by stomata. Gowing *et al.* (1993) addressed this issue by pulsing varying concentrations of ABA into cherry leaves for different durations and found both xylem [ABA] and the mass of ABA delivered had significant effects on the stomatal response. Different results were found by Trejo *et al.* (1995). They observed that the stomata of whole leaves of *Phaseolus acutifolius* were unresponsive to increases in J_{ABA} at 3 of 4 of the ABA concentrations tested. Only the last ABA solution, which had an abnormally high concentration, brought about a slight reduction in g_s. By contrast, increasing the concentration of the ABA solution (10-fold increases) fed into the leaf independently of J_{ABA} caused significant drops in g_s. Therefore, whole leaves were responsive to changes in xylem [ABA] but not changes of J_{ABA} over the range tested. When isolated epidermal strips were tested, their stomata responded to changes in both J_{ABA} and [ABA]. The response to [ABA] was particularly sensitive. An ABA concentration typical of the xylem of a well watered plant, induced a 50-60 % stomatal closure in epidermal strips. The authors reasoned that leaves must avert stomatal closure under well watered conditions through processes in the leaf mesophyll, including ABA catabolism,

sequestration and conjugation, which attenuate ABA sensitivity in whole leaves by restricting ABA access to the guard cells. Further, the same mesophyll ABA processes seemingly buffer changes in J_{ABA} more effectively than changes in xylem [ABA]. Consequently, measures of changing xylem [ABA] have higher physiological relevance since accumulation of ABA in the epidermis and ultimately stomatal behavior shows a closer dependence on xylem [ABA] than on J_{ABA}.

1.9 Theories of stomatal control based on mesophyll capacity for carbon fixation.

Trejo *et al.* (1995) suggested the many observations that show correlations between stomatal behavior and photosynthesis might be explained by a reduction in mesophyll activity. Typically, mesophyll activity is defined in terms of the capacity to fix carbon (photosynthetic rate). An important question in stomatal control is whether reduced mesophyll capacity for ABA catabolism leads to stomatal closure that limits photosynthesis or alternatively, whether reduced mesophyll capacity for photosynthesis controls stomatal behavior. The latter view originated with Wong *et al.* (1979). They theorized that water deficits reduce mesophyll capacity for carbon fixation resulting in an elevated intercellular CO_2 concentration (C_i). In turn, increased C_i provides negative feedback to the stomata and causes a reduction in g_s consistent with maintaining constant C_i. Their conclusions were drawn from observations of proportional change in carbon assimilation (A) and g_s during bouts of water stress.

As the rearranged equation for carbon assimilation shows;

$$C_i = (C_a - 1.6\ A/g_s)$$

(where C_a is the ambient CO_2 concentration) if A and g_s change proportionally and C_a is constant, then C_i must also be constant. Wong *et al.* (1979) reasoned that invariant C_i could mean assimilation is limited by mesophyll carbon fixation capacity rather than restrictions in the CO_2 supply caused by stomatal closure. Terashima *et al.* (1988) suggested that the proportional changes in A and g_s observed by Wong *et al.* (1979) may be explained by a patchy stomatal closure pattern which effectively acts like a loss of leaf area. Such a patchy or heterogeneous pattern of stomatal closure develops in response to exogeneous ABA or if the onset of leaf dehydration is rapid (Hirasawa *et al.* 1995) *et al.* (1988) pointed out C_i calculated from gas exchange measurements on an assumption of uniform stomatal closure is overestimated for leaves which display a patchy stomatal closure pattern. Since C_i is taken as an indicator of mesophyll activity, increased C_i can be misinterpretted as a sign of reduced mesophyll photosynthetic capacity (Terashima 1992; Mott 1995). Consequently, the model of Wong *et al.* (1979) which indicates g_s varies with mesophyll photosynthetic capacity could be based on artifactual results (Farquhar *et al.* 1987) A review by Kaiser (1987) indicates mesophyll photosynthetic capacity is relatively insensitive to short term dehydrations. Yet, g_s decreases in these instances, which indicates reduced mesophyll photosynthetic capacity is a not a prerequisite to stomatal closure.

1.10 ABA analogs and their potential for improving plant performance in the field.

ABA, unlike other classes of plant hormones such as auxin, cytokinins and derivatives thereof has found comparatively little use in agriculture (Loveys 1992). However, the role of ABA in the control and maintenance of stomatal function illustrated by studies of wilty ABA minus mutants (Bradford 1983; Neill and Horgan 1985) indicates it may be possible to manipulate ABA physiology to increase production and reduce mortality. Findings that reduced ABA in xylem and foliage of well watered vines was associated with increased stomatal conductance, carbon assimilation and growth, led Loveys (1992) to propose that manipulations that decreased ABA levels in circumstances where water supply is not limiting could enhance crop productivity. Sinclair *et al.* (1984) suggested that there is a greater opportunity to increase production in water limited conditions by increasing water use efficiency (WUE) which Rademacher *et al.* (1989) and Raschke (1975) have shown can be improved by external ABA application.

Transplanting plants from propagation houses to greenhouses or outdoors with drastic differences in humidity, irradiance and temperature, can cause wilt even when there is adequate water supply. This phenomenon of transplantation shock (inability to cope with increased evaporative demand) has been attributed to low ABA levels or poor response to endogenous ABA. The response to transplantation emulates the behavior of wilty mutants in the field. Quarrie (1982) determined that external ABA application temporarily restores the wild phenotype in ABA minus mutants. Loveys (1984) showed that during hardening for outplanting, greenhouse grown vines had significantly higher

concentrations of xylem ABA than unhardened clones. These facts suggest there is an opportunity to increase WUE in the field and diminish transplantation shock by external application of ABA.

The feature of ABA, apart from cost which has limited its use in field applications is the brevity of its half life, estimated at 3 hours (Harrison and Walton 1975). In part, ABA break down is the result of plant catabolic enzyme systems, but losses are exacerbated by rapid photoisomerization to an inactive 2-trans ABA isomer. Researchers have worked towards increasing biological efficacy of ABA by the synthesis of bioactive analogs which are less easily catabolized and persist longer *in vivo*. However, efforts to produce a useful analog have been hampered by the fact that most structural features of ABA including the carboxy group, 2-cis and 4-trans bonds in the dienoic acid side chain, 4′ keto group in the ring and 2′ double bond are required for biological activity (Millborrow 1983, 1990), leaving limited latitude for imposing structural changes that increase persistence without deleting activity. Still, significant antitranspirant activity has been attributed to several ABA derivatives (Sharkey and Raschke 1980; Loveys and Millborrow 1983, 1990; Rademacher *et al.* 1989) suggesting further structure-activity studies may eventually produce more stable variants.

Thesis objectives

My first research goal was to examine the recent hypothesis that stomatal behaviour is determined primarily by soil and root water status. It has been proposed that root water status is communicated to the shoots and stomates *via* the transpiration stream by means of a chemical signal, possibly abscisic acid (ABA), which increases in the roots

with soil water deficit. This hypothesis, which developed from observations of isohydric behaviour in the field as well as evidence gathered from split root and root pressurization experiments performed in the laboratory that showed g_s was independent of Ψ_l, has challenged the traditional view (eg. Ludlow 1980) that stomatal conductance is a function of leaf water status. Nearly all experiments which indicate stomata are controlled by root influences have been performed on herbaceous species. To determine if similar stomatal controls exist in non-herbaceous species, I examined stomatal regulation during soil drought in two woody species; Douglas fir (*Pseudotsuga menziesii* (Mirb) Franco) and red alder (*Alnus rubra* Bong.). Douglas fir is a coniferous species of considerable economic importance to British Columbia. Alder is a deciduous species, regarded as a nuisance species in coniferous forest regeneration programs since it grows rapidly in harvested sites and limits growth of coniferous species through light interception. In this study, the shoot water deficits due to drought were relieved either by restoration of the soil water content (rewatering) or by root pressurization using a pressure chamber. The results of this study have been published recently (Fuchs and Livingston, 1996) and are included here in chapter 2.

My second research objective was to examine the antitranspirant activity of 19 ABA analogs in clonal white spruce (*Picea glauca* (Moench) Voss) produced *in vitro* through somatic embryogenesis. The analogs to be tested represented variants obtained through systematic chemical changes in specific regions of naturally occurring (+)-(S)-ABA. Through analysis of the relative antitranspirant activity of different ABA analogs (structure-function analysis) I sought to; 1) determine which features of ABA are important to its antitranspirant bioactivity 2) determine which analogs may be of use in a treatment which can be applied in the nursery prior to outplanting for the purpose of reducing field mortality from water stress.

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INTRODUCTION to Chapters 2 and 3

It is generally accepted that short- and long-term drought stress constitute major limitations to agricultural and forest production. Although the effects of drought have been described extensively, the mechanisms underlying drought responses are still not well understood and have been the subject of vigorous debate (see, for example, Sinclair & Ludlow 1985: Kramer 1988: Passioura 1988: Boyer 1989, Lösch 1992). This debate has largely focused on four issues: (1) the usefulness of water potential as a measure of plant water status: (2) the relationship of leaf turgor to stomatal conductance (g_s) and growth; (3) the relative importance of hydraulic versus chemical effects of water stress on g_s and (4) the role of roots, as opposed to shoots, as primary sensors of water stress.

There is no doubt that plants in drying soil experience a decrease in bulk leaf water potential (ψ_l) and leaf turgor with a consequent reduction in g_s and growth. However, there have been numerous observations that roots in drying soils can affect shoot physiology independently of shoot ψ (Bates & Hall 1981; Livingston & Black 1987; Grantz 1990; Saliendra, Sperry and Comstock 1995). A number of experiments have demonstrated, by manipulative treatment of roots, that a reduction in g_s can be induced even when the shoot water status is maintained. This has led to the hypothesis that roots in dry soil produce a chemical message, possibly abscisic acid (ABA) (Zhang & Davies 1989, 1990; Khalil & Grace 1993), which is transmitted to the shoot via the transpiration stream and causes stomata to close, a change independent of the leaf ψ or turgor (Gollan, Passioura & Munns 1986; Davies & Zhang 1991; Tardieu *et al.* 1992). There have also been observations of reduced g_s in response to reduced hydraulic conductance but relatively constant ψ_l. The mechanisms underlying such a response are not clear (Meinzer & Grantz 1990; Sperry, Alder & Eastlack 1993).

The hypothesis that there is a feedforward response to root or soil ψ is not without critics. Boyer (1989) and Nonami & Boyer (1990, 1993) argue that ψ and its components play a fundamental role in stomatal behaviour, in that water entry into guard cells is determined by water potential differences, and that they cannot be dismissed as being physiologically irrelevant. In drying soils, they argue, tensions are transmitted in the xylem water column to the shoot, and these can alter the rate of growth of shoot tissues without initially altering the turgor of the growing tissue. This is because during cell enlargement a growth-induced water potential gradient favors water movement into the enlarging cells from the xylem. As the water potential of the shoot xylem falls in response to decreasing root potentials, the surrounding growing tissues can no longer take up water at the required rate and therefore growth becomes slower. Changes in shoot water potential will probably be too small to be observed because the greatest changes are in the xylem water potential. Both Kramer (1988) and Boyer (1989) suggest that, while it is possible to produce experimental conditions in which roots are stressed in advance of shoots (as in the split root experiments of Zhang, Schurr & Davies 1987; Zhang & Davies 1990), in the field shoots always have lower potentials than roots. This is because gradients must exist for transpiration to occur. Zhang and Davies (1990) argue, however, that in the surface horizons of very dry soil small roots dehydrate while large roots maintain turgor through osmotic adjustment. With prolonged drying, surface roots die and an increasing number of roots in deeper soil are exposed to water stress. This results in a corresponding increase in the intensity of chemical signals.

Most of the work examining drought-induced stomatal closure has been carried out on herbaceous species and few investigations have been conducted to examine such interactions in trees. Khalil and Grace (1993) presented evidence that stomatal conductance in sycamore seedling (*Acer pseudoplantanus* L.) was related to the concentration of ABA in the xylem. Wartinger *et al.* (1990) concluded that,

during a drying cycle, the range of maximum g_s of almond trees was dependent on the ABA concentration in the xylem sap. More recently, Jackson *et al.* (1995) investigated the hypothesis that it is the rate at which ABA enters the leaf, rather than the concentration of ABA, that determines g_s. In experiments on droughted conifer saplings, they showed that , whilst there was no statistical relation between g_s and leaf turgor or ABA flux, there was a negative exponential relation between g_s and ABA concentration in the xylem. However, in contrast, Saliendra *et al.* (1995) describe measurements on *Betula occidentalis* seedlings that suggest that chemical messengers originating from the roots do not play a central role in regulating droughted plants.

In chapter 2, I describe experiments on Douglas fir [*Pseudotsuga menziesii* (Mirb) Franco] and alder [*Alnus rubra* (Bong)] seedlings in which I test the hypothesis that in wet or dry soils g_s is independent of ψ_l. The tests were performed with a root pressure chamber, based on the design described by Passioura and Munns (1984), that effectively breaks the interdependence of root and shoot water potentials and allows the effects of each to be independently studied.

In chapter 3 I describe the experiment wherein a set of 19 different ABA-analogs were screened for their potential to alleviate transplant shock in nursery raised conifer seedlings. Conifer seedlings raised in nurseries are often susceptible to drought soon after planting because of poor root to soil contact and high evaporative demand (Marshall and Maki 1946; Blake 1983). Drought can result in reduced growth and high mortality and can limit successful reforestation. This problem is exacerbated by the fact that seedlings raised for reforestation typically are nurtured, before planting under an optimal regime of nutrient and water supply which can foster poor stomatal responsiveness to more demanding conditions experienced in the field (Loveys 1992).

Various pre-treatments have been tested for the purpose of improving seedlings field performance after outplanting. Subjecting greenhouse plants to water stress as a finishing-hardening treatment prior to outplanting increases their ability to respond to episodes of drought in the field (Kramer 1983). However, in general, because nurseries concentrate on achieving rapid growth to bring seedlings to a plantable size, they forgo hardening treatments. Also, the value of hardening treatments is limited in that they only provide protection against moderate levels of drought (Levitt 1980). As a result, silviculturists have sought outplanting pre-treatments which do not curtail greenhouse growth rate, yet confer drought resistance in the field. Possibilities include the application of chemical and physical agents with antitranspirant properties immediately before outplanting.

Antitranspirants have the potential to aid in seedling acclimation to the field by increasing plant water use efficiency (WUE), the ratio of carbon assimilation to transpiration over a defined period. More efficient use of limited soil water reserves gives seedlings an opportunity to establish roots in deeper, moister soil horizons to ensure continued water supply. Foliar agents that have been tested as antitranspirants include thin layer applications of waxes, resins and latexes which curtail water loss by blocking stomata. Chemicals which close stomata by their metabolic effects such as atrazine, phenylmercuric acetate, succinic acid and hydroxysulfonates have also been investigated. Most prospective antitranspirants have proven to be impractical for use on conifers due to their high costs, poor application efficiency, or phytotoxicity which reduces growth (Kozlowski 1979; Simpson 1984).

Abscisic acid, which is a natural hormone with potent antitranspirant properties and no toxic side effects, has attracted interest as a potential pre-treatment agent. Unfortunately, ABA is subject to rapid inactivation by photoconversion and metabolic degradation which limits its usefulness (Harrison and Walton 1975). The latter point is well illustrated by the findings of Ribaut *et al.*

(1996) who devised a novel methodology, combining GC-MS and scintillation counting, to deduce ABA turnover in maize roots. They found that only 40 % of the [^{3} H]-ABA incorporated in the apical zone, remained after 1 hour. This estimate is consistent with the findings of Jia *et al.* (1996) who calculated that ABA in the xylem sap of maize has a half life of 42 minutes.

ABA analogs, structural variants of naturally occurring ABA, with comparable bioactivity but greater persistence within the plant may have the potential to overcome the limitations of the natural form. Todoroki *et al.* (1995) found that fluorinated ABA analogs were as effective as natural ABA in evoking a stomatal response but were more persistent. They speculated that the longevity of the fluorinated analogs was due to their ability to resist the first step of catabolic degradation. Rational design of ABA analogs has been hampered by a lack of information about the shape of the receptor active site which binds the natural ligand. To date, no plant hormone receptor has been isolated and described by amino acid sequence or tertiary configuration. Therefore, the strategy used to evaluate ABA analogs has been limited to bioassay of variants which have been systematically altered, one structural moiety at a time (Blake *et al.* 1990; Churchill *et al.*1992; Windsor *et al.* 1993; Rose *et al.* 1996). Willows and Millborrow (1993) suggested that the scale of the screening process might be reduced by conformational analysis of analogs. They reason that the low energy conformation of ABA is the most probable and is therefore most likely to be the bioactive form. Consequently, analogs unable to achieve comparable conformations are unlikely to be bioactive and can be eliminated from the screening roster.

ABA promotes many physiological responses and consequently there are many bioassays by which structure-function relationships might be tested (Churchill *et al.* 1992; Todoroki *et al.* 1995). Rapid bioassays such as stomatal closure and transpiration assays are advantageous because they minimize

complications and ambiguities that arise due to the metabolic conversion of some analogs to ABA as noted by Walton (1983).

Blake *et al.* (1990) tested ten ABA analogs with similar structures to those evaluated in this study (4 analogs were the same between studies). They applied the analog solutions as root drenches to aeroponically grown black spruce, *Picea mariana* (Mill.) B.S.P. and determined that those analogs that brought about pronounced stomatal closure caused a higher level of stomatal responsiveness to stress in seedlings challenged by drought 7 days later. The increased drought response was attributed to a priming effect of the analog rather than residual amounts of the analog *in situ.*

In this study I surveyed the relative antitranspirant activity of 19 different ABA analogs that were applied to white spruce (*Picea glauca* (Moench) Voss), one of the most widely distributed conifer species in North America and the most important commercial species in Canada. My objectives were; 1) to determine aspects of structure important to bioactivity, information that could be applied to direct subsequent analog synthetic efforts, 2) to determine which analogs are of potential use as antitranspirants in a nursery pre-treatment aimed at increasing performance of outplanted seedlings, 3) to determine how efficiently natural ABA is absorbed into *Picea vs* a herbaceous species such as wheat, (*Triticum aesitivum* L.) through the roots. The efficiency of ABA absorption into *Picea* roots was of interest because preliminary work indicated it was necessary to apply a relatively high (10^{-3} M) concentration of ABA to roots of *Picea* compared to *Triticum* (10^{-5} M) (Dr P. Rose, Plant Biotechnology Institute-NRC, Saskatoon, personal communication) in order to induce a comparable stomatal response. This may indicate that *Picea* absorbs ABA poorly, is inherently less sensitive to ABA, or possibly both. Consequently, ABA absorption efficiency in both species was assessed as a first step in ongoing investigations to resolve these questions.

As in Blake *et al.* (1990) treatment plants were conditioned to an aeroponic environment, but in my study, analogs were administered through the roots aeroponically, rather than by root drench. An aeroponic system was adopted to avoid extraneous soil influences on analog uptake and chemistry. To my knowledge, two features of this study are unique; a) analogs were applied to clonal plant material generated *in vitro* by induction of somatic embryogenesis, b) whole seedling transpiration rate (E) and carbon assimilation rate (A) were measured continuously as analogs were applied. In their study, of black spruce, Blake *et al.* (1990) noted the response to analogs and even to ABA varied significantly between groups of seedlings, a result they attributed to genotypic differences. In the present study genotypic factors were eliminated through use of clonal material raised as a lot under identical growth conditions.

CHAPTER 2

The portion of my thesis work that has been published (Plant, Cell and Environment (1996) **19,** 1091-1098) is presented in this chapter.

Hydraulic control of stomatal conductance in Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) and alder (*Alnus rubra* (Bong)) seedlings. E.E. Fuchs and N.J. Livingston.

2.0 METHODS AND MATERIALS

Plant material

All experiments were conducted on 1-year-old Douglas fir and 2-3 month-old-alder seedlings. The Douglas fir seedlings were nursery-raised and were from a dry interior provenance of British Columbia, Canada. The alder seedlings were grown from seed and raised in the field facilities at the University of Victoria. At least 2 weeks prior to any experiment, seedlings were transplanted to PVC cylinders (0.4m high and 0.15 m I.D. and designed to fit inside a pressure chamber) filled with fine sand. The sand was held in place by a nylon mesh (NGG 52, 335 μm opening, 46 % porosity) at the bottom of each cylinder.

Gas exchange

Whole-seedling transpiration (E) and photosynthesis rates (P_n) were measured using the computer-controlled plant cuvette system described by Livingston *et al.* (1994). In this closed system, seedlings are enclosed in a polycarbonate cylindrical chamber (0.2m O.D. and 0.3 m long) with removable top and bottom plates.

A dual detector infrared gas analyzer (LI6262, Li-Cor Inc., Lincoln, NE, USA) is used to measure the vapor pressure and CO_2 concentration in the chamber. Vapor pressure in the chamber (e_a) is controlled by circulating air through a desiccant column supported on a digital balance. Air is passed through the column whenever e_a exceeds a predetermined set point. Transpiration rate is determined from the change in desiccant mass (measured with a resolution 1 mg) with time. Photosynthesis is measured by integrating the output of a mass flow controller used to inject CO_2 into the chamber to compensate for that assimilated by the plant. Stomatal conductance to CO_2 (g_c) is calculated as E/1.6 (A × D), where A is the total projected leaf area determined using a LI-3100 leaf area meter (Li-Cor Inc.) and D the vapor pressure deficit in the cuvette. Calculations indicated that seedling boundary layer conductances could be assumed to be negligible (Livingston *et al.* 1994) because of the very high rate of air circulation in the chamber (approximately 0.025 m^3-s^{-1}). This assumption is supported by data that showed that differences between needle and chamber air temperatures were consistently less than 0.1 ° C, even when the photosynthetic photon flux density (Q) was almost 2000 µmol m^{-2} s^{-1}. In all experiments, P_n and E were monitored continuously. Seedlings were maintained, unless stated otherwise, at air temperature (T) of 20 ° C, D was 1.04 ± 0.02 kPa and the CO_2 concentration was 350 ± 2.0 µmol mol^{-1}. Daylengths were 10 h with a Q of 1000 ± 5.0 µmol m^{-2} s^{-1} obtained using the light control system described by Livingston (1994).

Pressure chamber

The pressure chamber (Figure 1) consists of a stainless steel cylinder (0.609 m long, 0.201 m I.D. and 0.236 m O.D.) with carbon steel flanges (0.051 m thick and 0.325 m O.D.) at each end. Circular grooves machined in each flange accommodated rubber O-rings (0.0032 m thick, 0.298 m O.D.) that provide a pressure seal with carbon steel plates bolted to the flanges.

The bottom of the cylinder is sealed with a single plate (0.05 m thick and 0.325 m in diameter). Two plates (each 0.024 m thick, 0.325 m in diameter seal the top of the cylinder. Each of these plates has a 0.045-m-diameter central hole and can be split into hemi-circular halves. The hole in the lower plate is threaded to accommodate a cylindrical brass plug. This plug has a 0.02 m bore and can be split into halves along its longitudinal axis. Once a seedling has been lowered in the cylinder, the lower split plates are put into place and the brass plug fit and secured around the stem as close to the base of the shoot as possible. The upper split plates are then put in place.

A pressure seal around the seeding stem is formed by a soft silicon rubber disc and plug which are compressed as the brass split plug is tightened (Fig. 1). These pieces are made by injecting silicon rubber into plaster moulds coated with petroleum jelly and are cured at 70 ° C for 24 h. Two slotted sheet brass discs (0.043 m O.D., slot width 0.01 m) prevent damage to the seals as the brass plug is tightened.

Chamber pressure is measured with a PX-602 pressure transducer (Omega, Stamford, CT, USA) that has a 0-3.5 MPa range and ± 0.4 % (full scale) accuracy. Chamber pressure is controlled using a simple feedback control system. Measured pressure is continuously compared to a specified set point pressure. When the chamber pressure is less than the set point, gas (N_2) is injected into the chamber through a solenoid valve (S1 in Fig. 1). When the chamber pressure exceeds the set point, gas is release using

an additional solenoid valve (S2 in Fig.1). The pressurization or exhaust rate is controlled by varying the duration of the pulse driving the solenoids or by adjusting in-line needle valves (1R-S4 stainless steel, Whitey Co., Highland Heights, OH, USA). Chamber pressure can be controlled to ± 0.007 MPa. The chamber is equipped with an adjustable (2.4 to 5.15 MPa) proportional safety relief valve (SS-4R3A, Swagelock Canada Ltd).

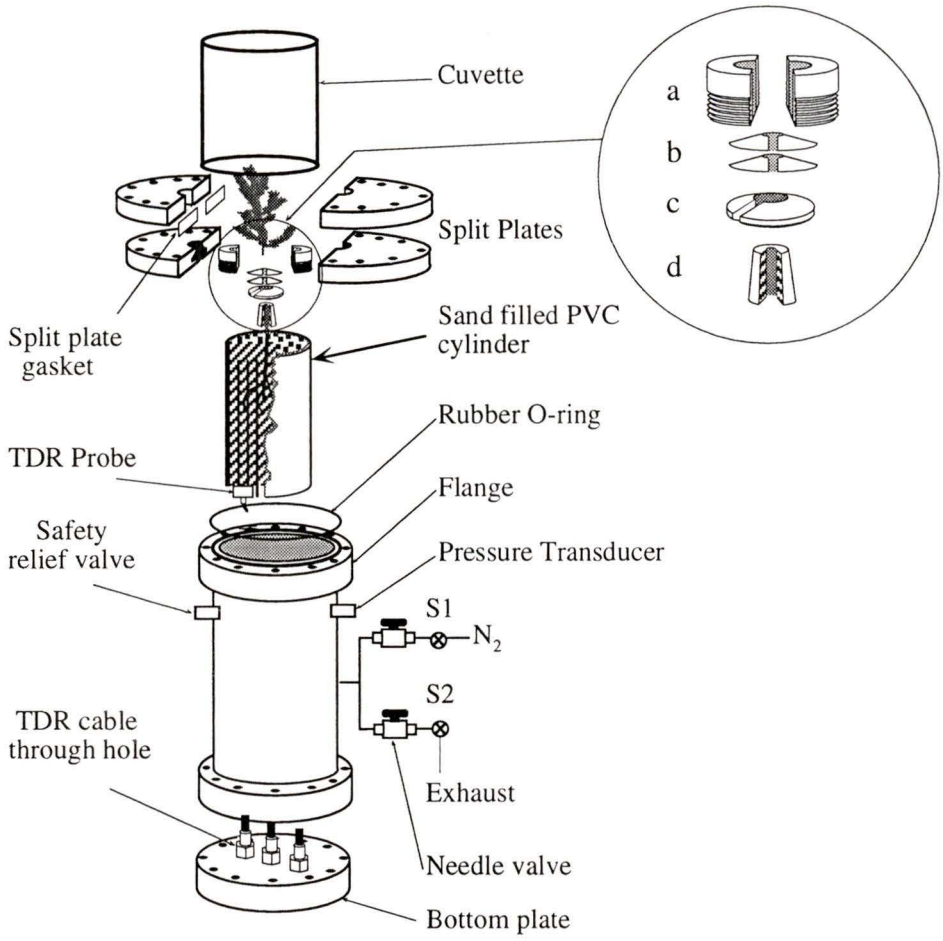

Figure 1. Schematic diagram of the pressure chamber (not to scale) Nitrogen gas enters through S1 and is exhausted through S2 where S1 and S2 are normally closed 110 V a.c. solenoid valves (SV-102, Omega, Stamford, CT, USA). The enlargement shows the components of the pressure seal: a) threaded brass split plug, b) sheet brass slotted discs, c) silicon rubber disc and d) silicon rubber plug. Silicon rubber gaskets (0.0032 m thick) fit between the lower set of split plates

Soil water content

Volumetric water content (θ_v) of the soil in a cylinder was measured daily using time domain reflectometry (TDR). Measurements were made using 0.4-m-long three-rod, single-diode probes (Hook *et al.* 1992). Remote diode shorting and differential waveform techniques were used to provide reliable detection of the two reflections that define the apparent dielectric constant of the soil (Hook *et al.* 1992). The probes, inserted into the soil from the bottom of the PVC cylinder, were approximately 2.0, 4.0 and 6.0 cm from the cylinder center and 120 ° apart. In some experiments, stripline probes were used (Hook *et al.* 1992). These had three segments, each 0.12m long, and provided a measure of water distribution along the length of the cylinder. Probe cables were passed through through-hole connectors (Swagelock Canada Ltd, Ont., Canada) in the bottom plate of the pressure chamber. Measurements of the electromagnetic propagation velocity along the TDR probes were converted to estimates of soil water content using the linear model of Hook and Livingston (1996). Measurement resolution and accuracy were 0.004 $m^3\ m^{-3}$ and 0.015 $m^3\ m^{-3}$, respectively (Hook and Livingston, 1995). Volumetric soil water content was also determined gravimetrically at the start of each experiment. Thereafter, θ_v was calculated as the difference between this initial value and daily transpired water as determined by the gas exchange system. There was generally excellent agreement between TDR and gravimetric determinations of soil water.

Experiments

Soil drying

Experiments were conducted to determine the response of Douglas fir seedlings to progressive soil drying. Seedlings (n = 5) were well watered for 1 week and then the soil water content was allowed to decline over the next 40-60 d. During this period P_n, E and g_c were measured continuously. The response to re-watering was also measured. In the first set of experiments, when a marked decline in P_n was first observed, daily transpired water was replaced overnight and seedlings monitored over the next 2 to 3 days. In the second set of experiments, enough water was applied to bring the soil water content back to the value measured at the start of the drought cycle. Seedlings were then monitored for at least 5 more days.

Root pressurization

In these experiments, the roots of Douglas fir (n = 6) and alder (n = 5) seedlings were encased in the pressure chamber. Each seedling was exposed to one drying cycle and E and P_n were measured continuously. During this period, seedlings were subjected to at least 10 short-term (1 to 2 h) or long-term (8 to 14 h) daytime root pressurizations. Typically, pressure was applied approximately 1 h after the lights had been switched on. The chamber pressure was increased linearly to a predetermined set point over 30 min. The response to de-pressurization was also evaluated; in this case, the chamber pressure was reduced over varying periods ranging from 5 to 60 min.

Experiments were also carried out on three additional Douglas fir and alder seedlings to determine whether the response to root pressurization would change if D was almost doubled to 2.06 kPa (Q and T were unchanged). During these experiments, θ_v was maintained between 0.06 and 0.074 $m^3 m^{-3}$. Seedlings were held for at least 4 d at each D.

In a final set of experiments, four Douglas fir seedlings were grown in dry sand (θ_v was maintained at 0.04 ± 0.013 $m^3 m^{-3}$) for over 120 d. At the end of this period, individual seedlings were placed in the pressure chamber and cuvette, and measurement of P_n and E made over the next 2 d without any chamber pressurization. For the next 3 d, the chamber pressure was held at 1-2 MPa for 8 h during the day and released at night.

2.1 RESULTS

Response to soil drying

All Douglas fir seedlings subjected to drought showed a pronounced decline in E, P_n and g_c; however, these parameters were remarkably constant during the first 40 d of soil drying. In every case, g_c only declined significantly (by more than 10 % relative to that measured when the seedlings were in wet soil) after θ_v had dropped to below 0.06 $m^3 m^{-3}$ (a soil water potential of approximately -1 MPa). A typical response to soil drying is shown in Fig. 2. Water release curves generated for the sand revealed that there was a sharp and almost linear drop in soil water potential (-0.1 to -2.0 MPa) as θ_v dried from 0.075 to 0.04 $m^3 m^{-3}$ (data not shown). Again, in every case, g_c and E declined before P_n (typically a week before, indicating that during this period P_n was not affected directly by

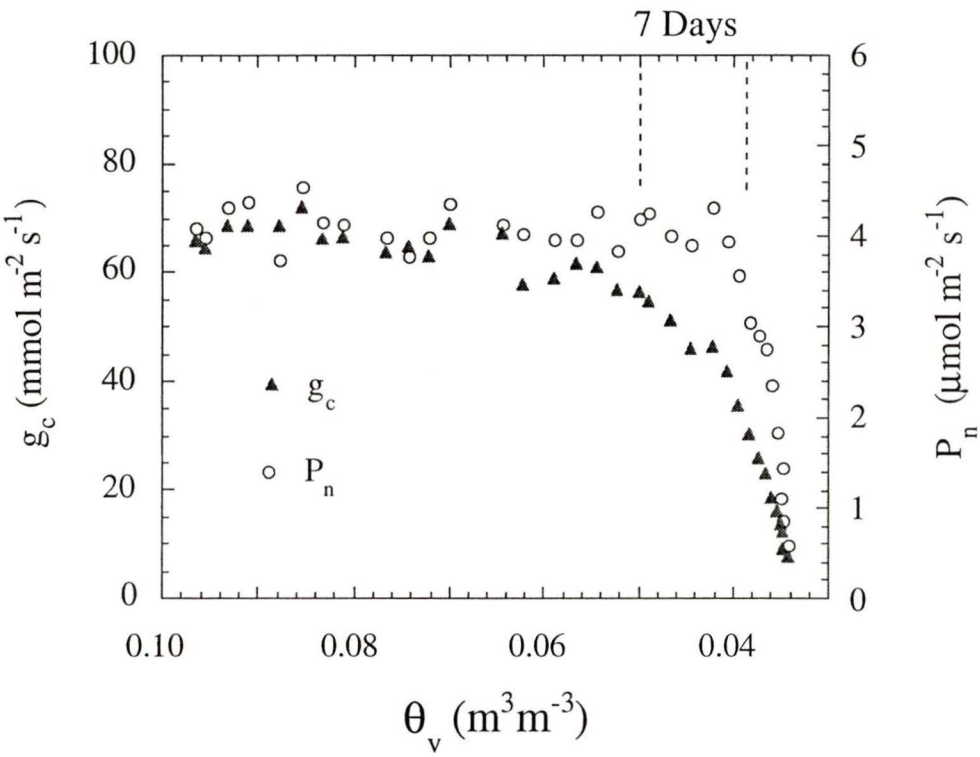

Figure 2. Stomatal conductance to CO_2 (g_c) and net photosynthesis (P_n) versus volumetric soil water (θ_v) content for Douglas fir. Each point represents the mean g_c or P_n

the water deficit. This increase in water use efficiency and maintenance of photosynthetic capacity suggest that the Douglas fir seedlings used in the study were well adapted to drought stress. The subsequent decline in P_n with further soil drying was presumably due to a stomatal limitation. During the period when there was a marked daily decline in both g_c and P_n, seedlings showed no measurable response to watering when daily transpired water (typically 4-6 ml) was replaced overnight. Presumably, this was because the added water was distributed over the whole soil volume and not concentrated at the roots. Time domain reflectometry measurements confirmed that the soil was at least 0.01 m^3 m^{-3} drier in the immediate vicinity of the roots than in the bulk soil.

All seedlings responded to watering when θ_v was raised overnight to the level recorded at the start of the experiment (approximately 0.1 m^3 m^{-3}). However, in every case it took at least 2-3 d before P_n and g_c returned to their pre-stress values. Mansfield & Davies (1985) suggest that such after-effects could be beneficial because they not only prevent a rapid expenditure of renewed water supply, which might be ephemeral, but may also improve average water use efficiency. There was no indication that the drought treatments caused any permanent damage to the photosynthetic apparatus of the plants.

Response to root pressurization

During the course of our study there were 144 experimental runs in which the root chamber was pressurized. In all but seven cases, regardless of the soil water content, vapor pressure deficit or species, seedlings showed a positive response (significantly increased g_c and P_n) to either short term (less than 2h) or long-term root chamber pressurization. Typically, g_c and P_n increased within 1 min of pressure being applied (Fig. 3) and reached their maximum values, for a given applied pressure, within 5 min of

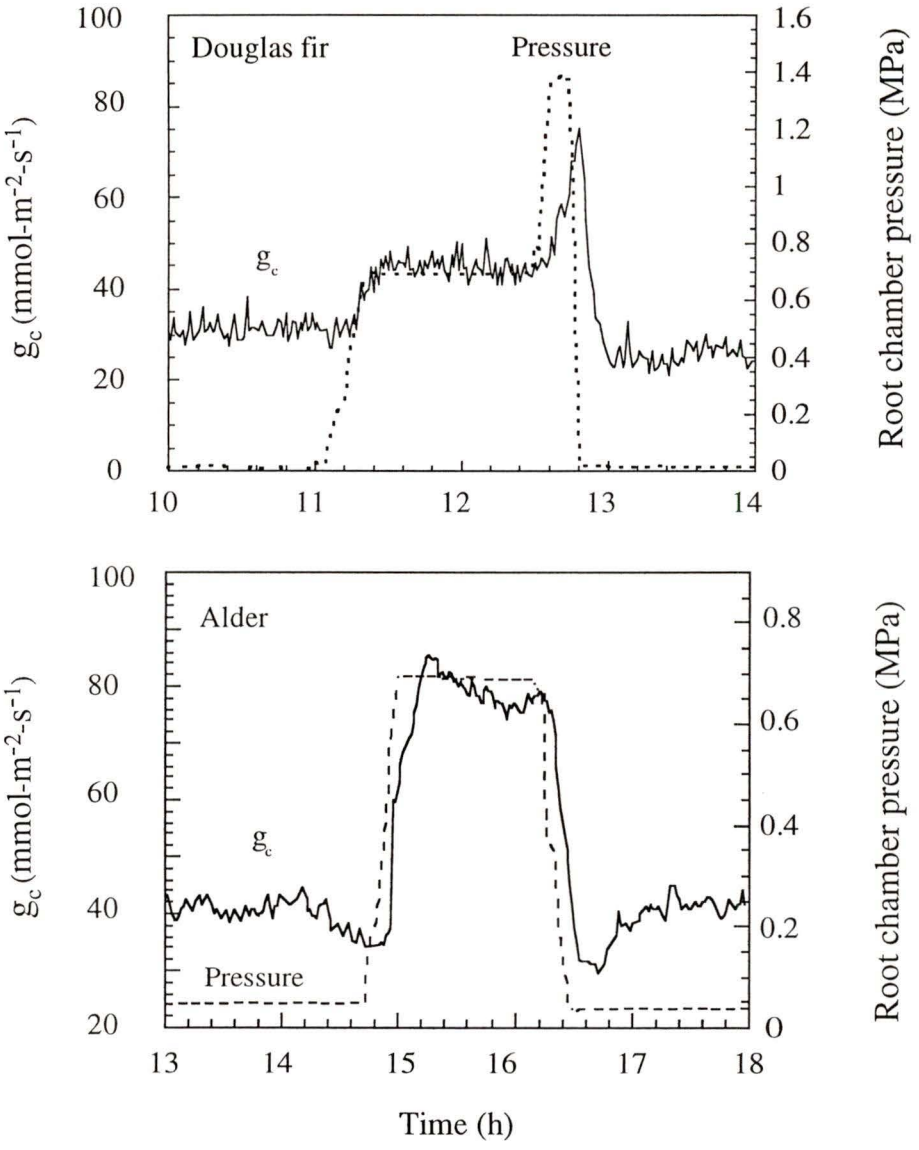

Figure 3. Time versus stomatal conductance to CO_2 (g_c) and root chamber pressure for a Douglas fir and an alder seedling. The soil water content was 0.04 m^3 -m^{-3} and 0.08 m^3 -m^{-3} for the Douglas fir and alder seedlings respectively.

that pressure being established. The response time to pressurization was independent of θ_v. Similarly, when the pressure was reduced there was a corresponding rapid reduction in P_n and g_c even after 14 h of continuous pressurization. In early experiments, before we had developed the final version of the pressure seal (Fig. 1) both g_c and P_n declined after sustained pressurization and would not recover to pre-pressurization values once the chamber pressure had been released. This led us to believe that g_c was controlled by chemical messenger from the root. However, close examination of the stem revealed damage caused by an incorrectly designed seal.

Figure 4 summarizes the effects, for Douglas fir, of root pressurization on mean daily g_c across a range of θ_v. Even in wet soils, g_c (and P_n ; data not shown) was increased (relative to that of the same unpressurized seedling) by up to 40 % after pressurization. In dry soils, g_c and P_n increased by as much as 4 times following pressurization; however, for $\theta_v < 0.04$ m^3m^{-3} , g_c and P_n were always significantly less than values obtained from well-watered unpressurized seedlings. This was even the case when the chamber pressure was equal and opposite to the soil water potential. There was no statistical difference in the responses (g_c, P_n, and E) to pressurization of seedlings that had been exposed to drought for over 120 d and those exposed to drought over the shorter term (45 to 60 d). The responses of alder seedlings to root chamber pressurization are shown in Fig. 5. Generally, the responses were similar to those found for the Douglas fir seedlings. However, chamber pressurization did not bring about as full a recovery in g_c and P_n. Also, the onset in the decline of g_c and P_n with soil drying occurred at a higher θ_v than in Douglas fir.

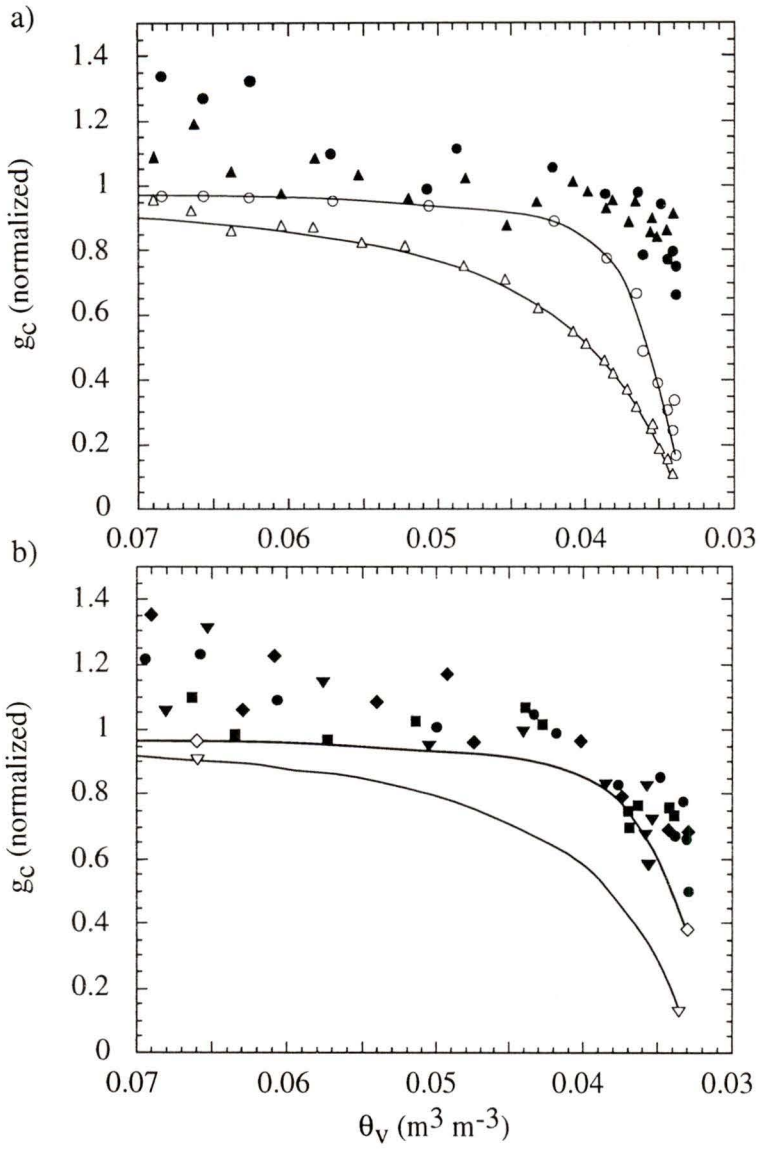

Figure 4. Soil water content (θ_v) versus stomatal conductance to CO_2 (g_c) normalized to the maximum measured over the course of the experiment. The symbols represent six different Douglas fir seedlings. The open symbols represent measurements made over 8 h when the root chamber was not pressurized, and the solid symbols represent measurements made over 2-8 h when the root chamber was pressurized. In (a) all data are given for two seedlings and lines were fitted by eye, while in (b) lines encompass the range of values obtained for four unpressurized seedlings.

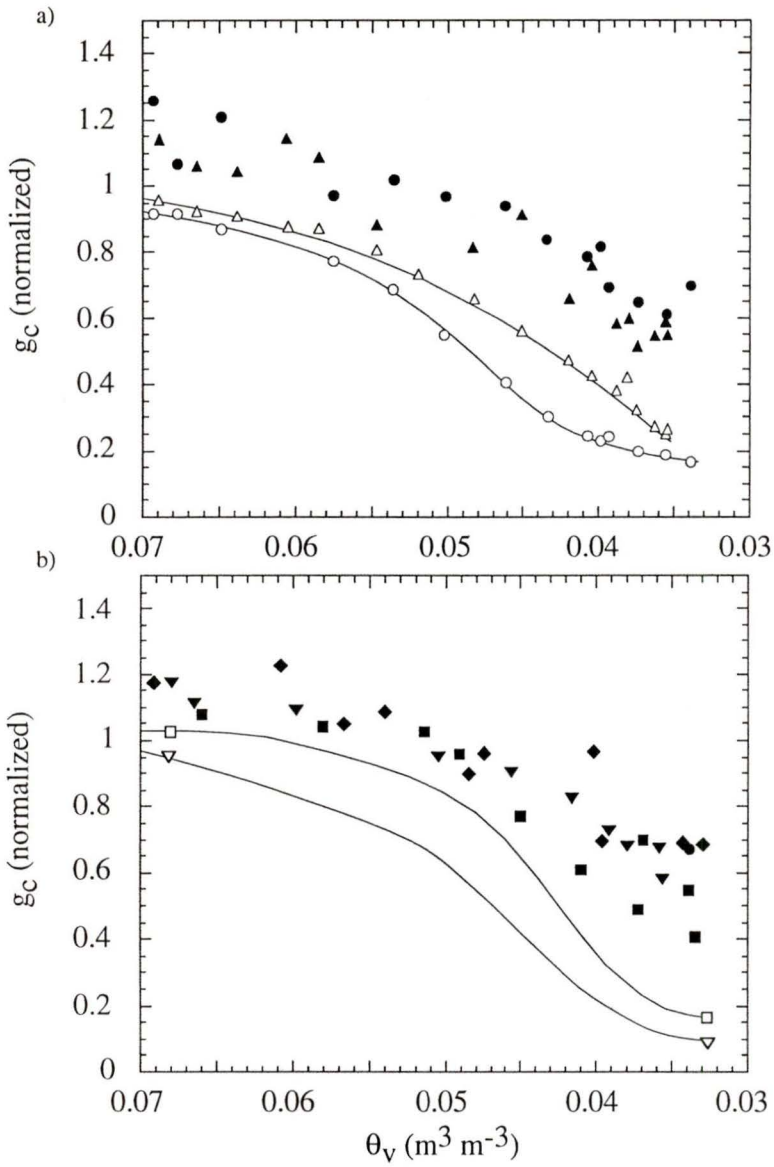

Figure 5. Soil water content (θ_v) versus stomatal conductance to CO_2 (g_c) normalizedto the maximum measured over the course of the experiment. The symbols represent five different alder seedlings. The open symbols represent measurements made over 8 h when the root chamber was not pressurized, and the solid symbols represent measurements made over 2-8 h when the root chamber was pressurized. In (a) all data are given for two seedlings and lines are fitted by eye, while in (b) lines encompass the range of values obtained for three unpressurized seedlings.

Response to increased D

In both species, increasing D from 1.04 to 2.04 kPa resulted in a pronounced decline in mean daily g_c (33% in Douglas fir and 26 % in alder). This was only partially reversed by pressurization of the root chamber (Fig. 6). There was a linear relation between applied pressure and g_c (normalized to the g_c measured when the seedling was unpressurized and held at 1.04 kPa) for both alder and Douglas fir (r^2 ranged from 0.63 to 0.66). When D was increased, the sensitivity of normalized g_c to increased pressure was almost halved in Douglas fir and declined by about 40 % in alder.

2.2 DISCUSSION

The root pressure chamber provides a direct means of determining the dependence of g_c on leaf water status. Soil pressurization does not significantly change the turgor pressure and cell volume of roots encased in the chamber. This is because the pneumatic and hydraulic pressures in the soil and roots are increased almost equally. However, shoots outside the chamber are subjected to an increase in hydraulic pressure alone, which results in a concomitant increase in cell turgor and volume. Therefore, any change in g_c brought about by root pressurization must be linked to changes in shoot rather than root water status.

Any change in chamber pressure involves both the very rapid propagation of pressure waves (up to the speed of sound in water, 1500 m s^{-1}), and changes in the much slower mass flow of water. Changes in leaf water status that result from pressurization might be confined to a relatively small population of cells (i.e. those that sense hydraulic

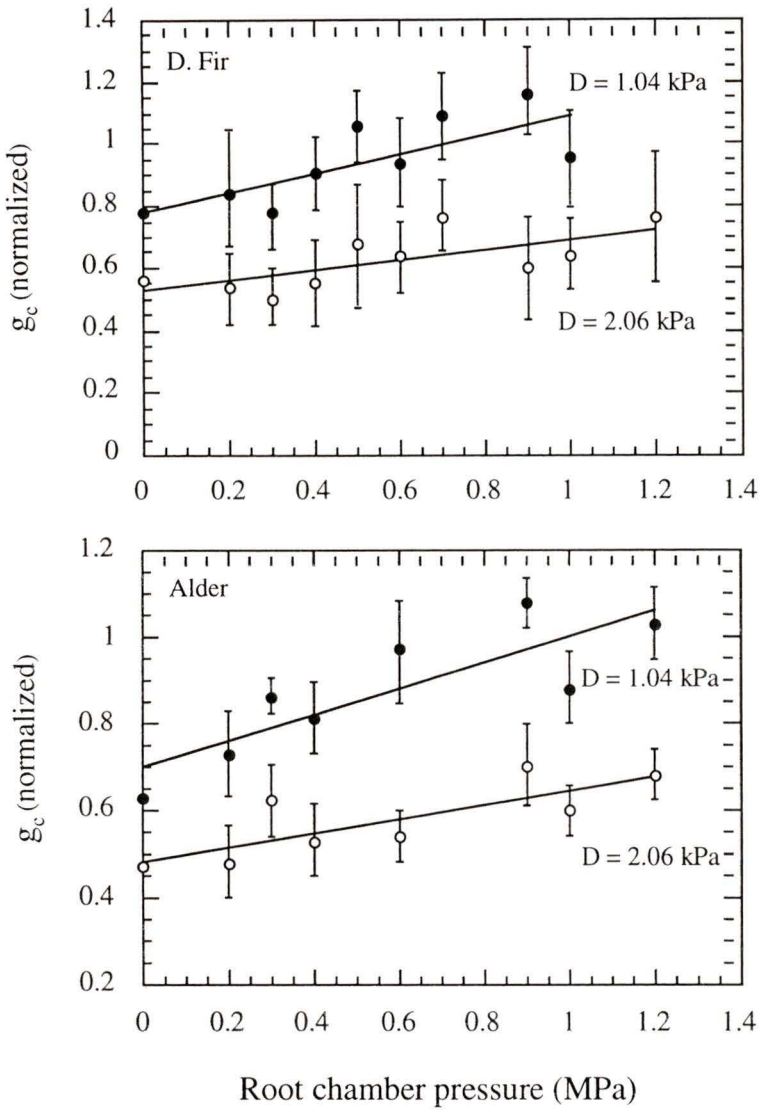

Figure 6. Root chamber pressure versus stomatal conductance to CO_2 (g_c) normalized to the maximum measured (unpressurized) for Douglas fir (D. Fir) and alder seedlings at two different vapour pressure deficits (D). Each point represents the average g_c measured over at least 2 h of three seedlings. The error bars indicate the standard deviation about the mean. The soil water content ranged from 0.06 to 0.074 $m^3 m^{-3}$. Douglas fir ; for D = 1.04 kPa, $y = 0.78 + 0.34 x$ ($r^2 = 0.62$), for D = 2.06 kPa, $y = 0.53 + 0.18 x$ ($r^2 = 0.54$); Alder; for D = 1.04 kPa, $y = 0.71 + 0.29x$ ($r^2 = 0.66$), D = 2.06 kPa, $y = 0.47 + 0.18 x$ ($r^2 = 0.66$).

signals) and might not necessarily be reflected by measurable changes in bulk leaf water potential. This is because any increase in ψ_l could be rapidly offset by an increase in g_c and hence E.

A number of experiments on herbaceous species that have employed a root pressure chamber have provided compelling evidence that g_c is not controlled exclusively at the leaf level. In these experiments, pressurizing the soil did not bring about any significant increase in g_c when plants were subjected to soil drought (Gollan *et al.* 1986; Schurr, Gollan & Schulze 1992).

In contrast, the results of our experiments on woody plants strongly support the hypothesis that leaf water status, at least in the short term, does indeed have an overwhelming influence on g_c and P_n. We have demonstrated that stomatal closure, brought about by either soil drying or increased D, can be very rapidly fully or partially reversed by pressurizing the root system. If this pressure is relieved, stomata almost immediately close. Recently, Saliendra *et al.* (1995) presented similar results for the woody plant *Betula occidentalis.* They also showed that stomatal closure in response to decreased hydraulic conductance (as a result of transverse cuts made on the stem base) could be reversed by root pressurization. There have been only a limited number of published experiments that have used the root pressure chamber to demonstrate the dependence, or otherwise, of stomatal conductance on leaf water status. However, to our knowledge, the results of these experiments all point to a difference in stomatal response (to root pressurization) between woody and herbaceous species. This is consistent with the notion that woody species, by virtue of their larger size, are less reliant on relatively slow moving root signals for short-term stomatal control.

Recent experiments, described by Whitehead *et al.* (1996), in which a 7-year-old *Pinus radiata* tree was subjected to a hydraulic shock by suddenly shading its lower foliage also support the notion of rapidly transmitted hydraulic signals. After the imposition of shade there was an almost immediate increase in g_c and P_n in the upper

(unshaded) part of the crown. When the shade was removed, g_c and P_n decreased instantaneously.

Saliendra *et al.* (1995), however, cite some unpublished data (J.P. Comstock) on the desert shrub *Hymenoclea salsola*, in which there was an increase in g_c in response to pressurizing the soil. They also present data for *Betula occidentalis* which show that, when the leaf-to-air vapor pressure deficit was *c.* 2.5 kPa, pressurizing the soil increased ψ_l but not g_c and in some cases caused it to decline. They attribute this latter effect to an increase in epidermal turgor leading to hydropassive closure of stomata.

We did not see any evidence of hydropassive closure in either Douglas fir or alder. Our results suggest that, while root pressurization always brought about an active increase in guard cell turgor, there must still have been significant gradients in water potential within the shoots (particularly at high D; Fig. 6) with the lowest potential at the site of evaporation. Hydraulic signals are likely to have been at their weakest at these sites because of the high hydraulic resistance offered by the mesophyll cells.

Our results do not exclude the possibility that chemical signals were generated in the roots and transmitted to the shoots via the xylem in response to soil drying. In fact, in separate experiments on a sub-sample of drought-stressed Douglas fir seedlings (data not shown), we found that the ABA concentrations in the xylem sap (determined by indirect ELISA) increased almost 4-fold, from a base level of about 100 $\mu mol\ m^{-3}$ in well watered seedlings. However, the ABA flux to leaves did not increase significantly because of the concomitant decline in whole -seedling transpiration rate.

The very rapid stomatal response to both increased and decreased root chamber pressure (Fig 3.), in contrast to the relatively slow response of drought-stressed seedlings to re-watering, strongly suggests that hydraulic signals overwhelmed any chemical signals transmitted from roots to shoots. Further, the fact that there were no statistically significant difference in response to changes in pressure between seedlings subjected to short- and long-term drought suggest that chemical messengers from the roots did not

increase stomatal sensitivity to soil drying by, for example, changing the guard cell turgor pressure threshold or 'set point' at which stomata close.

Our results do not, however, rule out the possibility of some interaction between hydraulic (physical) and chemical systems. Recent models of stomatal response to soil drying incorporate such an interaction (Tardieu 1993; Tardieu & Davies 1993).

There is a large body of evidence that points to the interaction of hydraulic and chemical systems in response to wounding (Malone 1993) or other perturbations. Certainly, the results of split root experiments on both herbaceous (e.g. Zhang & Davies 1990) and woody (Gowing, Davies & Jones 1990; Khalil and Grace 1993) species provide strong evidence that chemical messengers originating in the roots can, at times, play a dominant role in influencing g_c (although Saab and Sharp (1989) found drying whilst reducing leaf elongation in maize plants did not reduce g_s).

Saliendra *et al.* (1995) argue that it is misleading to refer to root chemical signals transported in the xylem stream to shoots as 'feedforward' responses to soil drying. They suggest that any treatment or event that brings about a change in soil water potential or hydraulic conductance will result in hydraulic signals being generated in the roots. These signals will be received much earlier than chemical messengers carried in the transpiration stream. Such signals could bring about a stomatal response, through the release of ABA sequestered in the leaf, or another leaf-level response as has been demonstrated by Chazen and Neumann (1994) in maize.

CONCLUSIONS

Root chamber pressurization brought about a very rapid increase in the stomatal conductance of drought-stressed Douglas fir and alder seedlings. We conclude that hydraulic signals completely over-rode any chemical signals that might have originated in the roots. Our results are in direct contrast to those obtained by others for herbaceous species using the same experimental approach. Split root treatments have generally not revealed fundamental differences in response between herbaceous and woody species, and have provided evidence that chemical signals from the roots can play a dominant role in determining g_c. We suggest that differences in response between herbaceous and woody species could be related to the method by which stress is imposed or relieved.

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CHAPTER 3

I. Structure-activity relationships of ABA-analogs based on their antitranspirant effects in clonal white spruce (*Picea glauca* (Moench) Voss).

II. Assessment of ABA-analog potential as aids in seedling establishment.

3.0 METHODS AND MATERIALS

3.10 Plant material

Interior white spruce

The interior white spruce seedlings (emblings) used in these procedures were produced from clonal somatic embryos (genotype U 144). The embryos were hormonally induced to form complete plantlets *in vitro* on solid agarose medium at the Forest Biotechnology Center of B.C. Research Inc., Vancouver, B.C., Canada. The emblings were subsequently transplanted into styroplug containers (410B container) filled with a 1:1 peat-vermiculite soil mixture in February and raised at Pelton Reforestation Ltd., Maple Ridge, B.C., under the nursery cultural regime described by Grossnickle *et al.* (1994). In August, emblings were transferred to environmental growth cabinets in Victoria, B.C. and maintained at an air temperature of 22 ° C, photosynthetic flux density (Q) of 400 μmol m^{-2} s^{-1} supplied by mixed fluorescent and incandescent lamps over a 16 h photoperiod. A 100 ppm (20-7-19) NPK-micronutrient solution was applied to the emblings at least every second day.

The emblings used to assess ABA analog bioactivity were selected by height (12-15 cm) and root collar (3.0-3.5 mm) to ensure size uniformity amongst test specimens.

Wheat

Wheat seedlings (cultivar; Katepwa) were grown from seed provided by Dr. P. Rose, (PBI, Saskatoon). Seeds were planted at a depth of 2 cm in 10mm x 130 mm glass test tubes filled with vermiculite. Two or three seeds were added to each tube. The vermiculite was moistened (not saturated) with distilled water to initiate seed germination which occurred 2-3 days later. Water was added as needed throughout the subsequent growth period to keep the vermiculite well moistened. Seedlings were grown at a temperature of 22 ° C, Q of 300 μmol m^{-2} s^{-1} with a 16 h photoperiod. After 10-12 days of growth (post-germination) seedlings were large enough to be handled conveniently and provided a practical amount of biomass to analyze after (±)-[^{3}H] ABA incorporation.

3.11 Embling transfer to an aeroponic environment and acclimation.

The roots of emblings lifted from styroplug containers were intimately bound with the planting medium and together formed a dense plug. The roots were separated from the planting medium by carefully pulling apart the plugs and rinsing them with a low pressure flow of tap water. The separation process however, inflicted some root damage. Fine root hairs of the absorbing roots (Johnson-Flanagan and Owens 1984) were likely to have been particularly susceptible to damage. Since root hairs contribute significantly to absorptive capacity (Nobel 1983) there was some concern ABA analogs may be absorbed inefficiently. Therefore, prior to

analog testing, emblings were given a 10-14 day recuperative period in an aeroponic environment to enable root hair reformation.

The apparatus constructed to facilitate root recovery is shown in figure 1. It consists of two main components; a) an ultrasonic mist generator and b) a root cuvette. The cuvette, made from plexiglass, consists of a cylinder (0.015 m O.D., 0.137 m I.D. and 0.018 m height), a bottom plate (0.020 m square, 0.008 m thickness) and a detachable top plate (0.018 m diameter, 0.004 m thickness). The bottom plate has a central hole which fits over the male extension of the mist generator. The top plate has 4 opening holes to accomodate the emblings (one per hole). Cleaned emblings were fitted at the root to shoot transition with a collar made from a number 5 rubber stopper. Collared emblings were inserted into the cuvette top plate with their roots projecting into the cuvette interior (figure 1) The collar functions as an embling holder and a seal against mist leakage from the cuvette. A nutritive mist is formed from a small reservoir of half strength Hoaglands solution over the ultrasonic head of the mist generator. Roots were held at 25 ° C by running tap water though a coil of copper pipe (0.065m O.D.) inside the cuvette.

The ultrasonic mist generator is a modified humidifier (model JUH-730, Jutan International, Toronto, ON, Canada). The stock mist container supplied with the humidifier was replaced by the aeroponic cuvette. No other modifications were made.

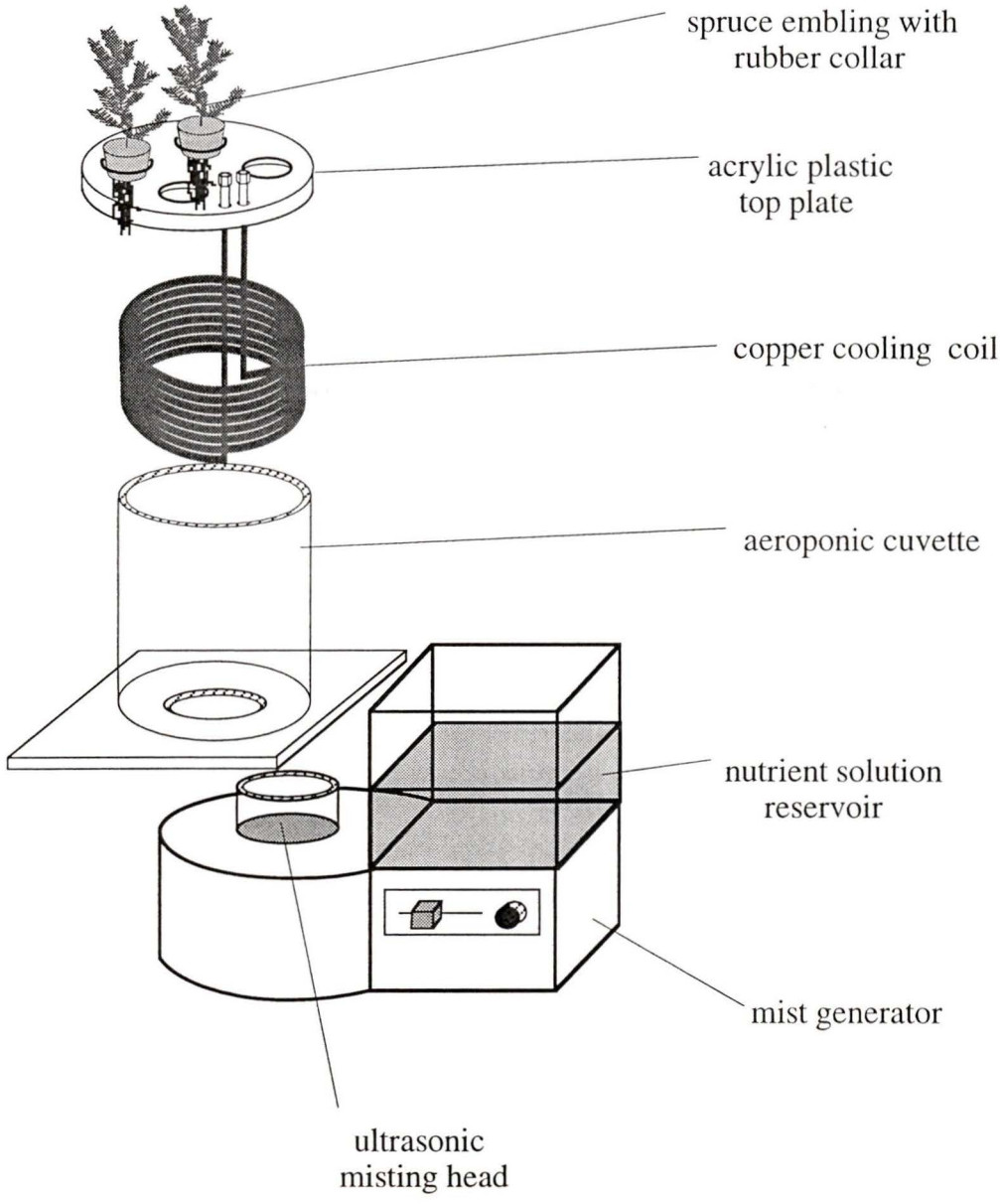

Figure 1. The ultrasonic mist generator with removable top plate. A copper coil maintained the air temperature inside the cuvette at 25 ° C.

3.12 Incorporation of ABA analogs by root misting.

Figure 2 illustrates the root mister used to apply ABA analog solutions to embling roots. Its frame consists of 3 steel rods (0.0063 m diameter, 0.060 m length) attached to circular top and bottom plates of plexiglass (0.020 m O.D., 0.008 thickness). The frame supports the components of the root mister which include; 1) a root misting vessel in which embling roots receive a spray of analog solution 2) a reservoir for the analog solution and 3) a solution delivery pump (Chem-feed Model C-630-P, Blue White industries, Westminster, CA, USA). The root misting vessel is made of plexiglass tubing (0.075 m O.D., 0.065 m I.D., 0.017 m length), the top of which fits into a central hole in the top plate. The bottom of the misting vessel has a PVC end cap drilled through with a drain hole (0.01 m dia) which permits analog solution running off the roots to flow back to the solution reservoir via a connecting tube. Nitex mesh (NGG 52, 335 mm opening, 46 % porosity) over the drain hole traps debris rinsed from the roots and prevents its accumulation in the delivery pump.

The solution reservoir is a PVC plastic pipe (0.015 m O.D., 0.0142 m I.D., 0.020 m length) with glue - on end caps. Contents of the solution reservoir flow to the pump via a tube extending from the bottom of the reservoir. The pump delivers the solution to two misting heads (Baumac, # 19- 2020, St Louis, MO, USA) inserted in the root misting vessel (see enlargement of Figure 2) All plumbing connections of the root mister are made with Dekoron tubing (0.0635 m O.D., type 1300 Dekeron Instrument Components, Aurora, OH, USA). The root mister was installed in the whole plant cuvette system described in appendix I.

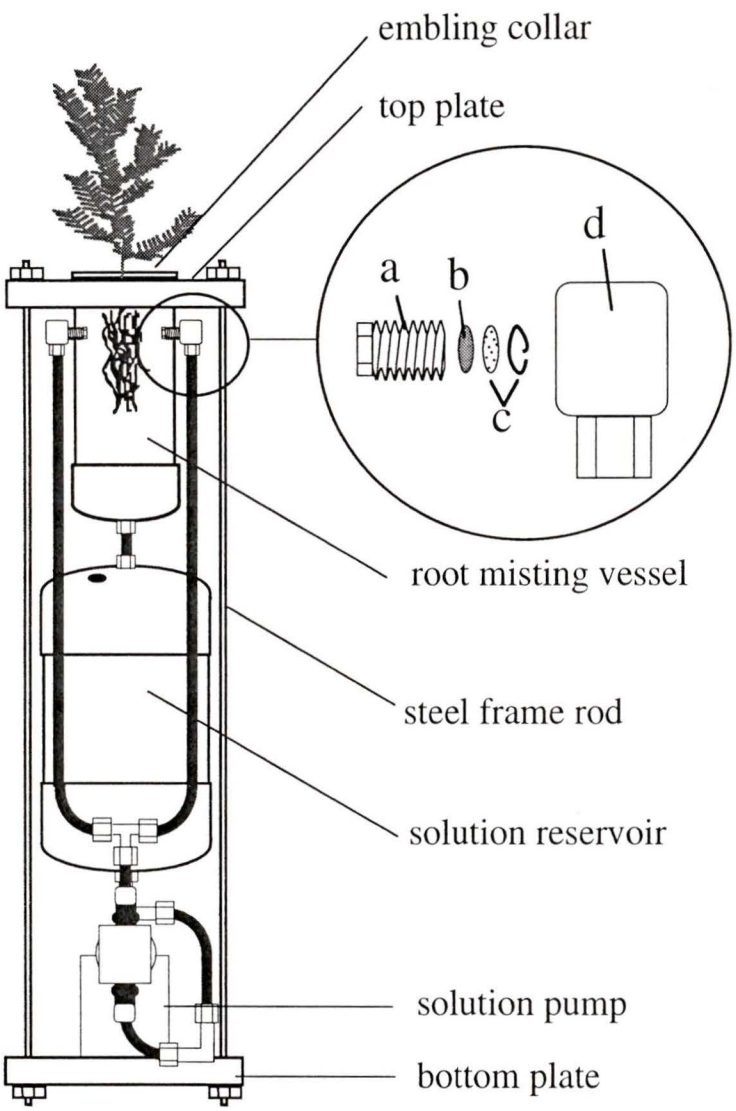

Figure 2. The aeroponic root mister (not to scale). An analog solution (10^{-3} M) held in the solution reservoir flowed into the pump below via a connecting tube. Embling roots in the root misting vessel received the analog solution as a fine spray from two misting heads (see enlargement). The enlargement shows; a) brass misting head b) nylon mesh debris filter c) filter retainer d) nylon plumbing connection.

3.13 Embling and wheat seedling installation in the root mister

Emblings were removed from the root recovery apparatus. The rubber collar at the root-shoot transition was removed and replaced with another collar made from 2 identical, slotted, circular pieces of polycarbonate plastic (radius = 0.038 m , 0.003 m thick). A seal around the stem was made with Plasticine. The collared embling was installed in the root mister with its roots suspended between the misting heads as shown in figure 3. The embling collar functions as a lid to prevent spray leakage during mister operation. Misting with half strength Hoagland's solution was started immediately after embling installation. The installation of wheat seedlings into the mister was the same as for emblings. The wheat seedlings were installed as a 4-6 seedling bundle to provide workable amounts of tissue for ABA uptake efficiency experiments.

3.14 ABA analogs

A selection of 19 ABA analogs to be screened for antitranspirant bioactivity were synthesized at the Plant Biotechnology Institute (PBI, Saskatoon) and kindly provided by Dr. S. Abrams and Dr. P. Rose. The analogs are shown by their structural formulas in figure 3 and are referred to by their PBI designations throughout this text. All of the analogs tested are racemic about the chiral C-1′ center with the exception of PBI-58, and PBI-145, which are optically pure (+)-(S)-ABA and its (-)-(R) enantiomer, respectively.

Analogs differ from one another by changes made in 6 structural regions of S-ABA as shown in figure 4. Changes were made systematically, in one region at a

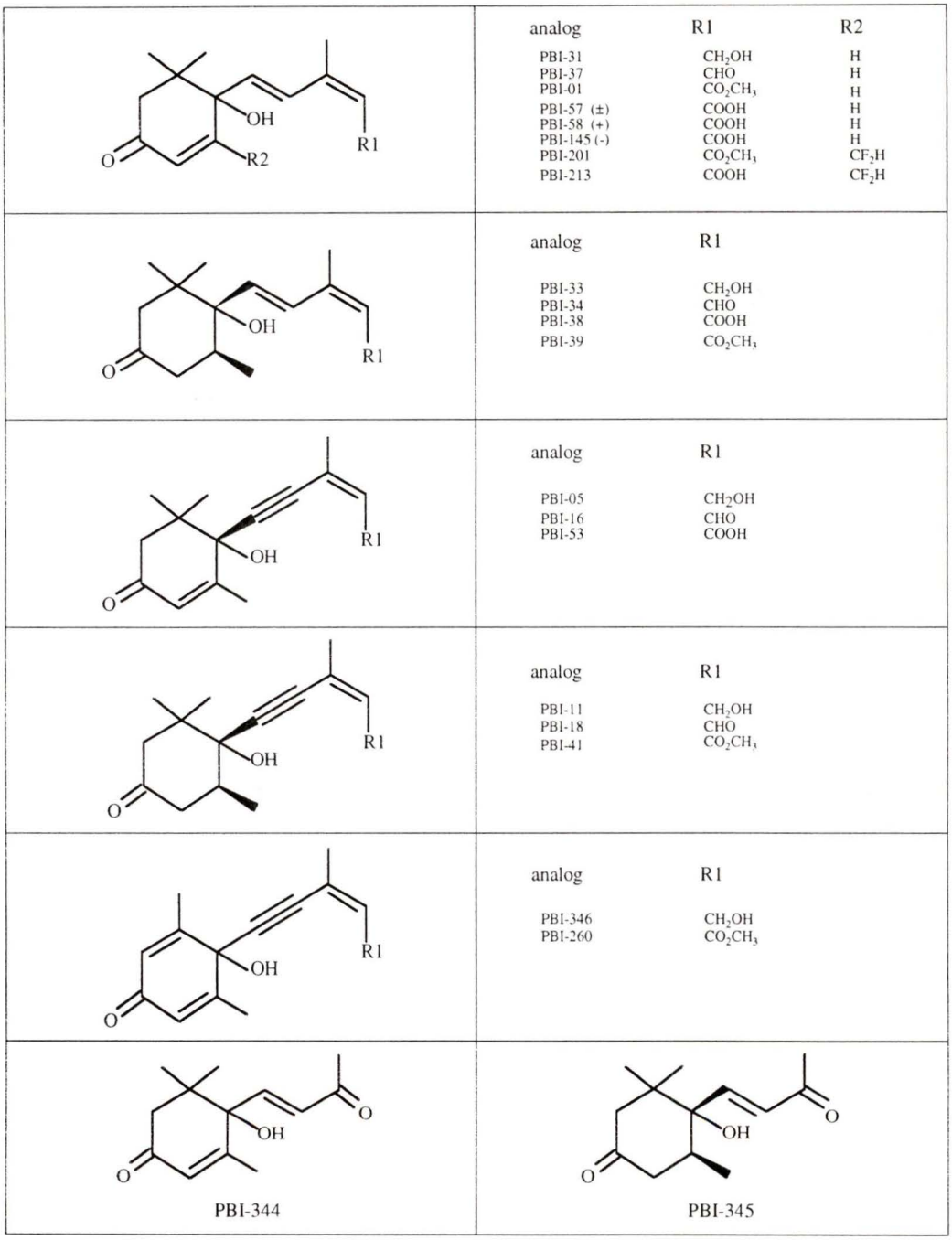

Figure 3. The ABA analogs tested for antitranspirant bioactivity . All analogs were racemic with the exceptions of PBI-58 & 145 which are optically pure S (+) and R (-) - ABA respectively.

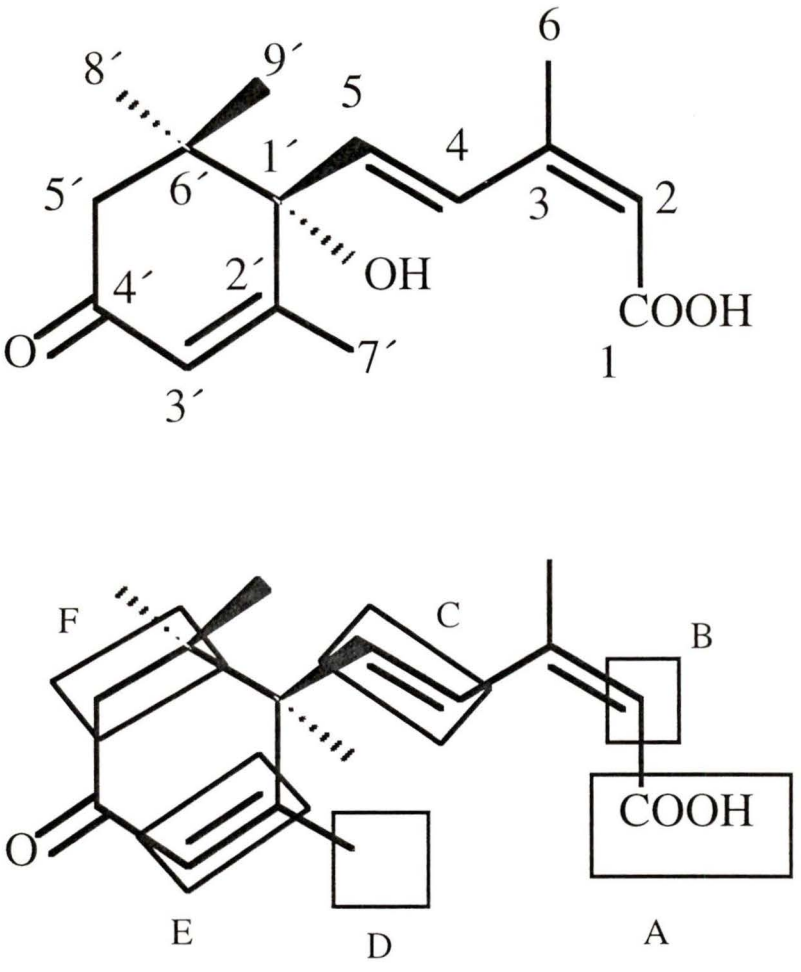

Figure 4. Structural formula of S-ABA showing the conventional numbering scheme above. Outlined regions on the molecule below indicate the regions that were altered to the produce the various ABA-analogs shown in figure 3.

time, to determine the contribution of particular structural attributes to bioactivity. The alterations were; 1) the normal acid functionality at C-1 was changed to the corresponding aldehyde, alcohol or methyl ester, 2) the side chain bond order at C-4, C-5 was changed (trans double bound replaced with an acetylenic triple bond), 3) ring saturation was reduced (C-2′, C-3′ double bond was replaced with a single bond) or increased (the C-5′, C-6′ single bond was replaced with a double bond which required removal of one of the C-6′ methyl groups 4) or the C-7′ methyl group was fluorinated to form CHF_2. The structural alteration made outside of this systematic scheme accounted for the two remaining analogs and consisted of a two carbon truncation of the normal 2-cis-4-trans pentadienoic side chain to form the α - ionone derivatives, PBI-344 and PBI-345.

3.15 Analog activity trials

Prior to analog activity trials, the appropriate concentration at which to apply analog solutions to *Picea* emblings was determined. This assessment was performed by monitoring the antitranspirant response to (±) ABA solutions applied at 3 concentrations, 10^{-5} M, 10^{-4} M and 10^{-3} M with respect to the (+) enantiomer. Three applications were performed at each concentration.

Analog solutions were prepared by dissolving 20-25 mg of analog in 0.5-1.0 ml of 95 % v/v EtOH followed by dilution with distilled water to bring the concentration of the (+) enantiomer to 10^{-3} M. Precautions were taken to reduce analog exposure to light throughout these procedures to reduce potential loss of bioactivity due to cis-trans photoisomerization. A solution of 1% EtOH/H_2O v/v served as a negative control.

The embling shoot was enclosed in a whole plant cuvette system (Livingston *et al.* 1994) which allowed precise control of the shoot environment and continuous measurement of A and E. Throughout the experiment shoots were maintained at 24 ° C and provided with a photosynthetic photon flux density of 1000 µmol m^{-2} s^{-1} using the light control system described by Livingston (1994). The vapor pressure and CO_2 concentration inside the cuvette were 1.3 kPa and 330 µmol mol^{-1} respectively. After the lights were turned on, A and E were allowed to reach steady state values and monitored for 1-2 h to establish their pre-treatment baseline values. The nutrient solution in the root mist reservoir was then replaced with an analog solution. Root misting was briefly interrupted (< 30 s) during this procedure. The embling was allowed to absorb the analog solution over 10 h. At least 3 replications were performed for each analog. Stomatal conductance (g_s) was calculated as E/(L x D), where L is the total projected leaf area and D is the vapor pressure deficit in the cuvette. Estimates of L were based on a linear relationship established between leaf dry weight (DWl) and embling leaf area (figure 5). The leaf area of the emblings used to establish the relationship was measured with a LI-3000 leaf area meter (Li-Cor Inc., Lincoln, NE, USA). DWl was measured after leaves had been dried for 24 h at 72 ° C. The coefficient of variation for the relation between L and DWl was $r^2 = 0.995$ with a SE of ± 3 % .

3.16 Analog bioactivity data acquisition and analysis

Data from the whole plant cuvette system were recorded at 30 s intervals using acquisition and control software (Workbench P.C. Strawberry Tree Inc., Sunnyvale, California, USA) as described in Livingston *et al.* (1994). Average values of A,

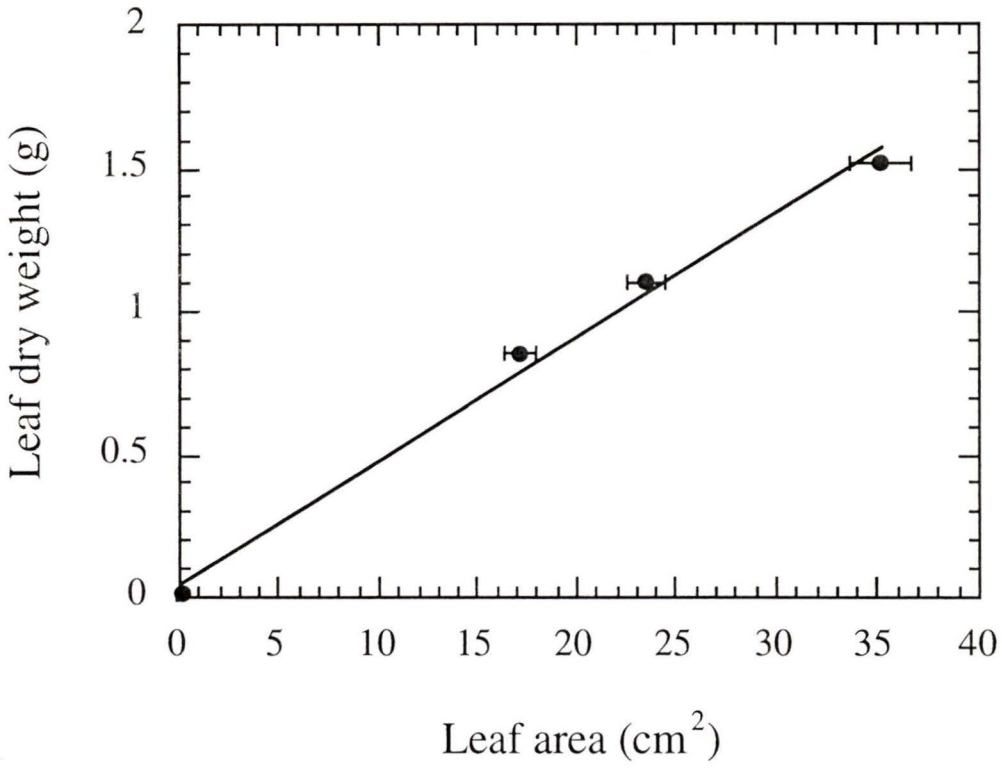

Figure 5. The relationship between the projected leaf area and leaf dry weight of white spruce needles; $y = 0.042 + 0.043x$ ($r^2 = 0.995$) Values are the averages ± SE of four samples.

WUE and g_s over 30 minutes were determined after 3, 5 and 10 h of analog absorption and expressed as a percentage of their pre-treatment, initial values (normalization). Normalized treatment values were compared to the control by ANOVA. The null hypothesis was tested for $p < 0.05$ and 0.10 significance levels.

In addition to determining the bioactivity of individual ABA analogs, the activity of specific analog groupings was also determined. The set of test analogs were grouped and compared according to the scheme shown in figure 11. This analytic scheme allowed the effect of changes in; a) C-4, C-5 side chain bond order (acetylenic *vs* dienoic), b) ring saturation and c) C-7′ fluorination (difluorinated *vs* non-fluorinated) to be examined. The activity of the different analog groups was tested for significant differences relative to the control and each other by a paired t-test at $p < 0.10$. The group analysis included all racemic test analogs but omitted optically pure (+) ABA and (-) ABA as well as the analogs with a truncated side chain, PBI-344 and PBI-345. Optically pure analogs were excluded from the analysis to eliminate the activity effects of C-6 chirality since it could not be incorporated equally as an activity factor between groups. The analogs with a side chain truncation were excluded since they differed from other groups by more than a single structural feature which precluded the possibility of a meaningful activity comparison. Structure - activity relationships were deduced from antitranspirant activity shown by analogs individually as well as analog groups.

3.17 Incorporation of ± [^{3}H] ABA

The relative efficiency of ABA absorption into roots and shoots of *Picea* and *Triticum* was assessed by measuring the uptake of tritiated (±)-ABA applied to

the roots with the aeroponic root mister described above. Labelled (±) [^{3}H]ABA (69 Ci/mmol) (Amersham, Arlington Heights, IL, USA) was added to 0.30 l of freshly prepared, half strength Hoagland's solution to impart an activity of approximately 1.4 x 10^5 dpm/ml. The (±) [^{3} H]ABA was tritiated at stable 3′, 5′ & 7′, positions which would be unlikely to back exchange with H_2O under the pH conditions of the organic extraction protocol described below (Dr. P. Rose; personal communication). Spruce emblings or wheat seedlings were installed in the aeroponic root mister and treated as described in section 3.16. The labelled solution was poured into the reservoir of the aeroponic root mister within 30 minutes of being made and the pump was activated to begin root misting. The rates of transpiration and carbon assimilation were measured continuously during root misting. Emblings (or wheat seedlings) were misted until they had absorbed volumes approximating their pre-treatment fresh weights (5.0 to 15.0 ml of labelled solution, determined from total transpiration data).

After (±) [^{3} H]ABA incorporation, roots were washed with dH_2O to rinse off any unabsorbed labelled ABA. The whole plant was flash frozen in N_2 (l) and lyophilized. Roots were divided from shoots and processed separately thereafter. Root and shoot dry weights were measured. Tissues were then ground into fine powder by mortar and pestle and (±) [^{3} H]ABA was extracted with organic solvents.

3.18 ABA extraction and scintillation counting

Weighed amounts of powdered plant tissue (0.2-0.5 g) were placed in 50 ml plastic centrifugation tubes and extracted in 3 x 20 ml of 80 % (v/v) MeOH/H_2O : 0.5%

(v/v) glacial AcOH using a reciprocating wrist shaker. Successive extraction intervals were 1.0 hr, 0.5 hr and 0.2 hr. Centrifugation between extractions was for 8 min at 3000 × g . Supernatants from each centrifugation were pooled and evaporated to the aqueous phase under reduced pressure. The aqueous sample was adjusted to 20 % (v/v) MeOH/H_2O with 100 % MeOH and adjusted to pH = 3.0 with 4 M HCl. Samples were cleaned by reverse SPE (solid phase extraction) using 1.0 g pre-packed C-18 silica cartridges (Supelco, St Louis, MO, USA). The sample was loaded on to an SPE cartridge and eluted with 3-4 bed volumes of 60 % (v/v) MeOH/ H_2O : 0.5% (v/v) glacial AcOH. The eluted volume was collected and evaporated to dryness under reduced pressure. Evaporation vials containing the dried sample were rinsed 3 times with 100 % MeOH. The pooled sample rinses were reduced to a final volume of 1.0 ml under N_2 gas. Triplicate 200 µl aliquouts of sample were counted for [^{3} H] by liquid phase scintillation counting.

3.19 Determination of (±)-[^{3}H]-ABA uptake efficiency

The efficiency of ABA uptake into root or shoot tissue was calculated as the ratio of the scintillation count of a tissue extract to the total count of the solution absorbed into the tissue sample. The count of the solution absorbed into the tissue sample in turn was calculated as (E_{total} × activity of the misting solution × (tissue dry weight/total embling dry weight)), where E_{total} represents the total volume of water transpired during incorporation. The calculated uptake efficiency was corrected for the amount of radiolabel lost during tissue extraction. The required correction factor was determined from the efficiency with which the extraction protocol recovered a known quantity (1.5 -1.6 x 10^6 cpm) of (±)-[^{3}H]-ABA that

was added to cold (unincorporated) samples (n = 3) of lyophilized *Picea* needles which weighed 0.5-0.6 g.

3.1 RESULTS

A typical time course of the change in g_s in *Picea* resulting from the application of ± ABA solutions ranging in concentration from 10^{-5} M to 10^{-3} M with respect to the (+) enantiomer is shown in figure 6. Stomatal conductance was not significantly affected by the 10^{-5} M solution, whereas the 10^{-4} M solution reduced g_s by nearly 10 % after 10 h. The 10^{-3} M solution caused a decrease in g_s of 50 % and consequently, 10^{-3} M concentrations were used for subsequent analog activity trials. It was felt that because the analogs were likely to be less active than natural ABA, a relatively high concentration of analog had to be applied in order to resolve any activity differences between analogs and establish structure-function relationships.

Table 1 summarizes the results of (±) [^{3} H]ABA uptake efficiency and recovery trial. In *Picea* (n = 2) the uptake efficiency of shoots (2.8 ± 0.6 %) exceeded root uptake efficiency (0.6 ± 0.13 %). By comparison, higher uptake efficiencies were found in both the roots and the shoots of *Triticum* (n = 2). Shoot uptake efficiency in *Triticum* was 6 ± 2 % and root uptake efficiency was not significantly different. The efficiency with which (±) [^{3} H]ABA was recovered from samples (n = 3) of *Picea* needles by means of the extraction protocol was 56 ± 5 %

Figure 7 shows a typical change in g_s, A and WUE over time following the application of a relatively active analog. Since the vapor pressure deficit inside the cuvette was held constant, E and g_s showed identical trends. Figure 8 shows the

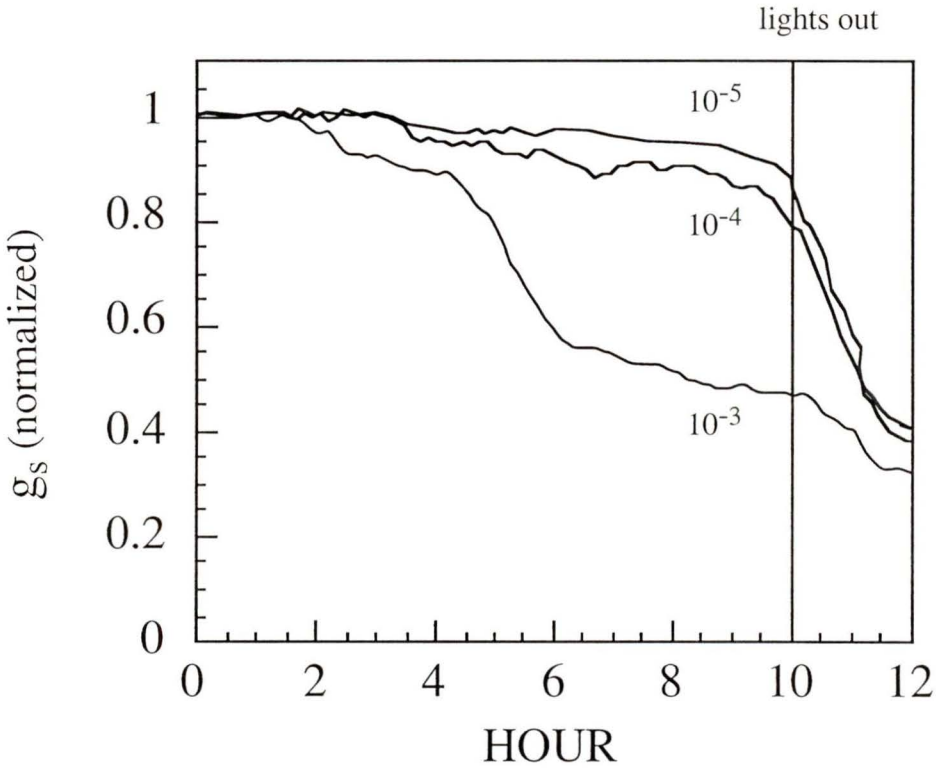

Figure 6. The change in stomatal conductance (g_s) of *Picea* emblings over time resulting from aeroponic application of 10^{-5} M, 10^{-4} M & 10^{-3} M (±) ABA solutions. The stomatal conductance is normalized with respect to the initial stomatal conductance measured before ABA was applied (g_{so}). The vertical line indicates when the lights were turned off.

	Test	Species	Number of Samples	Shoot	Root
a	ABA uptake efficiency	*Picea*	n = 2	(2.8 ± 0.6) %	(0.6 ± 0.13 %)
		Triticum	n = 2	(6 ± 2) %	(6 ± 2) %
b	ABA recovery	*Picea*	n = 3	(56 ± 5) %	-

Table 1. a) The efficiency of (±) [^{3}H]-ABA uptake into the roots and shoots of *Picea* and *Triticum* from an aeroponically applied solution. b) The average amount of (±) ^{3}[H]-ABA recovered from *Picea* needle samples (n = 3) by means of the extraction protocol.

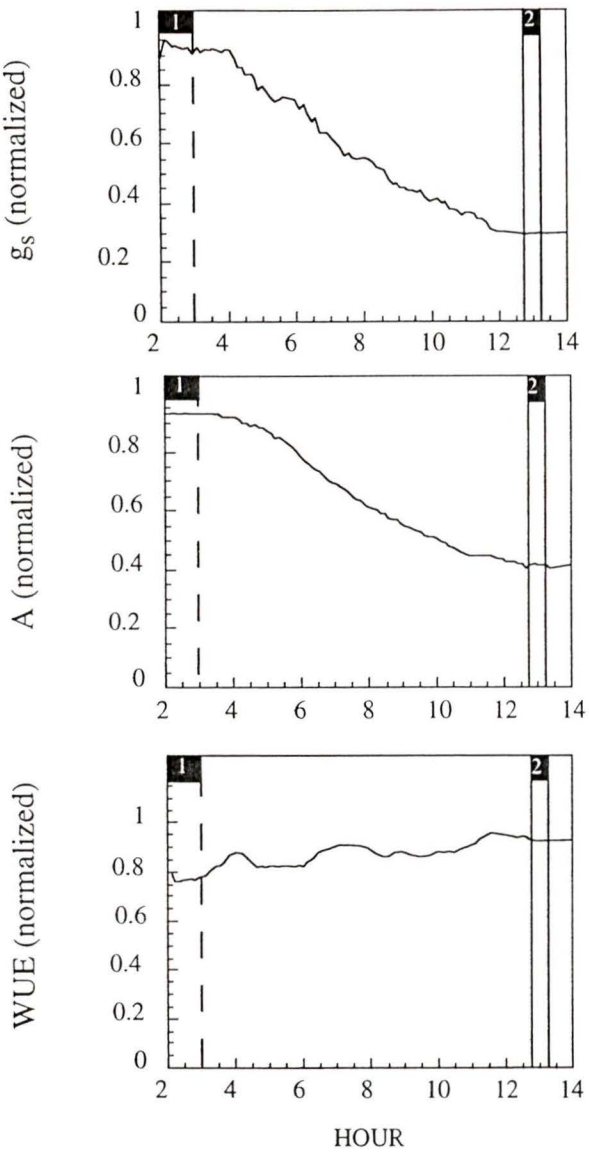

Figure 7. Change in stomatal conductance (g_s), carbon assimilation rate (A) and water use efficiency (WUE) (normalized with respect to pre-treatment values) over time after application of an active ABA analog , PBI-05. The dashed line indicates when the analog was applied. The highlighted region, (1) shows the 1-2 h period over which pre-treatment baseline values were calculated. (2) indicates the 0.5 h interval used to detemine mean values of g_s , A, and WUE after 10 h of analog treatment. Mean values after 3 and 5 h of analog treatment were also calculated over (0.5) h intervals.

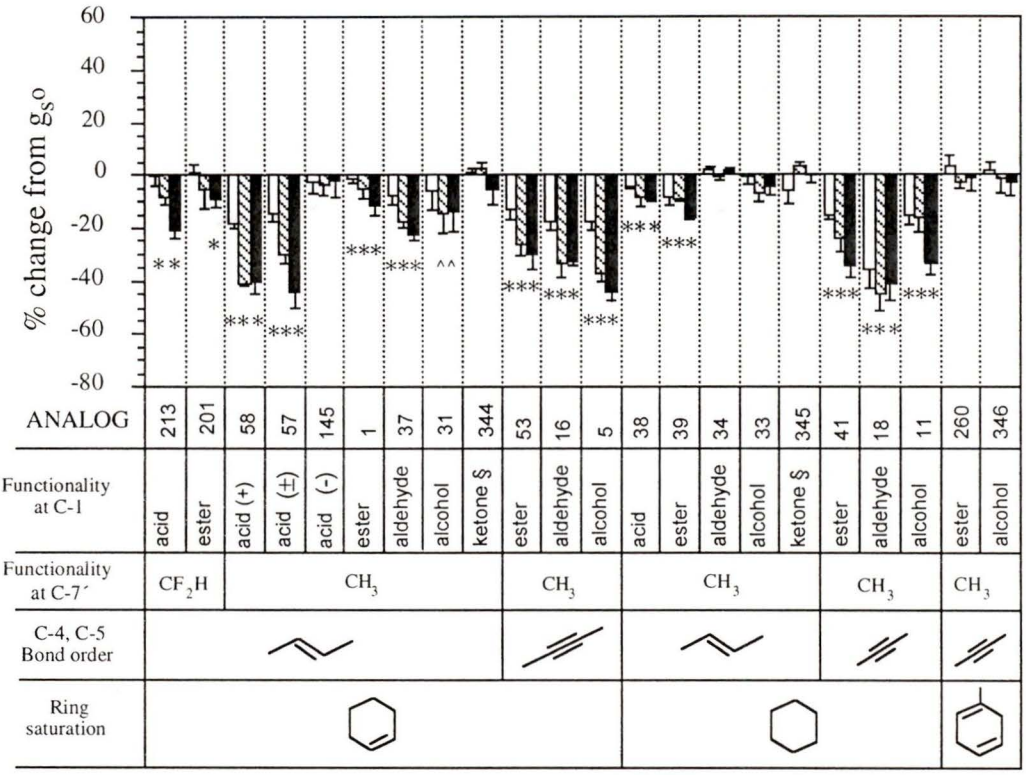

Figure 8. The effects of ABA analogs on stomatal conductance (g_s) after □ 3h ▨ 5h & ■ 10 h of aeroponic treatment. The respective change in g_s caused by each analog is given as the percent change relative to g_{so}, the stomatal conductance measured prior to analog application. The percentages shown represent the change relative to the control. Statistically significant differences between the treatment and the control g_s at any interval (3, 5, 10 h) are denoted by * (significant at $p = 0.05$) and ^ (significant at $p = 0.10$), determined by the ANOVA. The bars represent the means of ($n = 3$) replicates ± SE. The § indicates analogs with a side chain which is 2 carbons shorter than the normal ABA side chain

percent reduction in g_s caused by various analogs after 3, 5 and 10 h of continuous treatment. Analogs have been grouped according to their structural similarities to allow the effects of structural variation to be seen more easily and thereby establish structure-function trends. The reduction in g_s resulting from analog treatment over 10 h ranged from a low of 2 % for the unnatural (-) ABA enantiomer, (PBI-145), to 44 % for the cyclohexenone acetylenic alcohol, (PBI-05). Significant reductions in g_s ($p < 0.10$ or $p < 0.05$) were found for 15 of the 19 analogs

Natural (+) ABA (PBI-58) and (±) ABA (PBI-57) solutions each had 10^{-3} M concentrations of the (+) enantiomer and produced comparable reductions in g_s (41% for (+) ABA *vs* 43% for (±) ABA), but only after a 10 h treatment period (figure 8). After 5 h of treatment, the g_s reduction caused by racemic ABA lagged behind that for (+) ABA by 27 % . Therefore, racemic ABA appeared to be as active as (+) ABA, but exerted its effect more slowly.

Effects of analog structural variations

Change in the C-4, C-5 bond order

The dienoic side chain bond order was varied by conversion of the natural C-4, C-5 trans double bond to an acetylenic linkage. Comparison of dienoic and acetylenic analogs which were otherwise structurally identical, showed the change to an acetylenic bond order generally increased antitranspirant activity. The activity increase resulting from the side chain conversion to an acetylenic linkage was unaltered by reduction of the ring C-2′, C-3′ double bond and occurred in both cyclohexenone and cyclohexanone analogs. For example, amongst the cyclohexenone

variants the reduction in g_s (figure 8) due to the dienoic analog *vs* its acetylenic counterpart was (14 % : 44 %) for the C-1 alcohols, PBI-31 and PBI-05 and (22 % : 32 %) for the C-1 aldehydes, PB1-37 and PBI-16. The dienoic C-1 ester, PBI-01, reduced g_s by 12 % but the acetylenic C-1 ester, PBI-53 reduced g_s by 30 %.

Similarly, amongst the cyclohexanone variants, the reduction in g_s caused by dienoic analogs *vs* their acetylenic equivalents were (16 % and 34 %) for the C-1 esters; PBI-34 and PBI-18, respectively. The dienoic C-1 aldehyde, PBI-34, had no significant effect on g_s while the acetylenic C-1 aldehyde, PBI-18 reduced g_s by 41 %. Similarly the C-1 alcohol, PBI-33 had no significant effect on g_s but the acetylenic alcohol, PBI-11 reduced g_s by 33 %.

Cumulatively these results indicated that the trans C-4, C-5 double bond was not essential to analog activity since its loss by conversion to an acetylenic bond enhanced rather than decreased analog bioactivity.

Change in ring saturation

A. Increasing ring saturation

I. Effect on acetylenic analogs.

Reducing the natural C-2′, C-3′ ring double bond to form a fully saturated ring did not consistently increase or decrease the antitranspirant effects of acetylenic analogs. Instead, analog activity varied with C-1 functionality. For example, a comparison of cyclohexanone to cyclohexenone acetylenic analogs with the same C-1 functionality, shows the corresponding esters (PBI-53 *vs* PBI-41) resulted in

nearly equal reductions in g_s of 32 % and 34 %, respectively, whereas the cyclohexenone aldehyde, PBI-16, which reduced g_s by 32 %, was less active than its cyclohexanone counterpart, PBI-18, which caused a 41 % reduction in g_s. For corresponding alcohols, the activity trend was reversed; the cyclohexenone, PBI-05, reduced g_s by 43 % while the cyclohexanone, PBI-11, reduced g_s by 33 %.

II. Effect on dienoic analogs.

Reduction of the C-2′, C-3′ ring double bond decreased antitranspirant bioactivity of most dienoic analogs. The effect was most evident between the related C-1 acids, PBI-57 and PBI-38, but also occurred between C-1 aldehydes and C-1 alcohols (figure 8). For the C-1 acids, the cyclohexenone, PBI-57, reduced g_s by 43 %, whereas the cyclohexanone, PBI-38, brought about only a 9 % reduction in g_s . Likewise, for aldehydes, the cyclohexenone, PBI-37 decreased g_s by 22 % while the cyclohexanone, PBI-34 had no significant effect on g_s. For the C-1 alcohols, the cyclohexenone, PBI-31 reduced g_s by 14 % compared to a non-significant reduction of 4 % caused by the cyclohexanone, PBI-33. In contrast, reduction of the ring double bond increased activity slightly amongst dienoic C-1 esters; the cyclohexanone C-1 ester, PBI-39 reduced g_s by 16 % compared to the cyclohexenone C-1 ester, PBI-01 which reduced g_s by 12 %.

B. Effect of decreasing ring saturation.

Ring saturation was decreased through the addition of a second double bond at the C-5′, C-6′ position. Two acetylenic analogs, PBI-260 and 346 had this ring structure (figure 3). The ester, PBI-260, decreased g_s by 17 %. By comparison, acetylenic C-1 esters, PBI-53 and PBI-41, with more saturated rings, caused larger decreases in g_s of 30 % and 34 %, respectively. Similarly, the C-1 alcohol, PBI-346, was less active as an antitranspirant than other acetylenic C-1 alcohols with more highly saturated rings like PBI-05 and PBI-11. Therefore, the general effect of adding the second ring double bond was a reduction in bioactivity which was more pronounced in the alcohol than the ester.

C-7′ functionality change

The natural C-7′ methyl group was difluorinated in two analogs; PBI-213 and PBI-201. The C-1 acid, PBI-213 reduced g_s by 21 % compared to a 44 % reduction caused by its non- fluorinated equivalent, (±) ABA. In contrast, the fluorinated C-1 ester, PBI-201, and its non-fluorinated equivalent, PBI-01, caused nearly equal reductions in g_s of 9 % and 12 % respectively.

Change in C-1 functionality

Changing the C-1 acid to either a methyl ester, aldehyde or alcohol generally reduced antitranspirant activity amongst any group of analogs which differed solely by their C-1 functionality. The ester of the dienoic cyclohexanone analogs, PBI-39,

which showed slightly higher activity than the equivalent acid, PBI-38, was an exception to this trend.

Acetylenic analogs with a cyclohexanone ring (41, 18, 11) were less susceptible to loss of antitranspirant activity by reduction of the C-1 functionality than their dienoic counterparts. The acetylenic aldehyde, PBI-18, retained particularly high activity and reduced g_s by 41 %. Likewise, the ester (PBI-41) and alcohol (PBI-11) retained strong antitranspirant influences compared to esters and alcohols of analogs with dienoic side chains, (PBI-01, 39, 31, 33).

The relative activity of C-1 esters and C-1 alcohols showed some dependence on ring saturation. Amongst cyclohexenone analogs, the alcohols (PBI-05 & PBI-31) reduced g_s more than the esters (PBI-53 & PBI-01). The reverse was true amongst cyclohexanone or cyclohexadiene analogs; esters (PBI, 39, 41, 260) reduced g_s more than alcohols (PBI-33, 11, 346).

Neither of the analogs which had a truncated side chain, PBI-344 and 345, caused a significant reduction in g_s .

Carbon assimilation

Figure 9 shows the change in (A) for each analog. Significant reductions in A (after 10 h) were observed for 13 analogs ($p < 0.1$ or 0.05). Analogs with acetylenic side chains caused relatively large reductions in A compared to the other analogs. The acetylenic cyclohexenone analogs reduced A more than the acetylenic cyclohexanone variants; mean reductions in A caused by each group were 30 ± 3 % and 20 ± 3 %

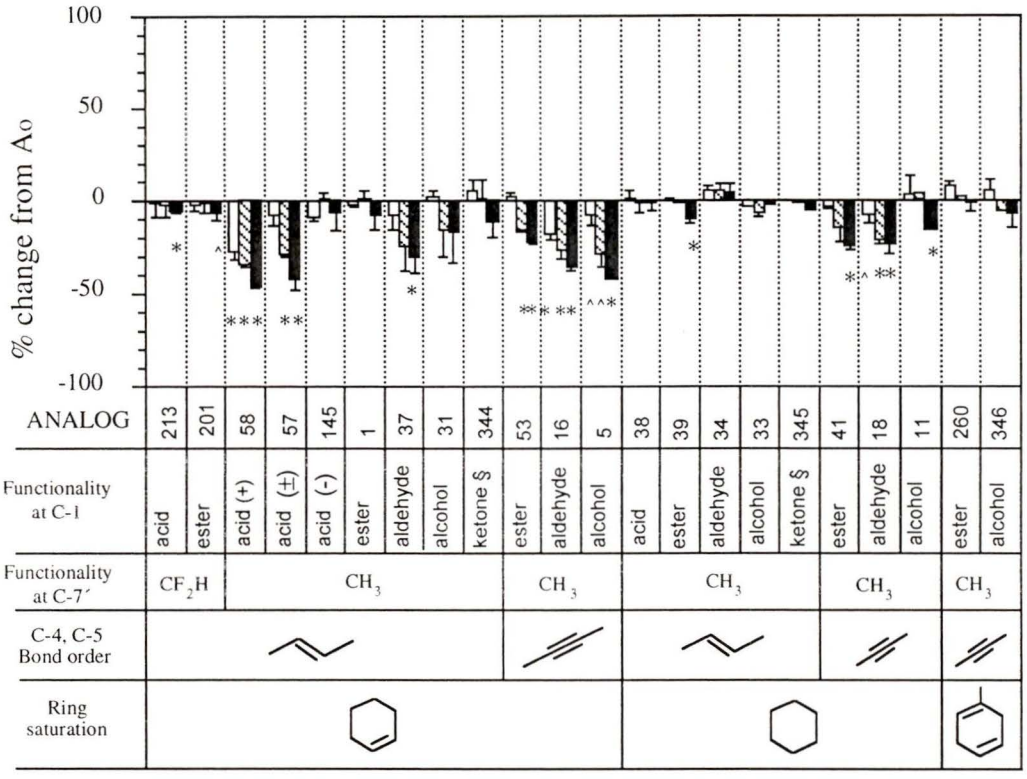

Figure 9. The effects of ABA analogs on carbon assimilation rate (A) after □ 3 h ▨ 5h & ■ 10 h of aeroponic treatment. The respective change in A caused by each analog is given as the percent change relative to A_o, the carbon assimilation rate measured prior to analog application. The percentages shown represent the change relative to the control. Statistically significant differences between the treatment and the control A at any interval (3, 5, 10 h) are denoted by * (significant at $p = 0.05$) and ^ (significant at $p = 0.10$) as determined by the ANOVA. The bars represent the means of ($n = 3$) replicates ± SE. The § indicates analogs with a side chain which is 2 carbons shorter than the normal ABA side chain.

respectively. Amongst the analogs which had a dienoic side chain, only PBI-37 and PBI-39 reduced A significantly. Generally analogs had a less pronounced effect on A than E. This is becauses there is a single resistance to water loss which is imposed by the stomata. Therefore any change in g_s will bring about an equal change in E. However, this does not apply to A because of the additional resistance to carbon fixation imposed by the mesophyll which is large relative to the stomatal resistance. Consequently, any change in g_s will bring about a proportional change in A but A is not affected as much as E.

Water use efficiency

Figure 10 shows the change in WUE found for each analog. Six analogs increased WUE significantly ($p < 0.1$) after 10 h. Four of the six analogs which increased WUE significantly were C-1 esters. The largest increases were caused by the acetylenic esters, PBI-53 and PBI-41, which increased WUE by 20 % and 22 % respectively. Several other analogs reduced WUE, but these reductions were not significant.

Analog activity by structural groupings

All analog groups (1-7) reduced g_s significantly in relation to the control (figure 11a) with the exception of group 3, which included the cyclohexadiene analogs. The lack of activity displayed by group 3 indicates that analog bioactivity is reduced by the insertion of a second double bond at the C-5′-C-6′ position.

The most active analogs groups (1 and 2) had an acetylenic side chain and were significantly more active than their dienoic counterparts, groups 4 and 5, respectively. This indicates that analog activity was significantly increased by the C-4, C-5 bond order conversion to an acetylenic linkage.

No significant reduction of activity occurred between the acetylenic groups, 1 and 2, as a result of elimination of the C-2′, C-3′ ring double bond. In contrast, loss of the same ring double bond between the dienoic groups, (4 and 5), resulted in a significant activity decrease. This suggests that the activity lost through the reduction of the ring double bond can be offset by the presence of an acetylenic side chain.

Group 7, which included the C-7′ fluorinated analogs, was found to be less active than its non-fluorinated counterpart, group 6, but not significantly so (figure 11a). Therefore, analog activity may not have been affected by fluorination of the C-7′ methyl group.

All analog groups reduced A significantly ($p < 0.10$) compared to the control with the exceptions of groups 3 and 5 (figure 11b). Generally, the reduction in A observed for an analog group correlated well with the reduction in g_s observed for the same group. Embling WUE was increased by all analog groups, however, only group 7 (fluorinated analogs) increased WUE significantly ($p < 0.10$) .

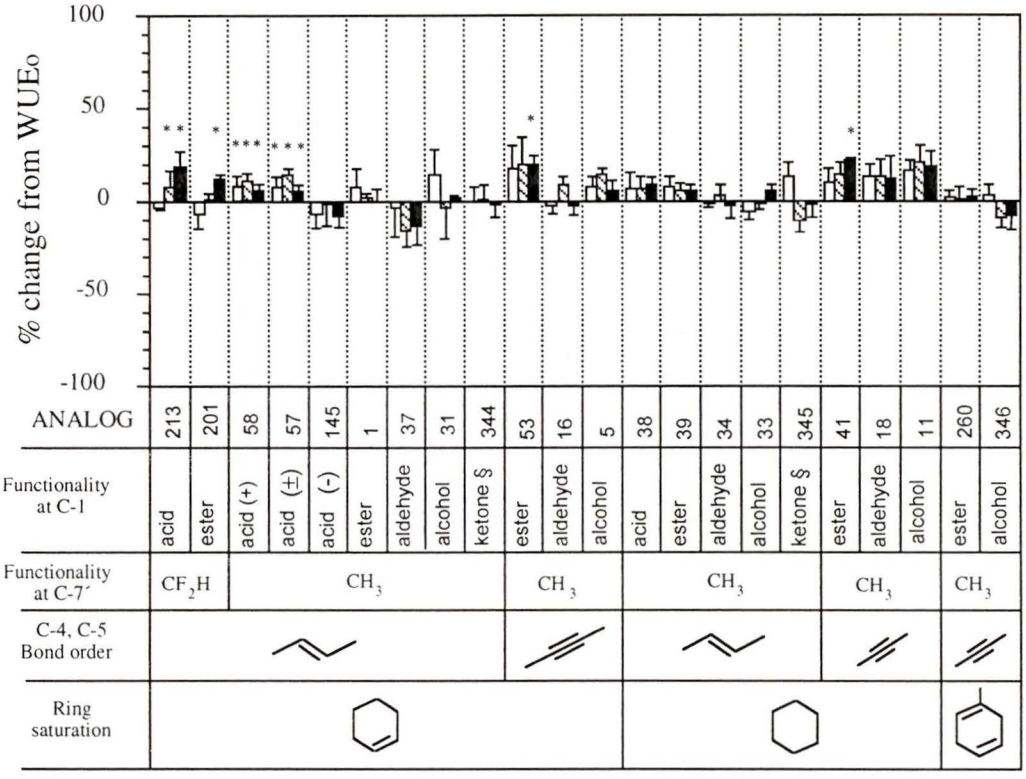

Figure 10. The effects of ABA analogs on water use efficiency (WUE) after □ 3h ▨ 5h & ■ 10 h of aeroponic treatment. The respective change in WUE caused by each analog is given as the percent change relative to WUEo, the water use efficiency measured prior to analog application. The percentages shown represent the change relative to the control. Statistically significant differences between the treatment and the control WUE at any interval (3, 5, 10 h) are denoted by * (significant at $p = 0.05$) and ^ (significant at $p = 0.10$) as determined by the ANOVA. The bars represent the means of ($n = 3$) replicates ± SE. The § indicates analogs with a side chain which is 2 carbons shorter than the normal ABA side chain.

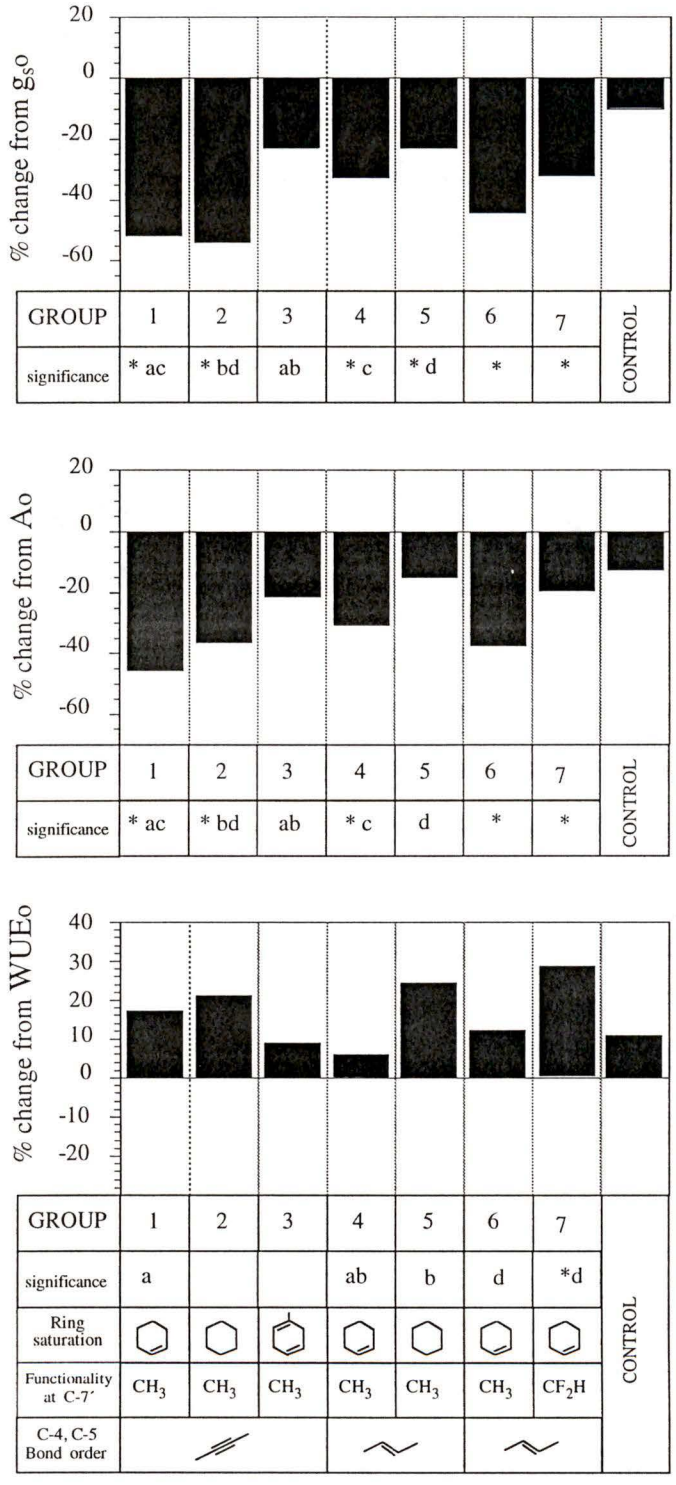

Figure 11. Mean percent change in g_s, A and WUE caused by ABA analog groups 1-7 and the control after 10 h of treatment. Each group included test analogs with the structural attributes shown in (c). Means differ significantly between groups associated by matching letters. A significant difference from the control is denoted by (*).

3.2 DISCUSSION

The results reveal several analog structure-function activity trends. Activity was greatly diminished in dienoic analogs by reduction of the C-2′, C-3′ ring double bond to form cyclohexanone analogs. The acid and ester within this group retained some activity but in the aldehyde and ester, activity almost disappeared. Several studies have reported that an actual or potential C-2′,C-3′ ring double bond is required for analog activity (McWha *et al.* 1973; Orton & Mansfield 1974; Walton 1983). My results do not support this contention but are consistent with the results of Churchill *et al.* (1992) who found dienoic cyclohexanone analogs retain some portion of ABAs activity in promoting freeze tolerance in suspension culture bromegrass.

A bond order conversion of the C-4, C-5 side chain double bond to an acetylene linkage increased analog activity (figure 11a). The activity increase was most conspicuous when compared to the low activity of the dienoic cyclohexanone analogs. The presence of an acetylene bond in the C-4, C-5 position restored activity lost by reduction of the C-2′, C-3′ ring double bond. Apparently, bond order manipulations in these different locations can result in offsetting activity changes.

The amount of activity promoted by C-4, C-5 bond order conversion to an acetylene linkage depended on ring saturation as well as C-1 functionality. Amongst cyclohexenone analogs, an acetylene bond in the C-4, C-5 position increased the activity of esters and alcohols more than the aldehydes in relation to their dienoic counterparts. However, amongst cyclohexanone analogs, acetylenic acids and aldehydes gained more activity than acetylenic esters and alcohols did in relation to their dienoic equivalents. Churchill *et al.* (1992) also reported that ring saturation

and C-1 functionality acted synergistically to modulate the activity increase resulting from a C-4, C-5 bond change to a triple bond .

The insertion of a second ring double bond at the C-3′, C-4′ position has been shown to reduce activity in various growth bioassays (Sondheimer and Walton 1970; Mousseron-Canet *et al.* 1970). My results show that the addition of a second ring double bond at the C-5′, C-6′ position also reduces bioactivity significantly ($p < 0.10$). Therefore addition of extraneous double bonds into the ring may generally diminish activity. This view is supported by the findings of Bittner *et al.* (1977) who found ABA analogs with variously substituted aromatic rings were invariably less active than ABA.

PBI-344 and PBI-345, which both have the unique feature of a truncated side chain, displayed insignificant activity. Reports on the antitranspirant effects of analogs with truncated side chains vary. Walton (1983) reported that analogs with truncated side chains which resembled PBI-344 and PBI-345 were either inactive or displayed low activity, which is consistent with my findings. However, Coke *et al.* (1975) reported that (+) vomifoliol, which occurs naturally and resembles PBI-344 (the compounds differ only in that vomifoliol has a C-1 hydroxyl instead of the C-1 ketone of PBI-344) closed stomata as readily as ABA. They noted, however, that PBI-345 was inactive. Taken together these results indicate that the response to truncated analogs probably varies between species and is dependent on the specific features of the analog.

Analog activity was affected by C-1 functionality. Acids were generally more active than similarly substituted esters, aldehydes and alcohols although amongst highly active acetylenic analogs these differences were often small. Amongst low activity dienoic cyclohexanone analogs, the ester, PBI-39 was more active than the acid, PBI-38. This could reflect more efficient absorption of the ester coupled with reduced catabolic breakdown (Walton 1983). The ester, which is less polar than the acid and unable to form an anion would be expected to be more

permeable to membranes and not subject to compartmentation in alkaline traps; factors which would speed absorption and aid delivery to the site of action. For the other analog esters (PBI-201 and PBI-01) which showed less activity than their corresponding acid despite presumably higher accumulation near the stomata, lower activity could be explained by a slow rate of hydrolysis to form an active compound. Ester activity has been attributed to hydrolytic release of ABA (Walton and Sondheimer 1972) although it is not clear if hydrolysis is a general requirement for ester activity (Churchill *et al.* 1992).

Abscisyl aldehyde (PBI-37) and abscisyl alcohol (PBI-31) were moderately active which is consistent with findings of Churchill *et al.* (1992) but differs from results reported by Orton and Mansfield (1974) who observed high antitranspirant activity for these compounds. Abscisyl aldehyde is the immediate precursor to ABA and its activity has been attributed to the formation of ABA rather than direct action (Taylor *et al.* 1988). Similarly, activity of the abscisyl alcohol is likely the result of a slow conversion to form ABA along a shunt path from the abscisyl aldehyde (Rock *et al.* 1991). Acetylenic aldehydes and alcohols showed high activity compared to abscisyl aldehyde and abscisyl alcohol. Since these analogs have comparable polarity and cannot form the membrane impermeant carboxylic anion, differences in their activity are unlikely to be related to differences in absorption rate or compartmentation. Instead, abscisyl alcohol and aldehyde may be less active because the rate of C-1 oxidation to form the acid group required for activity (Walton 1983) is slow relative to ABA catabolic processes (Churchill *et al.* 1992). Conversely, for acetylenic aldehydes and alcohols, ABA catabolic processes are slow relative to the formation of the active C-1 acid. Therefore, activated acetylenic analogs may have greater persistence *in vivo* which enhances their antitranpirant effects.

Both C-7′ difluorinated analogs (PBI-213 & 201) exhibited lower activity than ABA which is consistent with previous findings (Hampson *et al.* 1992). Todoroki

et al. (1995) argued that fluorinating ABA at catabolic oxidation sites should block catabolism without affecting activity because H and F are sterically very similar. They confirmed this view for analogs fluorinated at the 8′ major oxidation site; such analogs had activity equal to ABA and increased persistence. My results are inconsistent with these findings. The 7′ site is a minor catabolic oxidation site (Hampson *et al.* 1992). Therefore, by the arguments presented by Todoroki *et al.* C-7′ difluorinated analogs such as PBI-213 & 201 should have shown comparable activity and slightly greater resistance to catabolism than racemic ABA. It is unlikely that the C-7′ difluorinated analogs were less active than racemic ABA as a result of differences in transport, sequestration, catabolism or steric differences. However, F is a highly electronegative atom which contributes to the formation of a strong bond dipole. Conceivably, a strong bond dipole in the C-7′ position reduces the binding efficiency between the receptor and agonist, leading to reduced activity. Lack of a similar activity reduction through fluorination at C-8′ could mean that the C-7′ site is relatively more important to receptor coupling than the C-8′ site.

The natural enantiomer, (S)-(+)-ABA and racemic ABA displayed comparable bioactivity. However, (+)- ABA achieved its full effect much more rapidly. The delayed response to (±)-ABA occurred even though (+)-ABA and (±)-ABA solutions had equal concentrations of the active (+) enantiomer. This may indicate that (+)-ABA was absorbed from the racemic ABA solution at a reduced rate which delayed its accumulation and action in the embling. Slow absorption of (+)-ABA from the racemic solution could have occurred if ABA entered embling roots primarily through an ABA selective membrane bound carrier. Astle and Rubery (1980, 1983) provided evidence for such a carrier for ABA in the root tips of *Phaseolus*. They determined that the carrier was specific for the impermeant ABA anion. Recently, Windsor *et al.* (1994) found that the ABA carrier of *Daucus* is also stereoselective for the (+) ABA anion but that the (-) ABA anion could occupy the carrier receptor transiently and inhibit uptake of (+)-ABA. The presence of a

similar carrier in *Picea* roots may explain the dynamics of the response to (±)-ABA *vs* (+)-ABA. However, ABA carriers have been found in herbaceous species only. The presence of similar carriers in woody species has not yet been established. Moreover, a recent study indicates that the absorption of ABA into *Picea* roots is not carrier mediated but occurs by passive diffusion (S. Kaul, University of Victoria, personal communication). The study showed the rate at which tritiated ABA was absorbed into the roots was unaffected when the concentration of unlabelled ABA in the solution applied to the roots was increased. This result argues against carrier mediated ABA uptake because unlabelled ABA would have competed for carrier sites and reduced the absorption rate of tritiated ABA.

ABA uptake efficiency experiments determined ABA was absorbed into *Picea* roots with a mean efficiency of 0.6 ± 0.1 % and into *Picea* shoots with a mean efficiency of 2.8 ± 0.6 %, for ($n = 2$) samples. In *Triticum*, the mean uptake efficiency into both roots and shoots was 6 ± 2 %, determined for ($n = 2$) samples. The uptake efficiencies found for *Triticum* tissues and *Picea* shoots were comparable to those determined in a more extensive study on the same species (S. Kaul, unpublished data). However, the *Picea* root uptake efficiency found here was much lower than the uptake efficiency of ≥ 25 % found in the other study, even though the same methods of assessment and plant stock were used in both studies. The lower uptake efficiency is probably inaccurate and the inaccuracy may be the result of cold storage of the Picea root extracts at -20 ° C prior to further extraction steps and scintillation counting. An effect of cold storage on radiolabel recovery is suggested by the fact that the other tissue extracts (from *Triticum* roots and shoots and *Picea* shoots) were not subjected to cold storage and uptake efficiencies that were consistent with those from the other study were found from these extracts.

A 10^{-3} M ABA solution had to be applied to *Picea* roots to cause a 50-60 % reduction in g_s. In *Triticum*, a comparable reduction in g_s can be achieved with an ABA solution which is 100-fold less concentrated (S. Kaul; personal

communication). Although the uptake efficiency of ABA into *Triticum* shoots was found to be 3-4 times higher than the uptake efficiency that was found for *Picea* shoots, the increase in uptake does not fully account for the response of wheat to ABA concentrations that are 100 times lower than those required to induce stomatal closure in *Picea*. This indicates that *Triticum* may be inherently more sensitive to ABA than *Picea* . It is plausible these species differ with respect to their sensitivity to ABA. Kim *et al.* (1984) showed a difference in sensitivity to ABA between apple and cherry and Quarrie (1983) indicated that sensitivity to ABA varies even between different genotypes of wheat.

II. Analogs of potential use in a nursery pre-treatment

Several of the test analogs were highly active antitranspirants. The analogs that caused the largest reductions in E and simultaneously increased WUE are most likely to improve seedling establishment on reforestation sites.

Analog treated seedlings with low E and elevated WUE are expected to show better survival rates after outplanting despite a reduction in A which limits growth. This is because these seedlings should maintain a more favorable water balance for a longer period, which reduces stress and preserves root turgor and some degree of root growth that is necessary for seedling establishment. Grossnickle and Reid (1984) reported that newly planted seedlings showing root growth were less stressed and better able to maintain normal physiological processes. Therefore, it is anticipated that the main advantage of analog usage is that they may improve root growth under marginal environmental conditions. Their utility would be limited if they were too ephemeral in effect to fulfill this function. Therefore, in addition to conducting short term analog activity tests, which can only serve to detect analogs

of prospective use, it is imperative to perform field tests which assess analog persistence *in vivo* and their ability to increase seedling survival to ultimately evaluate their usefulness.

The increase in WUE promoted by various analogs is shown in figure 10. The C-7′ difluorinated analogs both increased WUE significantly but PBI-213, the C-1 acid also reduced g_s by over 20 % whereas PBI-201, the C-1 ester reduced g_s only by about 10 % which limits its usefulness. In general the acetylenic analogs were very active antitranspirants and reduced g_s by 30 - 45 % relative to the control after 10 h. Amongst the acetylenic-cyclohexenone analogs, the C-1 ester, PBI-53, caused a large and significant increase in WUE of about 20 % whereas the other acetylenic-cyclohexenones showed relatively small increases in WUE. The acetylenic-cyclohexanone analogs increased WUE by about 20 % on average and also reduced g_s significantly as a group, therefore any of these analogs is likely to provide an effective seedling treatment. Therefore, based on their combined ability to reduce E and increase WUE, the analogs which may be of the greatest use in reforestation efforts are; PBI-11, 18, 41, 53 , and 213.

Recently, Grossnickle *et al.* (1996) reported that *Picea* seedlings, that were treated with ABA analogs which differed from ABA by the oxidation level at the C-1 carboxyl, maintained a shoot Ψ that was 50 % higher than controls after three cycles of moderate drought. Under optimum environmental conditions they found the same analogs had only a short term effect on A and g_s, probably due to rapid degradation since ABA catabolism is more rapid at elevated shoot Ψ (Trejo *et al.* 1993). This indicates that the analogs would not impose an undesirable delay on seedling growth if environmental conditions become favorable soon after planting.

Conclusions

Analog activity was determined from the change in seedling gas exchange resulting from application of analog solutions to the roots. Consistent changes in seedling gas exchange resulted from each type of analog structural change. The effects of structural alteration on analog bioactivity were similar to those reported by other studies. The deviations between my findings and previous reports may reflect the effect of analogs within the different plant species that were used in each study. A finding of unambiguous structure-function relationships which were comparable to those of other reports indicates the method used to assess analog activity was accurate and reliable. Therefore, the testing method provided an effective means of screening analogs which could potentially aid reforestation by reducing water stress in planted seedlings. At least 5 of the analogs tested may be useful as reforestation aids based on their combined ability to reduce seedling E and improve WUE. Further research efforts to develop ABA-analogs for reforestation purposes should be undertaken to examine which active analogs affect seedlings most persistently. Analog persistence needs to be evaluated because analogs with a longer lasting effect will reduce water stress in planted seedlings more effectively. Analog uptake into seedlings must also be improved. Experiments showed relatively high analog concentrations were required to affect the seedlings. It may be possible to improve the efficiency of analog uptake into seedlings by means of combined chemical-physical treatments currently under investigation.

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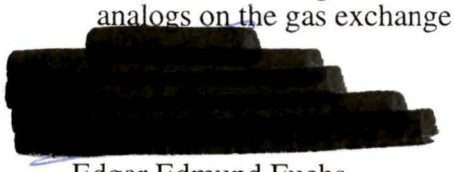
Author 

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