The effects of chemical preconditioning on physiological changes in drought-stressed plants

By

Md. Anisul Islam

A thesis submitted in conformity with the requirements for the degree of Master of Science in Forestry Graduate Department of Forestry University of Toronto

© Copyright Md. Anisul Islam 1999
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-45432-0
FACULTY OF FORESTRY
University of Toronto

DEPARTMENTAL ORAL EXAMINATION FOR THE DEGREE OF
MASTER OF SCIENCE IN FORESTRY

Examination of Mr. Anisul ISLAM

Examination Chair's Signature: 

We approve this thesis and affirm that it meets the departmental oral examination requirements set down for the degree of Master of Science in Forestry.

Examination Committee:

Date: June 7, 1998
The effects of chemical preconditioning on physiological changes in drought-stressed plants

Graduate Department of Forestry, University of Toronto

Abstract

The use of antisenescence compounds to precondition seedlings was studied in white pine (Pinus strobus L.). In a preliminary study, the effectiveness of eight compounds were tested by their ability to prevent desiccation-induced membrane leakage in Phaseolus vulgaris. When fed through the xylem and roots, Ambiol, spermine and aminoethoxyvinylglycine prevented the decline in water potential under drought in white pine seedlings. These compounds also ameliorated gas exchange, reduced membrane leakage and inhibited ethylene production under drought. Ambiol stimulated osmotic adjustment and inhibited transpiration. Spermine increased elastic adjustment, enhanced photosynthesis and water use efficiency. These results showed that preconditioning improved water relations and gas exchange. The ability of these compounds to delay key senescence reactions in white pine was shown by the reductions in ethylene production and membrane leakage. Preconditioning with antisenescence agents may thus be a viable option to enhance survival and growth of seedling transplants.
ACKNOWLEDGEMENT

I express my gratitude to my supervisor Dr. T. J. Blake for his consistent support and guidance throughout my study period. I truly appreciate that he has given me the foundation and the opportunity to broaden my knowledge in forest tree ecophysiology. I am also grateful to the members of my supervisory committee, Drs. D. N. Roy and J. R. Malcolm for their valuable comments and suggestions on my work. Without their help and support this work could not have been completed.

I would also like to thank the members of the Physiology Group: Bob Ocran, Thomas Teklemariam and Ferit Kocacinar for their encouragement and contributions. Special thanks go to Dr. L. R. Rajasekaran for his generous help and suggestions.

I am indebted to Khulna University, Bangladesh, for granting me the study leave and Canadian Commonwealth Scholarship and Fellowship Program for financial support. The financial assistance from the School of Graduate Studies, University of Toronto, is also gratefully acknowledged.

Finally, I would like to acknowledge the encouragement that I received from my parents, my wife Sharmin and my son Rashad.
# TABLE OF CONTENTS

**ABSTRACT** .......................................................................................................................... ii  
**ACKNOWLEDGEMENTS** ........................................................................................................ iii  
**TABLE OF CONTENTS** ......................................................................................................... iv  
**LIST OF FIGURES** .............................................................................................................. vii  
**LIST OF TABLES** ................................................................................................................ vii  
**LIST OF ABBREVIATIONS** .................................................................................................. x  
**CHAPTER I: GENERAL INTRODUCTION** ............................................................................. 1  
**CHAPTER II: REVIEW OF LITERATURE** ............................................................................. 4  
  
2.1 Introduction ....................................................................................................................... 4  
2.2 Drought tolerance and avoidance .................................................................................. 4  
2.3 Leaf senescence ............................................................................................................... 7  
2.4 Role of antisenescence, antitranspirant and antioxidant compounds ............................. 11  
  
2.4.1 Spermine (Polyamine) ................................................................................................. 11  
2.4.2 Ambiol (Antioxidant) ................................................................................................. 14  
2.4.3 Cytokinin (Kinetin) .................................................................................................... 15  
2.4.4 Salicylic Acid .............................................................................................................. 16  
2.4.5 AVG (Aminoethoxyvinylglycine) ................................................................................ 17  
2.4.6 CaCl₂ (Calcium chloride) ............................................................................................ 17

iv
LIST OF FIGURES

Figure 3.1. Effect of various concentrations of spermine on desiccation-induced electrolyte leakage (%)........................................................................................................... 29

Figure 3.2. Effect of various concentrations of AVG on desiccation-induced electrolyte leakage (%)........................................................................................................... 29

Figure 4.1. Effect of different chemical compounds on midday needle pressure potential of white pine seedlings after 11 days of drought........... 44

Figure 4.2. Effect of different chemical compounds on relative water content at zero turgor (RWC⁰) of white pine seedlings........................................... 45

Figure 4.3. Effect of different chemical compounds on apoplastic water fraction (%) of white pine seedlings................................................................. 46

Figure 4.4. Effect of different chemical compounds on osmotic potential at full turgor (π⁽¹⁰⁰⁾) of white pine seedlings....................................................... 47

Figure 4.5. Effect of different chemical compounds on osmotic potential at zero turgor (π⁰) of white pine seedlings......................................................... 48

Figure 4.6. Effect of different chemical compounds on bulk modulus of elasticity (ε) near full turgor of white pine seedlings................................. 49

Figure 4.7. Effect of various compounds on water use efficiency (WUE) of white pine seedlings after 11 days of drought............................................. 50

Figure 4.8. Effect of various compounds on net photosynthesis (μmol m⁻² s⁻¹) of white pine seedlings after 11 days of drought................................. 50
Figure 5.1. Effect of different chemical compounds on midday needle pressure potentials of white pine seedlings after 16 days of drought........ 66

Figure 5.2. Effect of different chemical compounds on ethylene production of white pine seedlings after 16 days of drought.................. 67

Figure 5.3. Effect of different chemical compounds on drought-induced membrane leakage of white pine seedlings after 16 days of drought....... 68

Figure 5.4. Effect of different chemical compounds on net photosynthesis (μmol m⁻² s⁻¹) of white pine seedlings after 16 days of drought................ 69

Figure 5.5. Effect of different chemical compounds on transpiration rate (μmol m⁻² s⁻¹) of white pine seedlings after 16 days of drought............... 70

Figure 6.1. Schematic diagram showing the sequence of hypothetical changes that occur in preconditioned and unconditioned white pine seedlings under drought.............................................................. 78
LIST OF TABLES

Table 2.1. A summary of senescence-retarding effects of polyamine in different plant species. .......................................................... 12

Table 3.1. Effects of various senescence inhibitors on membrane leakage in dehydrating leaves of Phaseolus vulgaris. .................................................. 25

Table 4.1 Effect of xylem feeding with various compounds on water relations parameters of well watered white pine seedlings for 11 days, after terminating xylem feeding. ............................................................. 51

Table 4.2 Effect of xylem feeding with various compounds on water relations parameters of drought-stressed white pine seedlings for 11 days, after terminating xylem feeding. ............................................................. 52

Table 6.1 Effects of different chemical compounds on physiological processes under drought. ........................................................................ 75
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varepsilon_{\text{max}}$</td>
<td>Bulk modulus of elasticity</td>
</tr>
<tr>
<td>$\pi^0$</td>
<td>Osmotic potential at zero turgor</td>
</tr>
<tr>
<td>$\pi^{100}$</td>
<td>Osmotic potential at full turgor</td>
</tr>
<tr>
<td>$\psi_x$</td>
<td>Xylem Pressure Potential</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>AVG</td>
<td>Aminoethoxyvinylglycine</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>Cs</td>
<td>Stomatal Conductance ($\text{cm}^3\text{ s}^{-1}$)</td>
</tr>
<tr>
<td>DAP</td>
<td>1,3-Diaminopropane</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EL</td>
<td>Electrolyte leakage</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detector</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HO'$_2$</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MPa</td>
<td>Megapascals</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>O$_2$'</td>
<td>Superoxide ions</td>
</tr>
<tr>
<td>OH'</td>
<td>Hydroxy radical</td>
</tr>
</tbody>
</table>
PAs  Polyamines
PGR  Plant growth regulator
Pn   Net Photosynthesis (μmol m⁻² s⁻¹)
PV   Pressure Volume
RNA  ribonucleic acid
RWC  Relative water content (%)
RWC⁰ Relative water content at zero turgor
SAM  S-adenosylmethionine
SAR  Systemic acquired resistance
Spd  Spermidine
Spm  Spermine
TBA  Thiobarbituric acid
TCA  Trichloroacetic acid
TI   Transpiration rate (μmol m⁻² s⁻¹)
tRNA Transfer ribonucleic acid
WUE  Water use efficiency
Va   Apoplastic water fraction (%)
CHAPTER I

General Introduction

Growing demand for wood and fiber has led to the establishment of large-scale plantations all over the world. Environmental stress, particularly drought, leads to early senescence and high mortality rates of transplanted seedlings. According to a recent estimate, one third of all transplanted conifer seedlings do not become established (Hearnden et al., 1992). Such losses threaten the long-term productivity of conifer plantations. Some species, for example white pine, are susceptible to competition for light and moisture (Wendel and Smith, 1990) which slows their early growth rate.

Drought is one of the main causes of slow growth in woody plants (Kramer, 1986). When sustained, drought causes the senescence of leaves and eventually, the whole plant. Leaf senescence is characterized by an increase in proteolysis, breakdown of chlorophyll, permeability of membranes and elevated levels of hydrogen peroxide (Trippi and Thimann, 1983). The ability of some pretreatments to delay leaf senescence could help to sustain tree and forest productivity.

Since membranes are the primary sites of desiccation injury (Levitt, 1980), they play a vital role in survival and growth of plants under drought. Fan and Blake (1994) observed that the loss of membrane integrity increased electrolyte leakage in droughted black spruce seedlings. During drought, dehydration caused a decline in sterols, lipids and their ratio, suggesting that drought increases membrane leakage by altering membrane constituents (Zwiazek and
Blake, 1990b). The loss of membrane integrity leads to the loss of control over key regulatory and receptor functions, which eventually results in whole-plant senescence (Crow et al., 1993). Drought also increases membrane permeability through photooxidative phenomena that accelerate senescence (De Luca d'Oro and Trippi, 1987). Drought increases ethylene production and the increase in membrane permeability in droughted plants also correlates with an increase in ethylene concentration (Hipkins and Hillman, 1985). The rate of net photosynthesis and level of key PGRs (plant growth regulators) also decline under drought, and the resulting depletion of carbohydrate reserves (Winston, 1990) and decreased synthesis or flow of cytokinin from the roots (Nooden and Letham, 1985) lead to senescence.

Since seedlings are currently grown under optimal conditions in greenhouses or nurseries, plantation failure is common with some conifers. To increase the success rate of plantation establishment, seedlings need to be preconditioned to withstand the rigors of the harsh planting sites. Seedlings can be hardened using mild stress or a range of chemicals (van den Driessche, 1992; Warren, S. 1996; MScF thesis, University of Toronto). However, the use of mild stress has not been very successful as a preconditioning regime. For example, such a preconditioning had no effect on the growth of red spruce (Seiler and Cazell, 1990) and even increased mortality rates in Corsican pine seedlings (Guehl et al., 1993). Hence, it has become necessary to find alternative approaches using a range of chemicals that may be more effective and can provide a cheap and precise method of hardening seedlings.
Pretreatment of seeds (Darlington et al., 1996; Rajasekaran and Blake, 1998) and excised leaves (Borrell et al., 1997) with growth regulators has been found to enhance growth or retard senescence respectively. However, the effect of such chemical pretreatments on whole plant senescence has not been studied in detail. Therefore, seedling pretreatment by chemicals with antisenescence, antitranspirant and antioxidant properties may increase drought tolerance in white pine seedlings by protecting membrane integrity.

The objectives of this study were two-fold: 1) to determine whether plant drought resistance can be enhanced through the use of appropriate chemical treatments that delay senescence; and 2) if so, to study their mode of action.

Chapter II reviewed the relevant literature, and Chapters III, IV and V described the methods and results of several experiments, which are written as journal papers. Chapter III was a preliminary study that used excised bush bean leaves to determine the most effective compounds and concentrations. The effectiveness of the compounds was determined by their ability to prevent desiccation-induced membrane leakage. Chapter IV dealt with the effects of different compounds on shoot-water relations of white pine seedlings under drought. Chapter V focused on the effect of various compounds on different senescence-related parameters. Chapter VI presented the conclusions of the study and summarized its contribution to the field of study.
CHAPTER II

Review of Literature

2.1 Introduction

The chapter will review the adaptive mechanisms that allow woody plants to withstand water stress and, the role of physiological changes in drought-induced senescence. Three major areas will be considered. Firstly, dehydration tolerance and avoidance adaptations will be compared. Secondly, the senescence process will be reviewed. Finally, the ability of different chemical compounds to delay senescence under drought will be considered.

2.2 Drought tolerance and avoidance

Drought is the major environmental constraint on tree growth and forest productivity. As the stress develops, plants exhibit a cascade of responses involving biochemical and morphological adjustments occurring at different time scales (Larcher, 1995). Levitt (1980) divided drought tolerance into two categories: dehydration tolerance and dehydration postponement (avoidance). Similar categories, with different terminology, have also been used by others (Turner, 1986; Kozlowski et al., 1991; Larcher, 1995). Dehydration tolerance refers to the ability to maintain turgor at lower water potentials. By contrast, dehydration postponement involves activating those mechanisms that enable the plant to maintain tissue water content under drought imposed by air and soil dryness. This may be achieved by improved uptake of water from the soil, reduced water loss, or increased water storage within the plant (Larcher, 1995).
Reduction in water loss under drought is often accomplished by leaf shedding (Kramer 1980). Stomatal closure also prevents water loss in many species under drought (Levitt, 1985; Steinburg et al., 1990). However, stomatal closure also reduces carbon dioxide uptake which can have a detrimental effect on plant productivity (Blake and Tschaplinski, 1992). Under drought, plants may increase absolute root growth (Hays et al., 1991) or root growth relative to the shoot by increasing root to shoot ratio (Seiler and Johnson, 1984; Blake and Filho, 1988; Steinberg et al., 1990).

It has been observed that since faster growing poplar clones (Populus spp.) maintained a greater stomatal conductance and transpiration rate during drought than slower growing clones, they were more drought tolerant (Tschaplinski and Blake, 1989). Similar results were observed when faster growing black spruce (Picea mariana Mill. B.S.P.) families were exposed to osmotic stress (Tan et al., 1992a). By contrast, stomatal conductance was negatively correlated with growth rate of Eucalyptus grandis (W. Hill ex Maid.) under drought which allowed faster-growing clones to maintain a greater leaf area with increasing water stress (Blake and Filho, 1988). These contrasting results suggest that drought tolerance mechanisms vary depending on the species and their ecological requirements. In contrast to the eucalypt species, it was observed that black spruce families, some other conifers and many poplar species rely more on dehydration tolerance (Tan et al., 1992b; Fan et al., 1994). It is not clear which type of mechanism is more efficient in energetic terms. McCree and Richardson (1987) could detect no clear productivity advantage
between dehydration tolerance mechanisms and those that postponed dehydration.

Osmotic and elastic adjustment are dehydration tolerance mechanisms, that permit turgor (result of hydrostatic pressure in cells which occurs when cell pressure balances the difference in water potential between the environment around the cells and the cytoplasm) and growth to be maintained under lower water potential, which will enhance survival under drought (Kramer, 1980). Elastic adjustment refers to modifications in cell walls that render them more elastic (Blake and Tschaplinski, 1992). A more elastic cell wall can shrink during tissue dehydration and tends to maintain the cell’s turgor. Change in cell elasticity in response to drought has been reported in poplar (Tschaplinski and Blake, 1989), white spruce (Koppenaal et al., 1991) and black spruce (Fan et al., 1994).

Plants also rely on osmotic adjustment for maintenance of turgor at low water potentials. Osmotic adjustment maintains cell water contents by increasing the osmotic concentration of solutes, which increases water uptake. The adjustment results from compatible organic solutes accumulating in the cytoplasm which lower the osmotic potential of the cytosol. Osmotic adjustment under drought has been observed in many forest species including western hemlock (Kandiko et al., 1980) and black spruce (Tan et al., 1992b). However, the degree of adjustment depends on the species and the duration or severity of the stress (Turner and Jones, 1980). Osmotic adjustment under drought has proven to be beneficial for the plants. Because compatible solutes accumulate in
the cytoplasm, enzyme function is maintained, the water content of the cell remains high and the concentration of regulatory ions is buffered against change. These changes maintain a positive turgor which allows a moderate amount of growth where none would otherwise occur (Meyer and Boyer, 1972; Michelena and Boyer, 1982).

2.3 Leaf senescence

Water deficiency is the most significant factor limiting early seedling survival and growth (Blake, 1983). Sustained water stress causes premature senescence. The physiological function of most multi-cellular organisms reaches a peak and then declines. This process of decline leading to death has been termed senescence (Nooden, 1980), with a distinction having been made between degenerative changes that lead to death (senescence), and those that do not necessarily cause death and physiological aging. Senescence can occur as a result of natural causes, such as those that occur seasonally and in response to environmental stress. Water stress is known to accelerate processes associated with senescence of plant parts and in whole plants (Aharoni and Richmond, 1978; Dwivedi et al., 1979). Water stress modifies chlorophyll and protein content (Vieira De Silva, 1976), affects photosynthesis and respiration processes (Bunce, 1982), and increases membrane permeability (Mukherjee and Choudhuri, 1981) which lead to senescence. These parameters can be used to measure senescence. Water stress also induces activation of enzymes, both
hydrolytic (Martin and Thimann, 1972) and oxidative (Mukherjee and Choudhuri, 1981; Kar and Misra, 1976).

Leaf senescence is usually quantified as i) breakdown of chlorophyll, ii) increase in hydrogen peroxide levels or iii) increase in membrane permeability (Thimann, 1980, Trippi and Thimann, 1983, Dhindsa et al., 1981). Water stress induces changes in cellular macromolecules that are associated with leaf senescence (Mukherjee and Choudhuri, 1981). The changes occurring in the leaves appear to be a primary cause of leaf senescence. Changes in membrane function occur in early stages of plant injury (Levitt, 1980) and are some of the first physiological changes detected under water stress.

Chloroplasts also show some of the earliest signs of physiological decline. Water stress led to an impairment of both chlorophyll and protein metabolism which show identical symptoms during senescence (Mukherjee and Choudhuri, 1981). Among these, the decrease in ribulose-1, 5-bisphosphate carboxylase – oxygenase (RUBPCase) activity is one of the first and most important. Correspondingly, there is an early loss of chloroplast ribosomes that appears to precede the decrease in cytoplasmic ribosomes. Although the chloroplasts start changing relatively early, they and other organelles may still be active when the tonoplast ruptures (Borton, 1966; Butler, 1967). Tonoplast breakdown releases hydrolytic enzymes, acids and toxic compounds that initiate cellular degradation (Matile, 1976; Nishimura and Beevars, 1978). However, Woolhouse and Batt (1976) suggested that cessation of chloroplast protein and nucleic acid synthesis
resulted in declining enzyme activities and the photosynthetic rate; which, in turn, led to plastid disintegration and visible senescence.

The decline in proteins during plant senescence is due primarily to changes in the cell chloroplast, while the final, more rapid decline involves changes in both the chloroplast and the cytoplasm (including other organelles) (Callow, 1974; Woolhouse, 1967).

The function of membrane proteins is affected by two processes during senescence. Free radicals generated during membrane lipid degradation attack amino acid residues of proteins including histidine, methionine and cysteine, causing configuration changes in these proteins (Nagy and Floyd, 1984; Wilson, 1985). These changes, in turn, have a deleterious affect on receptor function (Kirby and Green, 1980) of the cell which leads to senescence (Crow et al., 1993).

Lipids in functioning biomembranes maintain their fluidity, which allows enzymes, receptors and other structural components of membrane proteins to remain active (Magin et al., 1990; Viret et al., 1990; Kinnunen, 1991). During water stress and senescence, there is a decline in the membrane lipids (Zwijazek and Blake, 1990b), and in addition, the membrane protein content and their relative composition also decline. Protease activity increased during senescence (Peoples and Dalling, 1987), causing total membrane protein content to decline during senescence (Duxbury et al., 1991b). Proteins are subjected to proteolysis, which appear to be related to the generation of free radicals, especially superoxide anions (Duxbury et al., 1991). Studies by McRae et al. (1982) and
Kacperska and Kubacka-Zebalska (1985, 1989) have established a correlation between superoxide formation, an increase in ethylene production, and senescence which would explain the membrane deterioration during senescence in terms of drought-induced ethylene production. Senescence in plant tissues is accompanied by changes in membrane permeability (Dhindsa et al., 1991). Permeability changes have also been correlated with a increase in ethylene evolution (Hipkins and Hillman, 1985) and a decline in membrane lipids (Ferguson and Simon, 1973; Simon, 1974 and 1977; Zwiazek and Blake 1990b).

The process of senescence was also correlated with decreasing polyamine levels in intact bean and detached raddish leaves (Altman and Bachrach, 1981), oat protoplasts (Kaur-Sawhney et al., 1982), and detached leaves of barley (Coghlan and Walters, 1990) and rice (Chen and Kao, 1993).

The inhibition of ethylene evolution, lipid peroxidation, free radical formation and membrane stabilization are likely to be the key processes to defer drought-induced senescence in plants. Since Ambiol is an antioxidant (Santruœk and Krepelka, 1988), it could prevent membrane damage under drought. The maintenance of intact cellular membranes may also prevent other detrimental effects of water stress. Antioxidants can also reduce endogenous lipoxygenase activity in senescing tissues (Siedow, 1991). Polyamines, particularly spermine, are known antisenescence agents (Kaur-Sawhney et al., 1978). Exogenous cytokinin inhibits the degradation of chlorophyll and photosynthetic proteins (Richmond and Lang, 1957; Badenoch-Jones et al., 1996). Therefore, exogenous spermine, Ambiol and cytokinin may delay senescence under drought...
by: i) scavenging free radicals, ii) inhibiting lipid peroxidation, iii) limiting chlorophyll breakdown, or iv) inhibiting ethylene production. Specific actions of antistress and antioxidant compounds are reviewed in the following section.

2.4 Role of antisenescence, antitranspirant and antioxidant compounds

Under abiotic and biotic stress, plant growth regulators (PGR) trigger stress resistance mechanisms that minimize the impact of stress on the tree. Since these PGR induced changes appear to enhance resistance to subsequent stresses, they provide cross-protection which helps to sustain productivity under stress (Johnson, 1987).

2.4.1 Polyamines (Spermine)

Polyamines, particularly spermine, have been implicated in a number of senescence reactions in plants (Kaur-Sawhney and Galston, 1991; Tiburcio et al., 1994). They have been shown to: i) increase protein, RNA, and DNA synthesis in aging oat protoplasts (Altman et al., 1977; Kaur-Sawhney et al., 1980), ii) reduce RNAase activity and chlorophyll loss (Kaur-Sawhney and Galston, 1979), and iii) inhibit specific protase activity of senescing oat leaves (Kaur-Sawhney et al., 1982). DAP (1,3-Diaminopropane), a catabolic product of Spermine, has also been shown to inhibit protase activity, chlorophyll breakdown, and ethylene production in detached oat (Shih et al., 1982) and rice (Cheng et al., 1984) leaves.
Table 2.1. A summary of senescence-retarding effects of polyamine in different plant species.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of chlorophyll breakdown and ethylene production;</td>
<td>Oat</td>
<td>Shih et al., 1982</td>
</tr>
<tr>
<td>Inhibition of chlorophyll breakdown and ethylene production;</td>
<td>Rice</td>
<td>Cheng et al., 1984</td>
</tr>
<tr>
<td>Inhibition of ethylene;</td>
<td>Apple fruit tissue</td>
<td>Apelbaum et al., 1982</td>
</tr>
<tr>
<td>Inhibition of chlorophyll breakdown;</td>
<td>Barley leaves</td>
<td>Rodriguez et al., 1987</td>
</tr>
<tr>
<td>Inhibition of lipid peroxidation</td>
<td>Detached oat leaves</td>
<td>Borrel et al., 1997</td>
</tr>
</tbody>
</table>

Senescence is correlated with decreasing polyamine levels in intact leaves of bean and detached raddish leaves (Altman and Bachrach, 1981), oat protoplasts (Kaur-Sawhney et al., 1982), nodules of Vigna mungo (Lahiri et al., 1992), petunia flowers (Botha and Whitebread, 1992), detached leaves of barley (Coghlan and Walters, 1990) and those of rice (Chen and Kao, 1993). Membrane leakage and senescence during water stress are induced by ethylene production (Hipkins and Hillman, 1985). Ethylene evolution increased the rate of degradation of chlorophyll in Cucumis sativus cotyledons (Abeles and Dunn, 1989). PAs may retard senescence by inhibiting ethylene production (Suttle, 1981; Apelbaum et al., 1985) or by stabilizing nucleic acids and cell membranes against enzymatic
degradation and solute leakage (Kaur-Sawhney et al., 1978). Both ethylene and polyamines are formed from methionine via S-Adenosylmethionine (SAM). Higher SAM concentrations reduces endogenous levels of aminocyclopropylcarboxylic acid (ACC) and inhibit the conversion of ACC to ethylene (Even-Chen et al., 1982; Winer and Apelbaum, 1986). This process seems to require the presence of putrescine (Put) and higher Spermidine (Spd) to Spermine (Spm) ratio. An increase in the Put/Spm and Spd ratio, for example, favors ethylene formation whereas a decrease in this ratio prevents its formation. Exogenous PAs alter the Put/Spm and Spd ratio, causing the diversion of SAM from ACC to Spm and Spd. Several studies have shown that exogenous polyamine delay the senescence of excised leaves (Srivastava et al., 1983; Kar and Chouduri, 1986; Galston and Kaur-Sawhney, 1987). Polyamines also inhibit lipid peroxidation in osmotically stressed senescing tissues (Borrell et al., 1997).

The cationic nature of polyamine facilitates electrostatic bindings and H-bonding and allows them to become incorporated into negatively-charged nucleic acids and cell wall components, particularly phospholipids and proteins. The evidence seems to suggest that polyamines stabilize and organize the structure and activities of DNA and RNA (Altman et al., 1977; Kaur-Sawhney et al., 1980). Roberts et al. (1986) reported that exogenous spermine stabilizes membranes by inducing rigidification at the bilayer surface which, in turn, retards membrane deterioration (Liebermann and Wang, 1982).
2.4.2 Ambiol (antioxidant)

As a synthetic imidazole, Ambiol (2-methyl-4-[dimethylaminomethyl]-5-hydroxybenzimidazole dihydrochloride) is classified as an antioxidant (Santrucek and Krepelka, 1988). Its low molecular weight, aromatic structure and a hydroxy group in the aromatic ring suggest its antioxidant action. Since Ambiol is a 5-hydroxy derivative of benzimidazole, it is also a non-purine cytokinin (Fox, 1968). Ambiol may therefore have anti-senescence properties, and membrane preserving action like cytokinin, which prevents senescence in a range of plants including tobacco (Richmond and Lang, 1957; Winger et al., 1998).

Ambiol enhanced root and shoot growth in unstressed corn (Smirnov et al., 1984), pine and spruce (Vishnevetskaia et al., 1992), as well as in drought stressed canola, and soybean (Darlington et al., 1996) and pine (Vishnevetskaia et al., 1992). It reduced the transpiration rates of pine and soybean under drought (Darlington et al., 1996). Ambiol increased germination percentage (50%) and germination of black spruce seeds was accelerated by 4 days (Borsos-Matovina, V. 1997; MScF thesis, University of Toronto). It also increased root dry weight and total biomass in jack pine and decreased membrane leakage under drought in black spruce seedlings (Borsos-Matovina, V. 1997; MScF thesis, University of Toronto). Ambiol also increased stem elongation rate and water use efficiency and reduced transpiration, ethylene production and membrane leakage under drought in jack pine seedlings (Rajasekaran and Blake, unpublished data).
The increase in concentration of free radicals, such as super-oxide anion, singlet oxygen and hydroxyl, initiates senescence under stress (Dhindsa, 1982). Antioxidants protect plants by scavenging chain-carrying peroxyl radicals or by diminishing the formation of initiating lipid radicals. Imidazoles, such as Ambiol, donate a hydrogen atom (from their 2nd position) to oxygen radicals, which interrupts free-radical chain reactions. Like other antioxidants, Ambiol may reduce the level of endogenous lipoxygenase activity in senescencing tissues (Siedow, 1991) which suggests another possible mechanism by which Ambiol could delay senescence.

2.4.3 Cytokinins

Plant senescence can be retarded by cytokinins. Cytokinin action includes: i) interaction with tRNA metabolism, ii) effects on membrane permeability to mono- and di-valent ions, iii) localized induction of metabolic sinks, and iv) re-greening of senescing leaves (Letham, 1978).

Cytokinins retarded senescence in many plants including leaves of Xanthium (Richmond and Lang, 1957) and wheat (Person et al., 1957), asparagus stems (Dedolph et al., 1961), rose petals (Mayak and Halevy, 1970) and cut flowers (Mayak and Halevy, 1970).

Cytokinins inhibit many senescence reactions in chloroplasts (Dennis et al., 1967) and help to maintain protein and RNA content of detached leaves (Richmond and Lang, 1957).
As mentioned earlier, there is a rise in lipoxygenase activity during normal and induced senescence. Lipoxygenase causes the production of free radicals including superoxide, fatty acid, and peroxo radicals. Cytokinin lowers lipoxygenase and superoxide dismutase activities significantly in senescing leaves (Leshem, 1984).

Cytokinin involvement in free radical reactions can occur in several ways:
i) cytokinin may act as a direct free radical scavenger by extracting hydrogens of the α-carbon atom in the amine bond, resulting in amide formation, and ii) cytokinin may prevent free radical formation by inhibiting oxidation of the purines that releases superoxide and hydroxyl free radicals (Leshem, 1984).

2.4.4 Salicylic acid

Salicylic acid (2-hydroxybenzoic acid) is a natural plant product of willow bark that has been shown to increase plant height growth and stimulate adventitious root formation (Malamy and Klessig, 1992). Studies suggest that salicylic acid prevents rapid membrane depolarization, reverses ABA-induced stomatal closure (Rai et al., 1986), reverses leaf abscission, inhibits ethylene production through inhibiting ACC synthesis (Romani et al., 1989) and acts as an antisenescence agent (Raskin, 1995). Since ethylene increases membrane permeability under drought, exogenous application of salicylic acid could prevent membrane deterioration in stressed plants.
Salicylic acid also accelerated flowering, thermogenesis and induced systemic acquired resistance (SAR) in diseased plants (Malamy and Klessig, 1992).

2.4.5 AVG (aminoethoxyvinylglycine)

Ethylene production increases under environmental stress, including water stress (Yang and Pratt, 1978). Increased membrane permeability was correlated with increased ethylene concentration (Hipkins and Hillman, 1985). AVG prevents ACC synthesis (Abeles et al., 1992; Even-Chen et al., 1982), which inhibits ethylene production. Thus, exogenous AVG was expected to inhibit ethylene-induced membrane leakage under drought.

2.4.6 CaCl₂ (Calcium Chloride)

Calcium promotes ion transport across membranes (Epstein, 1961). It also helps to maintain membrane integrity. Ca²⁺ associated with the outside surface of the plasma membrane and, by acting as a divalent ligand, stabilized the plasmalemma (Thompson, 1988). Pretreatment with CaCl₂ decreased membrane permeability, prevented decline of chlorophyll and protein content and delayed senescence in Vigna catjang seedlings under drought. It also delayed senescence in oat leaves (Poovaiah and Leopold, 1973).
2.4.7 Abscisic acid (ABA)

Drought induces ABA accumulation in stressed plants (Creelman and Mullet, 1991) and exogenous ABA lowers transpiration by closing stomata (Mansfield et al., 1978). ABA and several synthetic analogs decreased transpiration rate and increased water use efficiency by up to 75% in black spruce after seven days of drought (Blake et al., 1990). Effects of ABA on membrane leakage are controversial. ABA reduced leakage of betacyanin and protected tonoplast membranes from osmotic stress induced by high polyethylene glycol concentrations (Pustovoitova, 1987). However, other work (Fan and Blake, 1994a) showed that ABA increased electrolyte leakage of black spruce, jack pine and *Eucalyptus grandis* seedlings.
CHAPTER III

Studies on the effects of different senescence inhibitors on dehydration-induced membrane leakage in bean leaves.

Abstract

The antisenescence properties of different compounds were determined by the ability of the compounds to prevent drought-induced membrane leakage. Although different senescence inhibitors have been tested for their ability to inhibit membrane leakage in agricultural crops, they have yet to be tested on white pine or any other conifers. We conducted a preliminary study using excised bush bean (*Phaseolus vulgaris* L) leaves to determine which compounds (Ambiol, AVG, Kinetin, Salicylic acid, ABA, CaCl₂, Spermine and Spermidine) inhibited membrane leakage and the most effective concentrations. Drought enhanced membrane leakage by up to 87% in 24h. Of the eight compounds tested, pretreatment with spermine (100 μg/l) and AVG (100 μg/l) were most effective and both significantly reduced membrane leakage by 15% under drought. A reduction in electrolyte leakage could protect plant membrane function in dehydrating leaves.

3.1 Introduction

Drought is a common cause of slow growth of woody plants during the growing season (Kramer, 1986). Dehydration causes several metabolic perturbations that leads to senescence. The primary targets of desiccation injury
to cells and organelles are membranes, which leak cellular contents into the cell apoplast. The resulting loss of control over key regulatory and receptor functions of the cell components leads to senescence (Crow et al., 1993). Senescence in plant tissues is accompanied by increased membrane leakage (Dhindsa et al., 1981). This is manifest by an increase in apparent free space and a loss of ability to retain solutes during ripening of fruits (Sacher, 1973) and senescence of green plant tissues (Farguson and Simon, 1973). Membrane permeability changes during senescence are also correlated with a simultaneous decline in membrane lipids (Simon, 1977) and sterols. Lipids and their ratio were found to decline in black spruce following dehydration (Zwiazek and Blake, 1990b). A relationship between membrane leakage and growth rate was suggested because membrane leakage was greater in the slow-growing progenies of black spruce. Apparently they were more severely stressed during drought compared to fast-growing progenies (Tan and Blake, 1993). Increases in membrane permeability under drought was also correlated with the increase in ethylene concentrations in dehydrating tissues (Hipkins and Hillman, 1985).

Since changes in membrane function occur in the early stages of plant injury, they are one of the first physiological changes detected under stress (Nilsen and Orcutt, 1996). They can therefore be used to quantify injury levels in stressed plants. Most commonly, changes in the electrical impedance (Grenum, 1980) and electrolyte leakage (Martin et al., 1987; Tan and Blake, 1993) have been used as a measure of drought injury in woody plants. However, membrane leakage has been found to be the most sensitive of several tested methods
(Zwiazek and Blake, 1991). The electrolyte leakage method has been used to measure membrane integrity under several environmental stresses, including cold (Palta et al., 1977), drought (Dlugokecka and Kacperska-Palacz, 1978; Zwiazek and Blake, 1991; Tan and Blake, 1993), and pollution (Zwiazek and Shay, 1988). Since changes in membrane permeability in plant tissues is accompanied by senescence (Dhindsa et al., 1981), changes in membrane leakage can be used to identify senescence delaying compounds (Mukherjee and Choudhuri, 1981).

Some phytohormone-related chemical compounds inhibit leaf senescence, suggesting they may preserve membrane integrity. The mechanisms of action of these compounds are not clearly understood. Chemical preconditioning with Ambiol (2 methyl-4dimethyl-5aminomethyl dihydrochloride), a synthetic antioxidant, reduced membrane leakage under drought in two conifer species black spruce (Borsos-Matovina, V. 1997; MScF thesis, University of Toronto) and jack pine seedlings (Rajasekaran and Blake, 1997 unpublished data). Ambiol has an aromatic structure which may allow it to function as an antioxidant and antitranspirant (Darlington et al., 1996). The possibility was therefore, considered that application of a synthetic antioxidant to unstressed plants may prevent subsequent oxidative damage to membranes under drought.

Polyamines (spermine and spermidine) inhibited lipid peroxidation in osmotically stressed cut oat leaves (Borrel et al., 1997). Since an increase in lipid peroxidation was correlated with increased solute leakage (Dhindsa et al., 1981), pretreatment with spermine and spermidine may reduce membrane leakage
under stress. Spermine also counteracted ethylene-induced increase in membrane permeability and decreased betacyanin leakage from beet roots (Naik and Srivastava, 1978). The anti-ethylene agent AVG (aminoethoxyvinylglycine) inhibited ethylene evolution by preventing ACC synthase (Abeles et al., 1992). Since this enzyme is an immediate precursor of ethylene, it may prevent the ethylene-induced increase in membrane permeability under stress. Kinetin (6-furfuryl aminopurine), a synthetic cytokinin, also delays senescence. Kinetin has been shown to inhibit the formation of free radicals, lipid peroxidation and ethylene (Leshem, 1984). The antisenescence properties of salicylic acid were correlated with inhibition ACC synthesis (Romani et al., 1989) and reversal of ABA-induced stomatal closure (Rai et al., 1986). Calcium chloride (CaCl₂) has membrane protective functions and it reduced membrane permeability in Vigna catjang (Endl.) seedlings under water stress (Mukherjee and Choudhuri, 1981) and delayed senescence in corn (Zea mays L.) (Poovaiah and Leopold, 1973).

The possibility was considered that anti-ethylene compounds (AVG, spermine and salicylic acid) particularly those having antisenescence properties (spermine and kinetin) or antitranspirant (AmbioI) properties may delay drought-induced membrane leakage which may eventually enhance the survival and growth of plants under drought.

3.2 Materials and Methods

Phaseolus vulgaris (var. contender) seeds were soaked for 4h in distilled water and sown in vermiculite. Seedlings were grown in a growth chamber
(PGW, Conviron Winnipeg, Man) at 24/22°C day/night temperature, 220μEin, m\(^{-2}\), sec\(^{-1}\) PAR and under a 16 h photoperiod. Seedlings were watered daily.

Pretreating excised bean leaves and allowing them to desiccate was suitable and useful to determine the effective concentration of antisenescence compounds for the following reasons: a) soil drying often have an uneven dehydration effect on individual plants; b) use of polyethylene glycol to induce drought has adverse physiological effects on plants; c) excision of leaves provided equal drying of leaves, and, d) helped to screen a wide range of concentrations of various antisenescence compounds.

Fully expanded leaves were excised on the 10th day after germination and pretreated for 20h in 10ml of aqueous solutions of different concentrations of various antitranspirant, antioxidant and antisenescence compounds (Sigma Chemical, Canada) viz., Kinetin (K: 0.01, 0.1, 1, and 10 μg/l), AVG (AVG: 1, 10, 100, and 1000 μg/l), Amniol (Amb: 0.01, 0.1, 1, and 10 mg/l), Spermine (Spm: 0.1, 1, 10, and 100 μg/l), Spermidine (Spd: 0.1, 1, 10, and 100 μg/l), Salicylic acid (SA: 0.1, 1, 10, and 100 μg/l), ABA (0.1, 1, 10 and 100 μg/l) and CaCl\(_2\) (0.1, 1, 10, and 100 μg/l). Following the pretreatment, leaves were allowed to desiccate for 20h at room temperature (21±1°C and RH 50-60%). Membrane leakage was determined when the leaves were at the incipient wilting stage (turgor loss point of bean is around –1.05MPa; Kim and Lee-Stadekmann, 1984) by immersing tissues in water and determining the increase in electrolyte leakage as described by Zwiazek and Blake (1990b). Briefly, leaves were washed, immersed in a glass vial containing 28 ml of deionized water, and incubated at
20±1°C for 24 h. Specific conductivity of the solution was measured using a Hi 8733 conductivity meter (Hanna Instruments Inc. Quebec). After measurement, the solutions were returned to their vials, sealed and then placed for 24 h in a hot air oven at 90±2°C to kill the tissues. The samples were left to cool to room temperature and the measurements were repeated. Results were expressed as a percent of total electrolytes leaked (i.e., relative to heat-killed leaves). Moisture loss was calculated as: (fresh weight – dry weight) / fresh weight*100 as described previously (Turner, 1981). For each of the compounds, five concentration levels were tested with appropriate controls and there were four leaf replicates per treatment.

A completely randomized experimental design was followed since leakage measurements were taken on leaves randomly sampled from the population in each treatment. Statistical analysis utilized a one-way analysis of variance (pairwise multiple comparison procedure; Student-Newman-Keuls method) using SigmaStat™, a statistical software program.

3.3 Results

Dehydration increased membrane leakage by up to 87% in Phaseolus vulgaris. Spermine and AVG caused a significant (p<0.05) reduction (15%) in membrane leakage compared with the controls (Table 3.1, Figures 3.1 and 3.2). Salicylic acid, kinetin, Ambiol, CaCl₂ and spermidine failed to reduce electrolyte leakage significantly (p<0.05).
Table 3.1. Effect of various senescence inhibitors on membrane leakage in dehydrating leaves of *Phaseolus vulgaris*.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Effective concentration</th>
<th>Membrane Leakage (%)</th>
<th>Reduction in membrane leakage (% over control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control ± SEM</td>
<td>Treated ± SEM</td>
</tr>
<tr>
<td>Kinetin</td>
<td>0.1μg/l</td>
<td>77.5 ± 6.64</td>
<td>73.4 ± 8.41</td>
</tr>
<tr>
<td>AVG</td>
<td>100μg/l</td>
<td>78.1 ± 2.62</td>
<td>63.7 ± 1.37*</td>
</tr>
<tr>
<td>Ambiol</td>
<td>10mg/l</td>
<td>70.7 ± 6.05</td>
<td>69.8 ± 2.8</td>
</tr>
<tr>
<td>Spermine</td>
<td>100μg/l</td>
<td>70.4 ± 1.26</td>
<td>60.8 ± 0.57*</td>
</tr>
<tr>
<td>Spermidine</td>
<td>1μg/l</td>
<td>65.9 ± 6.13</td>
<td>66.6 ± 1.99</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>100μg/l</td>
<td>62.4 ± 6.38</td>
<td>58.3 ± 1.59</td>
</tr>
<tr>
<td>ABA</td>
<td>1μg/l</td>
<td>85.3 ± 2.61</td>
<td>84.2 ± 3.36</td>
</tr>
<tr>
<td>CaCl2</td>
<td>100μg/l</td>
<td>75.3 ± 6.3</td>
<td>76.4 ± 3.31</td>
</tr>
</tbody>
</table>

* Significantly different from control at 0.05 level.

3.4 Discussion

Drought tolerance is important since it largely determines the survival and growth of trees. Drought caused oxidative membrane damage by altering enzyme function and lipid peroxidation (Sgherri and Navari-Izzo, 1995). An accumulation of lipid hydroperoxides preceded ethylene biosynthesis. Ethylene initiates changes that could result in cellular degradation and eventually senescence (Paliyath and Droillard, 1992). This starts with an increase in the
permeability of the tonoplast membrane through which hydrolytic enzymes, acids and toxic compounds leak (Mayak and Halevy, 1980).

The hypothesis for this study was that these chemical compounds protect cell functions by preventing membrane injury. If these compounds decrease electrolyte leakage in desiccated leaves they can be shown to be possibly used to harden plants against drought.

Spermine and AVG reduced membrane leakage under drought in this study. Polyamines accumulated in plants under osmotic stress (Flores and Galston, 1984). Since polyamines retarded senescence by inhibiting ethylene production (Suttle, 1981; Apelbaum et al., 1985), their ability to stabilize cell membranes could protect plants against enzymatic degradation and solute leakage (Kaur-Sawhney et al., 1978). Our results confirm this suggestion since membrane leakage declined in parallel with the reduction in moisture loss. The cationic nature of polyamines, which facilitates electrostatic bonding and H-bonding, allows them to become incorporated into nucleic acid (Kaur-Sawhney et al., 1980). Roberts et al. (1986) reported exogenous spermine stabilized membranes by inducing rigidification at the bilayer surface, which, in turn, retarded membrane deterioration (Lieberman and Wang, 1982).

AVG, an ethylene synthesis inhibitor (Arteca, 1996), reduced membrane leakage significantly (Table 3.1, Figure 3.2). Since AVG inhibits synthesis of ACC (Abeles et al., 1992), which is the immediate precursor of ethylene, it presumably prevented ethylene-induced membrane damage under dehydration. Spermine also prevented moisture loss in dehydrating tissues. When the moisture content
of the membranes declines below 20-30%, the attraction of the non-polar lipid ends causes their rearrangement and forms a hexagonal, leaky structure (Simon, 1978). On the other hand, proteins which are normally located close to one another can be separated by desiccation, causing a disruption of ion channels (Palta et al., 1982). When active ion transport function is lost under drought, ions move freely out of cells, resulting in an increase in membrane permeability (Sgherii and Navari-Izzo, 1995).

Though an antitranspirant role for Ambiol has been reported for other species (Beall et al., 1997, Darlington et al., 1996) including white pine (cf. Section 5.2.3), it failed to reduce moisture loss or membrane leakage in bean seedlings. There could be three possible explanations for the apparent discrepancy. First, Ambiol action is species specific and failed to reduce leakage in two eucalypt species (Warren, S. 1996; MScF thesis, University of Toronto). Secondly, the experiment was performed in the light. Since Ambiol is 5-hydroxy derivative of benzimidazole, it is a non-purine cytokinin (Fox, 1968). Like cytokinins, its ability to lower senescence may be inhibited by light (Mishra and Pridhan, 1973). Thirdly, compared to other studies, for example, Borrell et al., (1997), the duration of dehydration treatment in our study may have been too long to show an intermediate effect. Hence membranes may have been too desiccated to show and Ambiol action.

Since spermine and AVG reduced dehydration-induced membrane leakage, they may delay drought-induced senescence. Since these compounds
reduced membrane leakage significantly, they were further investigated in the following chapters (IV and V).
Figure 3.1. Effect of various concentrations of spermine on desiccation-induced electrolyte leakage (%). Bars show the standard error of the means. (n=4). Error bars with the same letter are not significantly different at p=0.05 as determined by one way ANOVA (pairwise multiple comparison procedure: Student-Newman-Keuls Method).

Figure 3.2. Effect of various concentrations of AVG on desiccation-induced electrolyte leakage (%). Bars show the standard error of the means. (n=4). Error bars with the same letter are not significantly different at p=0.05 as determined by one way ANOVA (pairwise multiple comparison procedure: Student-Newman-Keuls Method).
CHAPTER IV

The effect of xylem feeding with different chemical compounds on shoot water relations parameters in white pine (Pinus strobus L.) seedlings.

Abstract

To understand the physiological basis of their antistress and growth promoting actions, Ambiol, ABA, AVG and spermine were fed into the xylem of one-year old white pine (Pinus strobus L.) seedlings. The ability of these compounds to enhance shoot water relations was then compared under drought by withholding water from the soil for 11 days. Ambiol significantly increased osmotic adjustment and decreased the fraction of water in the apoplast. Spermine significantly increased bulk modulus of elasticity, net photosynthesis and water use efficiency. ABA and AVG failed to influence any cell water relations parameters under drought and they had no significant effects on transpiration rates. These compounds helped to maintain a positive turgor in white pine seedlings by increasing osmotic adjustment. The possibility that these compounds may enhance survival and growth of seedlings under drought is discussed.

4.1 Introduction

Drought is one of the main environmental stresses that slows growth of woody plants during the growing season (Kramer, 1986). A decline in stomatal conductance and net photosynthesis is usually associated with slow growth (Tan et al., 1992a; Hanson and Hitz, 1982), this suggests these and other
physiological changes under drought are major contributing factors to high mortality of transplanted conifer seedlings (Kramer, 1986; Blake and Sutton, 1987). Since young, succulent seedlings are transplanted from an optimal greenhouse environment to harsh planting sites, conifer seedlings need to be hardened against environmental stresses. Hence preconditioning treatment is an important requirement for successful reforestation (van den Driessche, 1991a; 1991b and 1992).

Seedlings can be preconditioned before outplanting using a mild environmental stress, such as drought, heat or a range of chemicals (Warren, S. 1996; MScF thesis, University of Toronto). A prior exposure to one stress increases plant tolerance to a later stress (Kramer, 1980; Kozlowski et al., 1991). For example, douglas fir and lodgepole pine seedlings showed increased survival rates after they were preconditioned by withholding water (van den Driessche, 1991a; 1991b and 1992).

When seedlings are preconditioned by drought, a number of changes occur in plants. Plants responses to drought usually involve stomatal reactions, but these may vary among species (Gebre and Kuhns, 1993). For example, stomata closed more rapidly following preconditioning of seedlings (Zwiazek and Blake, 1989). Preconditioning resulted in a decline in sterol : phospholipid ratios and also reduced electrolyte leakage under stress, compared to unconditioned plants (Zwiazek and Blake, 1990b). Stomata may also remain open during drought, as a result of osmotic or elastic adjustment (Oosterhuis and Wullschleger, 1987). These adjustments can occur in preconditioned plants.
under drought even when stomatal conductance changes were not observed (Levitt, 1985). Osmotic adjustment, refers to the active lowering of osmotic potential that results from an active accumulation of solutes under drought to maintain turgor (Turner and Jones, 1980). Osmotic adjustment also shows considerable genetic variation. Tschaplinski and Blake (1989a) observed that one fast growing *Populus deltoides* Bartr. X *P. balsamifera* L. (jackii 4) hybrid clone had a higher concentration of osmotically-active organic solutes and exhibited a greater decline in osmotic potential at both full and zero turgor, compared to another slower growing *P. deltoides* X *P. balsamifera* clone (jackii 7).

Osmotic adjustment is of vital importance for seedling survival and growth since it allows stomata to remain open and photosynthesis to continue to lower predawn leaf water potentials (Gebre and Kuhns, 1993). Osmoregulation also enables cell enlargement and growth to continue at water potentials that would otherwise be inhibitory (Turner and Jones, 1980; Kramer, 1983). Accumulation of sugars stabilizes membrane structure (Santarius, 1973; Hoekstra et al., 1989) and such osmotica protects plants against potentially damaging cellular dehydration (Giles et al., 1976).

A number of compounds have been found to act as antitranspirants. Polyamines and a number of natural and synthetic chemical compounds were found to possess antitranspirant and antisenescence properties suggesting they may be used to precondition seedlings against stress (Beall et al., 1997; Kaur-Sawhney et al., 1978). Polyamines accumulated in oat plants under osmotic
stress and exogenous polyamines retarded chlorophyll breakdown in the protoplasts and enhanced their ability to synthesize DNA (Galston and Kaur-Sawhney, 1990). Although spermine promoted needle growth and dry matter production in jack pine seedlings (Rajasekaran and Blake, 1999), its effect on drought tolerance has not been studied in conifers.

Application of abscisic acid (ABA) closes stomata and reduces transpiration rates (Zhang and Davies, 1990, Tardieu et al., 1992). ABA and several synthetic analogs decreased transpiration rate and increased water use efficiency in black spruce by up to 75% seven days after relief from drought (Blake et al., 1990). As an imidazole, Ambiol, (2-methyl-4[diethylaminomethyl] – 5-hydroxy-benzimidazole), falls into one of 13 classes of antioxidants (Santrucek and Krepelka, 1988). Ambiol may be an effective preconditioning agent since it increased growth of rape seed and soybean by 25-45% and reduced mid-day transpiration rate and total daily water usage under drought of soybean by about one-third (Darlington et al., 1996).

Water stress increases ethylene production (Aharoni, 1978; Yang and Pratt, 1978). Since AVG (aminoethoxyvinylglycine) inhibited ethylene synthesis and delayed senescence in cut flowers (Abeles et al., 1992) this suggests AVG could also prevent ethylene-induced membrane damages under drought. While the growth promoting effects of these compounds on whole plants have been reported, very little is known regarding their influence on drought tolerance and cell water relations in conifer seedlings.
The objective of the study was to examine the effect of these compounds (ABA, Ambiol, AVG and spermine) on cell water relations parameters under drought. Since application of ABA stimulated root growth of drought-stressed black spruce (Liu, Q. 1996; Ph. D. thesis, University of Toronto), the effects of these compounds on shoots physiology was investigated by feeding chemicals directly into the xylem to avoid compensatory effects elsewhere in the plants. The aim was to examine whether these compounds increase osmotic or elastic adjustment in shoots under drought. Pressure –Volume (PV) analysis was conducted to compare shoot water relations parameters of chemically treated and untreated white pine seedlings. Since these compounds promote growth, it was hypothesized that they may stimulate osmotic or elastic adjustment under drought, which will increase the range of water content over which plants can maintain turgor.

4.2 Materials and Methods

4.2.1 Plant materials and pre-treatment

White pine seedlings (seed source 42-42-0-00) were grown in a commercial potting mix of Pro-mix, NX, (Premier Inc. Ont.) under 20±2°C temperature and under a 16h photoperiod in the greenhouse at the University of Toronto. Seedlings were watered daily and fertilized weekly with 20:20:20 N-P-K fertilizer (Plant Products Ltd., Brampton, ON). Prior to starting the experiment, eighty seedlings of similar size (one-year-old) were placed into a controlled environment growth chamber (PGW, Conviron Winnipeg, Man) to acclimate for
seven days at 24/22°C day/night temperature, 220μEin, m⁻² s⁻¹ PAR and 16h photoperiod.

Before applying any treatment, five seedlings were taken to generate Pressure Volume (PV) curve and considered as control. Seventy five seedlings were then treated with 15ml of aqueous solutions of different compounds (Sigma Chem. Co. Canada) viz. abscisic acid (10μg/l), Ambiol (10mg/l), AVG (100μg/l), spermine (10μg/l) and deionized water. There were 15 seedlings per treatment. Chemicals were xylem fed by capillary action, using a cotton wick connected to a sealed 18ml, opaque plastic, non-reactive vial attached to the seedling stem. Deionized water was fed into the xylem to serve as control. Xylem feeding was carried for seven days and all the seedlings were watered daily. After completion of xylem feeding, cotton wicks were cut from the stem surface and the stems were sealed immediately with parafilm to prevent air entry. To examine whether the xylem feeding itself had any effect on water relations parameters, another set of PV curves were generated taking five seedlings from each treatment.

Drought was then imposed on one half of the seedlings by withholding water for 11 days. The remaining half of the seedling were watered daily. Midday needle pressure potentials of both stressed and non-stressed seedlings, five seedlings per treatment, were then measured using a pressure chamber (PMS Instruments, Corvallis, OR, USA). Finally, PV curves were constructed by taking five seedlings from each treatment from both the stressed and unstressed group of seedlings. Net photosynthesis and transpiration rate for both treated stressed and unstressed seedlings were measured for needles in the upper crown after
stress using a LI-6200 Portable Photosynthesis System (Li-Cor Inc., Lincoln, NE). Due to accumulation of CO₂ from operators working in the growth chamber, measurements of gas exchange were taken outside in a well-ventilated room at 22±1°C under 300μmol m⁻² s⁻¹ photosynthetically active radiation supplied by a Multi-Vapor lamp (MVR 1000/C/VBU, General Electric Co.). For calculation of gas exchange, needle area was determined by water immersion, using Archimedes' principle, as described previously (Johnson, 1984).

4.2.2 Pressure-Volume analysis

Shoot water relations were determined by pressure-volume analysis. Since sap expression method leads to an underestimation of the π¹⁰⁰ and π⁰ values (Tyree et al., 1978), the free transpiration method was used to generate PV curves from a series of paired fresh weight and pressure potential (ϕᵢ) using a Scholander-type pressure chamber (Schulte and Hinckley, 1985; Parker and Pallardy, 1986; Lassoie and Hinckley, 1991). Seedlings were rehydrated overnight for 15h at room temperature in the dark by submerging the pots up to the root collar and covering the shoot with plastic bags (Parker and Colombo, 1995). Aerators were inserted into the water to supply oxygen. Shoots were cut just above the xylem feeding point to reduce the error that may arise during pressing the xylem sap to the cut surface. Chamber pressure was increased and decreased following the precautions of Ritchie and Hinckley (1975), until water appeared at the cut surface that indicated the balance point. When 4-6 data points on the linear portion of the PV curve (plot of 1/ϕ versus relative water
content) were obtained, samples were placed in an oven at 70°C for 48h and dry weights were measured. From the plot of $1/\psi$ against RWC, osmotic potential at full turgor ($\psi_{100}$), zero turgor ($\psi^0$), relative water content at zero turgor (RWC$^0$), proportion of apoplastic water fraction and bulk modulas of elasticity were calculated between 100% and 95% of full turgor ($\epsilon_{\text{max}}$) as described by Colombo (1987). Relative water content of the samples were calculated as: $\text{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100$.

The experiment utilized a completely randomized experimental design and physiological measurements were taken on seedlings randomly sampled from the population. The location of each container was frequently changed to account for any minor environmental differences in the growth chamber. Use of standard condition (pot size, plant size, soil texture, irrigation control and environment control in the growth chamber) ensured that the effects produced were the result of treatments and not other factors. Since the design randomly allocated plants to treatments there was no restriction error (which can occur when randomization is restricted by blocking). For the above reasons, individual seedlings were considered to be true replicates, rather than sub-sampling units.

Data were analyzed using the one way analysis of variance (ANOVA) procedure of SAS (SAS Institute Inc., Cary, NC, USA). A least significant difference (LSD) test was performed for comparison of parameter means at the 5% significance level.
4.3 Results

Midday needle pressure potential ($\psi_1$) declined significantly (from -0.66 MPa to -0.86 MPa) after 11 days of drought in water-fed, control seedlings, compared with unstressed controls (Figure 4.1). However, treatment with ABA, Ambiol, AVG and spermine prevented the drought-induced decline in $\psi_1$.

Relative water content at zero turgor (RWC$^0$) increased (from 84.9% to 89.3%) in Ambiol treated, watered seedlings but decreased (from 89.3% to 85.5%) under stress (Figure 4.2). With the exception of Ambiol treated seedlings, apoplastic water fraction showed a similar significant increase after xylem feeding with all chemicals (Figure 4.3). The osmotic potential at full turgor ($\pi^{100}$) did not differ when measured after 11 days of drought (Figure 4.4). Ambiol-fed seedlings showed a significant increase in osmotic potential at full turgor (to -1.02 MPa) after 11 days under well-watered conditions, but declined to control values (-1.18 MPa) under stress. The osmotic potential at zero turgor ($\pi^0$) decreased significantly in Ambiol and spermine treated seedlings (Figure 4.5). The decline was from -1.49 to -1.86 MPa in Ambiol and from -1.39 to -1.53 MPa in spermine treated seedlings. The bulk modulus of elasticity at full turgor ($\epsilon_{\text{max}}$) increased from 10.0 MPa to 12.9 MPa) in spermine treated seedlings under stress but was not affected by any other treatment (Figure 4.6). Spermine significantly ($p<0.05$) increased net photosynthesis and water use efficiency (the ratio between net photosynthesis per unit water transpired; Blake et al., 1990) under drought (Figure 4.7 and 4.8). Transpiration rate under drought was not altered by
treatments compared to controls and there were no significant differences (p>0.05) (Appendix - B).

4.4 Discussion

Midday needle water potential declined significantly (p>0.05) by 31% in control (untreated) plants after 11 days of drought (Figure 4.1). All the tested compounds prevented this decline in $\psi_w$ during stress, compared with the untreated, unstressed controls.

Ambiol treatment resulted in significant (p<0.05) osmotic adjustment and osmotic potential at full turgor ($\pi^{100}$) and zero turgor ($\pi^0$) both declined (16% and 27% respectively) under drought, compared to their respective controls (Figure 4.4 and 4.5). The differences among three black spruce families in midday xylem pressure potential ($\psi$) were non-significant over one and three drought cycles. Droughted seedlings as a group had lower (p<0.1) predawn water potential in both drought cycles, compared to the watered controls (Tan and Blake, 1997). Although predawn $\psi$ may be a better indicator of tissue water relations adjustments under drought, the significant decline in midday water potential in non-treated stressed seedlings, compared with watered control supports this suggestion that chemical preconditioning promoted osmotic adjustments in white pine seedlings. The decline in midday water potential was almost totally reversed by ABA, AVG, ambiol and spermine, since needle water potentials remained close to values observed in the unstressed controls (Figure 4.1).
ABA, Ambiol, Spermine and AVG all exhibited antitranspirant actions, which would help to maintain also prevent the decline in water potential under drought. There was a statistically non-significant (p<0.05) reduction in transpiration rate under drought (16.6%) in ABA treated plants (Appendix- B). Blake et al., (1990) reported that treatment with ABA and synthetic analogs lowered transpiration rate of black spruce seedlings under drought, and one ABA analog increased water use efficiency by up to 75 percent.

Transpiration rate showed non-significant (p<0.05) decline by 33% following Ambiol treatment (Appendix - B). In other works, Ambiol reduced the midday transpiration rate and total daily water usage of soybean and increased growth of rape seed and soybean by 25-45% following seed treatment (Darlington et al., 1996).

Spermine significantly increased net photosynthesis and there was an increase by 40 % compared with controls after 11 days drought, hence, more carbon and energy would be available to support growth of spermine treated seedlings. Spermine also promoted needle growth and dry matter production in jack pine seedlings (Rajasekaran and Blake, 1998).

Needle pressure potentials declined in water fed (control) seedlings after drought (Figure 4.1), although their individual water relations components ($\pi^0$, $\pi^0$, and $\varepsilon$) showed no significant adjustment under drought (Figure 4.4, 4.5, and 4.6). Since osmotic potential did not change, a decline in turgor potential or an increase in matric potential or some other unmeasured components would
therefore be required to explain why $\psi_s$ did not decline in preconditioned seedlings.

White pine is a slow growing species in the early seedling stage (Wendel and Smith, 1990). A slow response in untreated seedlings may explain why cell water relations did not change under short-term drought. Osmotic and elastic adjustment was greater in faster-growing hybrid poplars clones (jackii 4) under drought, relative to closely-related but slower-growing clones (jackii 7) (Tschaplinski and Blake, 1989a). This suggests that osmotic adjustment in chemically preconditioned seedlings may promote growth rate under drought.

Although ABA and AVG treatment prevented the decline in needle pressure potential, these seedlings did not differ significantly in any cell water relations parameters. Other unmeasured changes may have helped to maintain $\psi$ and dehydration postponement action, e.g., early stomatal closure or more active root growth may have been involved since cell water relations parameters did not change.

In contrast to the other treatments Ambiol treated seedlings had high $\pi^{100}$, $\pi^0$, and RWC values in unstressed seedlings, but $\pi^{100}$ and $\pi^0$ declined significantly under drought (Figures 4.2, 4.4, and 4.5). Ambiol treatment also showed a significant increase in apoplastic water fraction and RWC and decrease in $\pi^{100}$ and $\pi^0$ in unstressed seedlings after xylem feeding where no such significant changes were observed in other treatments. Ambiol could have played an important role by shifting the apoplast to symplast water volume and
thereby helped the seedlings to osmotically adjust in stressed condition and de-adjust when unstressed.

Apoplastic water fraction increased significantly as a result of xylem feeding, both in droughted and well-watered seedlings (Figure 4.3), but there were no significant effect of xylem feeding itself on any other cell water relations parameters (Table 4.1 and 4.2; Figures 4.2, 4.3, 4.4, 4.5, and 4.6). An increase in apoplastic water fraction has been cited as a possible cell physiological response to leaf water deficits (Gunasekera and Berkowitz, 1992) although it is not clear how this could promote acclimation to water stress. Apoplastic water fraction increased in three genotypes of wheat under drought (Gunasekera and Berkowitz, 1992; Rascio et al., 1992). If apoplastic and symplastic water are in equilibrium, apoplastic water might act as a buffer for cell water during dehydration. The absolute values for this fraction bears no correlation since there appears to be a wide variation among species in the apoplastic water fraction (Tyree and Jarvis, 1982). However, the significant decline in apoplastic water fraction in Ambiol treated seedlings under drought indicates a 10% increase in the symplast water volume (Figure 4.3), which would be available to maintain cell turgor and cell functioning under stress (Gunasekera and Berkowitz, 1992).

According to terminology in common usage (Levitt, 1980; Kozlowski, 1991) the drought tolerance in woody plants has two components: dehydration avoidance (postponement) and dehydration tolerance. It is not clear how ABA and AVG prevented the decline in $\psi_k$, although this drought tolerance adjustment could have involved dehydration avoidance or metabolic changes unrelated to
the measured cell water relations components which measure tolerance of dehydration. The possibility will be considered in Chapter V that drought induce a stimulation of ethylene production which increases membrane leakage in the guard cells.

Several compounds show promise for hardening seedlings. Our results suggest that the significant increase in osmotic adjustment under drought in Ambiol treated seedlings may increase drought tolerance. Likewise the significant increase in bulk modulus of elasticity, net photosynthesis and water use efficiency may increase the drought resistance of spermine-treated seedlings.
Figure 4.1. Effects of different chemical compounds on midday needle pressure potential of white pine seedlings after 11 days of drought. Bars show the standard error of the mean (SEM). Values are the means of five seedlings. (where, CON = control, ABA = Abscisic acid, AMB = Ambiol, AVG = aminoethoxyvinylglycine and SPM = spermine treated seedlings). Means with same letters are not significantly (p<0.05) different.
Figure 4.2. Effects of different chemical compounds on relative water content at zero turgor (RWC\textsuperscript{a}) of white pine seedlings. Bars show the standard error of the mean (SEM). Values are the means of five seedlings. (where, CON = control, ABA = Abscisic acid, AMB = Ambiol, AVG = aminoethoxyvinylglycine and SPM = spermine treated seedlings). Treatments are: a) control (CON) (before xylem feeding), b) immediately after xylem feeding, c) unstressed and d) stressed after 11 days of drought. Means with same letters are not significantly (p<0.05) different. (n =5).
Figure 4.3. Effect of different chemical compounds on apoplastic water fraction (%) of white pine seedlings. Bars show the standard error of the mean (SEM). Values are the means of five seedlings. (where, CON = control, ABA = Abciscic acid, AMB = Ambiol, AVG = aminoethoxyvinylglycine and SPM = spermine treated seedlings). Treatments are shown in Figure 4.2.
Figure 4.4. Effect of different chemical compounds on osmotic potential at full turgor ($\pi^{100}$) of white pine seedlings. Bars show the standard error of the mean (SEM). Values are the means of five seedlings. (where, CON = control, ABA = Abscisic acid, AMB = Ambiol, AVG = aminoethoxyvinylglycine and SPM = spermine treated seedlings). Treatments are shown in Figure 4.2.
Figure 4.5. Effect of different chemical compounds on osmotic potential at zero turgor ($\pi^0$) of white pine seedlings. Bars show the standard error of the mean (SEM). Values are the means of five seedlings. (where, CON = control, ABA = Abscisic acid, AMB = Ambiol, AVG = aminoethoxyvinylglycine and SPM = spermine treated seedlings). Treatments are shown in Figure 4.2.
Figure 4.6. Effect of different chemical compounds on bulk modulus of elasticity ($\varepsilon$) near full turgor of white pine seedlings. Bars show the standard error of the mean (SEM). Values are the means of five seedlings. (where, CON = control, ABA = Abscisic acid, AMB = Ambiol, AVG = aminoethoxyvinylglycine and SPM = spermine treated seedlings). Treatments are shown in Figure 4.2.
Figure 4.7. Effect of various compounds on water use efficiency (WUE) of white pine seedlings after 11 days of drought. Bars show the standard error of the means. (n=5). Means with same letter are not significantly different at p=0.05 as determined by one way ANOVA (pairwise multiple comparison procedure; Student-Newman-Keuls method).

Figure 4.8. Effect of various compounds on net photosynthesis (μmol m^{-2} s^{-1}) of white pine seedlings after 11 days of drought. Bars are the standard error of the means. (n=5). Means with same letter are not significantly different at p=0.05 as determined by one way ANOVA (pairwise multiple comparison procedure; Student-Newman-Keuls method).
Table 4.1. Effect of xylem feeding with various compounds on water relations parameters of well watered white pine seedlings. Values are means (SEM) from 5 individual seedlings measured 11 days after terminating xylem feeding.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( \pi^{100} ) (MPa)</th>
<th>( \pi^0 ) (MPa)</th>
<th>RWC(^0) (%)</th>
<th>Va (%)</th>
<th>( \epsilon ) (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (control)</td>
<td>-1.22 (0.08)( ^a )</td>
<td>-1.78 (0.15)( ^a )</td>
<td>86.7 (1.6)</td>
<td>57.8 (2.7)</td>
<td>11.4 (1.2)</td>
</tr>
<tr>
<td>ABA</td>
<td>-1.11 (0.12)( ^ab )</td>
<td>-1.67 (0.15)( ^ab )</td>
<td>87.1 (1.4)</td>
<td>59.1 (5.4)</td>
<td>11.2 (1.4)</td>
</tr>
<tr>
<td>AMB</td>
<td>-1.02 (0.02)( ^ab )</td>
<td>-1.49 (0.03)( ^b )</td>
<td>89.3 (0.8)</td>
<td>65.9 (1.4)</td>
<td>11.4 (0.9)</td>
</tr>
<tr>
<td>AVG</td>
<td>-1.07 (0.03)( ^ab )</td>
<td>-1.52 (0.03)( ^ab )</td>
<td>89.3 (0.7)</td>
<td>63.7 (1.7)</td>
<td>12.5 (0.7)</td>
</tr>
<tr>
<td>SPM</td>
<td>-0.96 (0.02)( ^b )</td>
<td>-1.39 (0.04)( ^b )</td>
<td>89.5 (0.4)</td>
<td>65.3 (2.1)</td>
<td>10.0 (0.9)</td>
</tr>
</tbody>
</table>
Table 4.2. Effect of xylem feeding with various compounds on water relations parameters of drought stressed white pine seedlings. Values are means (SEM) from 5 individual seedlings measured 11 days after terminating xylem feeding.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( \pi^{100} ) (MPa)</th>
<th>( \pi^0 ) (MPa)</th>
<th>RWC(^{0} ) (%)</th>
<th>Va (%)</th>
<th>( \varepsilon ) (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (control)</td>
<td>-1.17 (0.03)(^{ab})</td>
<td>-1.61 (0.08)(^{ab})</td>
<td>88.9 (0.9)(^{a})</td>
<td>57.1 (3.9)(^{bc})</td>
<td>13.6 (1.1)(^{a})</td>
</tr>
<tr>
<td>ABA</td>
<td>-1.22 (0.09)(^{a})</td>
<td>-1.81 (0.11)(^{a})</td>
<td>84.9 (0.6)(^{b})</td>
<td>52.5 (4.7)(^{c})</td>
<td>10.0 (0.9)(^{b})</td>
</tr>
<tr>
<td>AMB</td>
<td>-1.18 (0.03)(^{a})</td>
<td>-1.86 (0.12)(^{a})</td>
<td>85.5 (1.3)(^{b})</td>
<td>59.7 (0.7)(^{bc})</td>
<td>10.6 (1.4)(^{ab})</td>
</tr>
<tr>
<td>AVG</td>
<td>-1.02 (0.03)(^{b})</td>
<td>-1.49 (0.06)(^{b})</td>
<td>88.7 (0.7)(^{a})</td>
<td>64.5 (1.1)(^{ab})</td>
<td>12.2 (0.8)(^{ab})</td>
</tr>
<tr>
<td>SPM</td>
<td>-1.03 (0.02)(^{b})</td>
<td>-1.53 (0.02)(^{b})</td>
<td>89.7 (0.3)(^{a})</td>
<td>68.9 (1.0)(^{a})</td>
<td>12.9 (0.6)(^{a})</td>
</tr>
</tbody>
</table>
CHAPTER V

Effect of chemical preconditioning on drought-induced physiological changes in white pine seedlings.

Abstract

Since Ambiol, AVG and spermine prevented membrane leakage under drought in beans, another study was conducted to ascertain their effect on this and other physiological changes in white pine. The effects of chemical preconditioning on the physiological responses of white pine seedlings to drought was studied by introducing Ambiol, AVG and spermine (10mg/l, 100μg/l and 100μg/l respectively) into the root-systems of seedlings for 48h. Drought, imposed by withholding water for 16 days, reduced xylem pressure potential, net photosynthesis and transpiration rate, and increased ethylene production and membrane leakage. Chemical pretreatment with these compounds prevented the decline in pressure potential under drought, and inhibited membrane leakage and ethylene production. Spermine treatment increased net photosynthesis under drought, compared to the stressed controls and Ambiol significantly reduced transpiration. Taken together, a reduction in ethylene-induced membrane leakage appeared to prevent visible senescence symptoms from developing under drought and allowed key physiological processes to continue when water became limiting.

5.1 Introduction

The reduction in growth in droughted plants is correlated with many physiological changes. Chlorophyll and protein content declined (Vieira De Silva, 1976) and photosynthesis was inhibited under drought (Bunce, 1982).
Membrane permeability also increased and resulted in a leakage of ions from cells and tissues (Mukherjee and Choudhuri, 1981, Zwiazek and Blake, 1990b). Increased ethylene production (Kacperska and Kubacka-Zebalska, 1985, 1989) and free radical formation (Dhindsa et al., 1981; Leshem, 1981; Leshem et al., 1981) resulted in a loss of membrane integrity (Thompson, 1988), lipid peroxidation (Dhindsa et al., 1981), and premature senescence (Brady et al., 1974) under drought.

Ethylene production increased at the climacteric in the ripening of fruits and in the senescing cut flowers (Celikel and Doorn, 1995). Inhibition of ethylene synthesis or action could delay the symptoms of senescence (Borocov and Woodson, 1989). Activated oxygen species, including O$_2^-$ or H$_2$O$_2$ react with lipids, caused peroxidation of unsaturated fatty acids in membranes and protein and enzyme denaturization (Leshem, 1981; Elstner, 1982, Monk et al., 1989). The accumulation of these free radicals initiated leaf senescence (Elstner, 1982). These activated oxygen species inactivated enzymes and, in the case of OH$^-$ or HO$^{-}$, they initiated lipid peroxidation (Asada and Takahashi, 1987; Halliwell, 1987).

Several phytohormones and chemical compounds inhibited leaf senescence and also preserved membrane integrity (Dhindsa et al., 1982). Pretreatment of plants with CaCl$_2$ (Mukherjee and Choudhuri, 1981; Poovaiah and Leopold, 1973), AgNO$_3$ (Abeles et al., 1992), cytokinin (Richmond and Lang, 1957; Wingler et al., 1998), polyamines (Borrel et al., 1997) retarded senescence.
Protection against oxygen radicals in stressed plants can be increased by antioxidants (Winston, 1990). These protect plants by scavenging chain-carrying peroxyl radicals or by diminishing the formation of initiating lipid radicals. Ambiol, a synthetic antioxidant, reduced membrane leakage under drought in black spruce (Borsos-Matovina, 1997; MScF thesis, University of Toronto) and jack pine (Rajasekaran and Blake, unpublished data) seedlings. An increase in free radicals including the super-oxide anion, singlet oxygen and hydroxyl ions initiated senescence under stress (Dhindsa, 1982). Imidazoles, such as Ambiol, interrupted free-radical chain reaction by donating a hydrogen atom (from their 2\textsuperscript{nd} position) to the oxygen radicals (Santrucek and Krepelka, 1988). Antioxidants also reduced the level of endogenous lipoxygenase activity in senescing tissues (Siedow, 1991) which suggests that exogenous Ambiol may delay drought-induced senescence.

Spermine also inhibited lipid peroxidation in osmotically stressed oat leaves (Borrel et al., 1997). Since lipid peroxidation was correlated with increased solute leakage (Dhindsa et al., 1981), pretreatment with spermine may also reduce membrane leakage in plants under drought. Spermine also counteracted ethylene-induced increase in membrane permeability (Naik and Srivastava, 1978).

Since AVG (aminoethoxyvinylglycine) inhibited the conversion of ACC to ethylene (Abeles et al., 1992), it may prevent membrane leakage and senescence under drought.
White pine is still commercially important in southern Canada. Treatments that enhance the resistance of seedling transplants to environmental stress will also promote growth. Since white pine is highly sensitive to biotic and abiotic stresses (Wendel and Smith, 1990), this study was therefore, conducted to determine whether antisenescence compounds prevent drought-induced injury in white pine seedlings possibly by inhibiting ethylene production and membrane leakage.

5.2 Materials and methods

5.2.1 Plant materials and pre-treatment

White pine seedlings (seed source 42-42-0-00) were grown in a commercial potting mix of Pro-mix, NX, (Premier Inc. Ont.) under 20±2°C temperature and a 16h photoperiod in the greenhouse at the University of Toronto. Seedlings were daily watered and fertilized weekly with 20:20:20 N-P-K fertilizer (Plant Products Ltd., Brampton, ON). Prior to starting the experiment, Forty (one-year old) seedlings of similar size were left to acclimate in a controlled environment growth chamber (PGW, Conviron Winnipeg, Man) for seven days at 24/22°C day/night temperature, 200-250µEin, m² s⁻¹ PAR under a 16h photoperiod.

Roots of seedlings were supplied with Ambiol, AVG and spermine (Sigma Chem. Co. Canada). White pine seedlings were grown in plastic pots (12.5X12.5X14cm) and were placed in plastic dishes (52X26X6cm) containing aqueous solution of above chemicals prepared at a concentration of 10mg/l,
100μg/l and 100μg/l respectively. There were 10 seedlings per treatment and chemicals were supplied as a root soak and solutions were aerated during the (48h) soaking treatment. After 48h of root-feeding, pots were fully drained. Drought was then imposed by withholding water to the remaining half of the seedlings for 16 days, while the other half were watered daily to field capacity.

5.2.2 Needle pressure potential

Midday xylem pressure potential (ψₐ) of the needles of both stressed and non-stressed seedlings, five seedlings per treatment, were measured using pressure chamber (PMS Instruments, Corvallis, OR, USA). Briefly, a group of needles in a fascicle were cut-off the stem and placed in the chamber with the cut end protruding from the lid. Since resin bubbles appear before the water droplet in most conifers and may indicate a false end-point (McGilvray and Barnett, 1988), hand lens was used to avoid such error and identify the water bubble accurately. The other precautions followed are described in Chapter IV.

5.2.3 Photosynthesis and gas exchange

Net photosynthesis and transpiration rate for both treated stressed and non-stressed seedlings were measured using a LI-6200 Portable Photosynthesis System (Li-Cor Inc., Lincoln, NE) after 16 days of drought. Due to accumulation of CO₂ from operators working in the growth chamber, measurements of gas exchange were taken outside in a well-ventilated room under similar conditions viz., 22±1°C and 300μmol m⁻²s⁻¹ of photosynthetically active radiation supplied by
a Multi-Vapor lamp (MVR 1000/C/VBU, General Electric Co.). Needle area was determined by water immersion, using Archimedes' principle, as described previously by Johnson (1984).

5.2.4 Membrane Leakage

Membrane leakage was determined by immersing needles in water and determining the increase in electrical conductivity as described by (Zwiazek and Blake 1990). Briefly, 10 needles were washed, immersed in a glass vial containing 28 ml of deionised water, and incubated at 20±1°C for 24 h on the laboratory bench. Specific conductivity (mmho cm⁻¹ or µS) of the solutions was measured using a Hi 8733 conductivity meter (Hanna Instruments Inc. Quebec). After measurement, the solutions were returned to their vials, sealed and then placed for 24 h in a hot air oven at 90±2°C to kill the tissues. These samples were left to cool to room temperature (20±1°C) and the measurements were repeated. Results were expressed as a percent of total electrolytes (i.e., those that leaked from heat-killed leafs).

5.2.5 Chlorophyll content

To measure the extent of chlorophyll degradation under drought, chlorophyll content was measured. Needles (0.5 gm) of treated stressed and unstressed seedlings were homogenized (10 ml of 80% acetone), centrifuged at 10,000g for 5 minutes, and absorbance of the supernatant was measured at 664nm and 647nm using an UV - 160 (Shimadzu) spectrophotometer.
Chlorophyll a and b and total chlorophyll was determined using the formulae of Jeffrey et al., (1974):

\[
\text{Chlorophyll a} = 11.73 E_{664} - 1.97 E_{647} \ \text{mg/l}
\]
\[
\text{Chlorophyll b} = 20.56 E_{647} - 5.42 E_{664} \ \text{mg/l}
\]
\[
\text{Chlorophyll (a + b)} = 18.59 E_{647} + 6.31 E_{664} \ \text{mg/l}
\]

5.2.6 Lipid peroxidation

Membrane damage attributable to peroxidation of the lipid component of membranes was estimated by the content of malondialdehyde (MDA), a decomposition product formed from oxidation of unsaturated fatty acids. MDA was estimated using the procedure of Heath and Packer (1968): 0.5 gms of needles were homogenized in 5 ml of trichloroacetic acid: water (TCA:H2O) 5:95(w/v) (Reagent A) and then centrifuged for 10 minutes at 8,000Xg. Two ml of the supernatant was combined with an equal volume of thiobarbituric acid reagent (0.5% TBA (w/v) in 20% TCA (w/v) (Reagent B) and heated at 95°C for 30 minutes. Absorbance was measured at 532nm and a non-specific absorbance at 600nm, and was substracted from the absorbance at 532nm to rectify the purity. MDA was calculated from the result using its extinction coefficient of 155mM⁻¹cm⁻¹ (Tarladgis et. al., 1962).

5.2.7 Ethylene evolution

Ethylene evolution was determined using a GC – 9A Shimadzu Gas Chromatograph with a Flame Ionization Detector (FID), a Porapak N (porous polymer beads) 80/100 column and 6' x 0.125" dimensions. The carrier gas was
helium, with a flow rate of 20ml/min. Detector gases were hydrogen and air (40 and 400ml/min, respectively). Injector and detector temperatures were 110°C and the oven (column) temperature (100°C) was run for at least 30 min prior to injection.

To measure ethylene evolution from white pine needles, 5 needles were enclosed in septum vials (2ml) and incubated for 2h at room temperature (22±1°C). A 1.0ml aliquot of the ethylene-containing atmosphere was injected into the gas chromatograph column for determination of ethylene production from needles of both treated and untreated seedlings. Peak retention time of ethylene was determined by determining peak area. The amount of ethylene was calculated with reference to a standard curve prepared using pure ethylene gas. Ethylene released was expressed as nl (g needle dry weight)^−1 h^−1.

5.2.8 Statistical analysis

A completely randomized experimental design, as described previously in Section 4.2.2 was followed. One way Analysis of variance (ANOVA) with pairwise multiple comparison procedure (Student-Newman-Keuls Method) was performed using a statistical software, SigmaStat™ (Jandel Scientific, CA).

5.3 Results

Compared to the stressed controls drought for 16 days significantly caused midday needle ψx to decline (Figure 5.1). These declined to −1.35 MPa in stressed control seedlings from −0.63 MPa in the unstressed control.
Pretreatment with Ambiol, AVG and spermine prevented significant (p<0.05) decline in pressure potential under stress.

Pretreatment with spermine significantly increased net photosynthesis (0.33 μmol/m²/sec) under drought compared to the control (0.16 μmol/m²/sec). Ambiol significantly reduced transpiration rate (0.07 μmol/m²/sec) under drought, compared to the untreated stressed controls (0.13 μmol/m²/sec) (Figure 5.4 and 5.5). AVG had no significant effect on net photosynthesis and transpiration under drought.

Drought significantly increased ethylene evolution from 18 in unstressed control to 38.3 nl/g needle dry weight h⁻¹ in stressed control seedlings. Pretreated with Ambiol, AVG and spermine significantly reduced ethylene evolution under, which declined to a third of pre-stress levels (Figure 5.2).

Drought significantly increased membrane leakage in the control (untreated) seedlings, compared to unstressed controls. Pretreatment with Ambiol, AVG and spermine significantly reduced membrane leakage (relative conductivity) under drought, compared to the stressed control (Figure 5.3). Ambiol, AVG and spermine treatment had no effect on MDA or chlorophyll content in either stressed or unstressed seedlings.

5.4 Discussion

Drought induced major changes in white pine seedling physiology. There was a significant decline in xylem pressure potential (111%) and net photosynthesis (205%) rates under drought relative to the fully watered control
seedlings. However, $\psi_k$ did not significantly decline (p<0.05) under drought in pretreated seedlings (Figure 5.1 and 5.4). Seedlings treated with spermine maintained significantly higher photosynthesis rate under drought. Pretreatment with Ambiol significantly reduced transpiration rate (46%), compared to the untreated stressed controls (Figure 5.4 and 5.5). This result confirms an earlier report that Ambiol reduced mid-day transpiration rate and total daily water usage in soybean under drought (Darlington et al., 1996).

Drought for 16 days increased ethylene production by 112%, compared to the unstressed control, as observed previously in other species (Aharoni, 1978; Guinn, 1976 and Wright, 1977). A peak (climacteric) of ethylene production is associated with the onset of cell tissue senescence (Paulin et al., 1986; Sylvestre and Paulin 1987; Sylvestre et al., 1989). These changes appear to explain the resulting increase in membrane permeability that occurs during drought (Dhindsa et al., 1981). Leakage of electrolytes and non-electrolytes under stress have been linked to ethylene production under stress (Irigoyen et al., 1992).

Permeability changes in membranes were also accompanied by losses of membrane phospholipids in senescing rose petals (Borocov et al., 1978) and black spruce seedlings (Zwiazek and Blake, 1990b). This suggests a possible sequence of senescence changes in membranes induced by ethylene. Ethylene production was also associated with activation of a lipoxygenase (Kacperska and Kubacka-Zelbalska, 1985), which increased lipid peroxidation. Peroxidation and ethylene production, although concomitant, are independent phenomena (Sylvestre et al., 1989).
The increase in membrane leakage paralleled ethylene evolution (Figure 5.2 and 5.3). In parallel to their higher \( \psi_w \) values under drought, pretreatment with antisenescence compounds caused significant reductions in both ethylene evolution and drought-induced membrane leakage under stress. Loss of membrane integrity is initiated quite early in senescence and prevention of membrane leakage will either slow or prevent senescence (Tiburcio et al., 1994).

There are three manifestations of membrane destabilization during senescence: i) rigidification of bulk membrane lipid, ii) the formation of gel phase lipid, and iii) the formation of non-bilayer lipid configurations (Thompson, 1988). This changes lead to membrane leakiness, advanced proteolytic activity, and the general loss of membrane function. Spermine stabilized the molecular composition of thylakoid membrane proteins and was able to preserve the structural integrity of the thylakoid membranes of oat (Besford et al., 1993; Tubircio et al., 1994). Rajasekaran and Blake (unpublished data) observed similar results, where spermine reduced drought-induced membrane leakage in jack pine seedlings. Spermine was an effective free radical scavenger and inhibited the superoxide-dependent conversion of ACC to ethylene (Drolet et al., 1986). Winer and Apelbaum (1986) suggested that polyamines including spermine inhibits ACC synthase since it is able to form a Schiff base with its cofactor, pyridoxal phosphate.

Ambiol significantly reduced membrane leakage under drought in white pine seedlings. An effect of Ambiol on membrane leakage would explain its growth promoting action under drought in canola and soybean (Darlington et al.}
A similar membrane-sparing action for Ambiol was also observed in drought and temperature stressed black spruce and jack pine (Borsos-Matovina, 1997; MScF thesis, University of Toronto). Since Ambiol and other compounds significantly reduced drought-induced ethylene production, a general antisenescence mode of action was indicated for these compounds.

Free radical-induced peroxidation of lipids caused membranes to deteriorate during senescence (Droillard et al., 1987). However, malondialdehyde (MDA) level did not increase in stressed seedlings in our study. Although treated seedlings appeared greener and exhibited growth flushes, chlorophyll contents in stressed seedlings were similar to levels in unstressed seedlings. The decline in chlorophyll content during leaf senescence is usually attributed to lipid peroxidation in the chloroplast membranes (Dhindsa et al. 1981; Irigoyen et al., 1992). Since chlorophyll content was slow to decline, in our own study, as observed previously (Innes, 1993), such a measurement appeared to have limited value as an indicator of stress. Lipid peroxidation was presumably less since MDA did not increase under stress.

Both the plasmalemma and the tonoplast can leak when synthesis of the key membrane constituents decline (Celikel and Droon, 1995). According to Celikel and Droon (1995), cell leakiness is not always related to lipid peroxidation. Hence, membrane leakage can occur in the absence of both lipid peroxidation and membrane phospholipid breakdown.

Stomatal closure has also been suggested as a cause of senescence (Thimann and Salter, 1979). However, since the decline in $\psi_r$ under drought
preceded stomatal closure, water stress was the primary cause of senescence in root-restricted alder seedlings under drought (Tschaplinski and Blake, 1985).

Loss of membrane function of guard cells may have prevented full stomatal closure under drought in untreated seedlings. The resulting dehydration when stomata failed to close (Figure 5.5) would explain the sequence of damage reactions observed in unconditioned white pine seedlings under drought.

Since ethylene evolution and membrane leakage were closely related, increase in ethylene could influence stomatal opening and closing patterns possibly through changes in membrane permeability.

Ethylene production is a key factor in the increase in membrane leakiness observed under drought. Pretreatment with spermine, AVG and Ambiol were able to significantly reduce ethylene production and transpiration rate and the combined effects reduced membrane leakage which delayed the appearance of senescence symptoms under drought in white pine seedlings.
Figure 5.1. Effects of different chemical compounds on midday needle pressure potential of white pine seedlings after 16 days of drought. Bars show the standard error of the mean (SEM). Values are the means of five seedlings. (where, □ = non-stressed and ■ = stressed seedlings, and CON = control, AMB = ambiol treated, AVG = AVG treated and SPM = spermine treated seedlings). Means with same letters are not significantly different at p= 0.05 as determined by one way ANOVA (pairwise multiple comparison procedure; Student-Newman-Keuls Method).
Figure 5.2. Effects of different chemical compounds on ethylene production of white pine seedlings after 16 days of drought. Bars show the standard error of the mean (SEM). Values are the means of five seedlings. (where, NDCON = undroughted control, DCON = droughted control, DAMB = Ambiol treated droughted, DAVG = AVG treated droughted and DSPM = spermine treated droughted seedlings). Means with same letters are not significantly different at $p=0.05$ as determined by one way ANOVA (pairwise multiple comparison procedure; Student-Newman-Keuls Method).
Figure 5.3. Effects of different chemical compounds on drought-induced membrane leakage of white pine seedlings after 16 days of drought. Bars show the standard error of the mean (SEM). Values are the means of five seedlings. (where, NDCON = undroughted control, DCON = droughted control, DAMB = Ambiol treated droughted, DAVG = AVG treated droughted and DSPM = spermine treated droughted seedlings). Means with same letters are not significantly different at p= 0.05 as determined by one way ANOVA (pairwise multiple comparison procedure; Student-Newman-Keuls Method).
Figure 5.4. Effects of different chemical compounds on net photosynthesis (µmol/m²/sec) of white pine seedlings after 16 days of drought. Bars show the standard error of the mean (SEM). Values are the means of five seedlings. (where, NDCON = undroughted control, DCON = droughted control, DAMB = Ambiol treated droughted, DAVG = AVG treated droughted and DSPM = spermine treated droughted seedlings). Means with same letters are not significantly different at p= 0.05 as determined by one way ANOVA (pairwise multiple comparison procedure; Student-Newman-Keuls Method).
Figure 5.5. Effects of different chemical compounds on transpiration rate (μmol/m²/sec) of white pine seedlings after 16 days of drought. Bars show the standard error of the mean (SEM). Values are the means of five seedlings. (where, NDCON = undroughted control, DCON = droughted control, DAMB = Ambiol treated droughted, DAVG = AVG treated droughted and DSPM = spermine treated droughted seedlings). Means with same letters are not significantly different at p = 0.05 as determined by one way ANOVA (pairwise multiple comparison procedure; Student-Newman-Keuls Method).
6.1 Introduction

White pine has been extensively used in reforestation, landscaping and stabilizing strip-mine spoils in North America (Wendel and Smith, 1990). Mature white pine trees endure extreme temperatures and desiccation over their natural distribution in the Great-Lakes St. Lawrence forest region. When used in reforestation, white pine seedlings are severely damaged by exposure to high light intensities and moisture deficits which lead to premature senescence (Stiell, 1985). In order to potentially increase the stress tolerance and competitiveness of white pine seedlings, chemical preconditioning agents were used as anti-ethylene agents to delay the key reactions in senescence. It was hypothesized that antisenescence agents preserve membrane integrity under drought, either by inhibiting ethylene production or by promoting turgor potential adjustment. Depending on the type of chemicals, both adjustments were observed in white pine.

6.2 Chemical preconditioning and drought tolerance

Previous exposure to drought increases plant tolerance to a later stress (Kramer, 1980). Water withholding is a widely used technique to harden plants in greenhouses and nurseries. Since withholding water does not influence each plant equally, the results of such conditioning are mixed. Drought preconditioning
has been found to be ineffective in conditioning some species (Seiler and Cazell, 1990; Frymire and Henderson-Cole, 1992) and increased mortality in Corsican pine seedlings (Guehl et al., 1993). Increased mortality in preconditioned seedlings can result when growth is depressed during the first growing season (van den Driessche, 1991a).

Chemical preconditioning offers a cheap, accurate and easy method of hardening seedlings. ABA and its synthetic analogs reduced transpiration under drought (Blake et al., 1990). Ambiol is a synthetic antioxidant that promoted growth of canola and soybean (Darlington et al., 1996) and reduced membrane leakage in jack pine seedlings under drought (Borsos-Matovina, 1997; MScF thesis, University of Toronto). However, since the effects of chemical preconditioning treatments on cell water relations and drought tolerance parameters has received less attention, a study was initiated using white pine, which is a highly stress-sensitive species. Since it was not clear which formulations and concentrations would be most effective in white pine, a preliminary study was conducted on bush beans (Phaseolus vulgaris L.).

6.3 Water Relations adjustments

The effects of different antistress compounds on shoot water relations parameters under drought were investigated. As observed in other conifers (Tan et al., 1992b), an active osmotic adjustment promoted dehydration tolerance in white pine seedlings (Chapter IV). Osmotic adjustment helps plant to maintain turgor at low water potential. Osmotic adjustment under drought is beneficial for
plants since it increases the accumulation of compatible solutes in the cytoplasm, which sustains enzyme function. The water content of the cell remains high as a result of osmoregulation which promotes turgor maintenance and growth where none would occur otherwise (Meyer and Boyer, 1972; Michelena and Boyer, 1982). Ambiol and spermine prevented the decline in water potential under drought (Chapter IV and V). Chemical preconditioning with Ambiol increased osmotic adjustment under drought (Chapter IV). Ambiol reduced the apoplastic water fraction by 10% under stress (Chapter IV). An increase in the apoplastic water fraction is a common physiological response to leaf water deficit which allows plants to acclimate to water stress (Gunasekara and Berkowitz, 1992). A reduction in apoplastic water fraction increases the availability of water, which increase the symplast water volume in the tissues and helps to prevent the decline in water potential by maintaining turgor. This in turn facilitates cell functioning under drought (Gunasekara and Berkowitz, 1992).

Spermine significantly increased bulk modulus of elasticity ($\varepsilon_{\max}$) under drought in white pine seedlings (Chapter IV). Elastic adjustment results from a modification of cell wall constituents that render them more elastic (Blake and Tschaplinski, 1992). A more elastic cell (i.e., a lower $\varepsilon_{\max}$) allows a tissue to shrink during dehydration without loss of turgor. Increases in cell elasticity (i.e., a decline in $\varepsilon_{\max}$) under drought were observed in poplar (Tschaplinski and Blake, 1989b), white spruce (Koppenaal et al., 1991) and black spruce seedlings (Fan et al., 1994). However, the apparent decline in elasticity in spermine
preconditioned seedlings may be a result of their higher turgor potentials, since elasticity decreases at higher turgor pressures (Kim and Lee-Stadelmann, 1984).

6.4 Gas Exchange adjustments

Spermine increased net photosynthesis and water use efficiency (WUE) in stressed seedlings compared to the untreated stressed controls (Chapter IV and V). The ability of spermine treated seedlings to continue photosynthesis under drought could compensate for the temporary losses of carbon resulting from osmoregulation (Figure 4.1 and 4.2), respiration and repair processes. Higher photosynthetic levels would supply additional carbon and energy to support growth of spermine-treated plants. The resulting increase in WUE would allow metabolism to continue as water availability decline under drought which would produce more competitive seedlings and should enhance growth rates after drought relief.

Ambiol significantly reduced transpiration in stressed seedlings compared to the stressed control (Chapter V). This suggests that Ambiol regulated stomatal closure and enhanced drought avoidance. Early stomatal closure also appeared to be more important in dehydration avoidance in Eucalyptus grandis clones (Blake and Bevilacqua, 1995), whereas conifers rely more on tolerance of dehydration (Blake and Tschaplinski, 1992).

The significant effects of different chemicals on physiological processes under drought is summarized in Table 6.1.
### Table 6.1 Effects of different chemicals on physiological processes under drought.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Effects</th>
</tr>
</thead>
</table>
| Spermine | Net photosynthesis (+)  
Water use efficiency (+)  
Bulk modulus of elasticity (+)  
Osmotic potential at zero turgor (+)  
Pressure potential (+)  
Ethylene production (-) and  
Membrane leakage (-). |
| Ambiol   | Osmotic potential at full turgor (+)  
Osmotic potential at zero turgor (+)  
Pressure potential (+)  
Transpiration rate (-)  
Apoplastic water fraction (-)  
Ethylene production (-) and  
Membrane leakage (-). |
| AVG      | Pressure potential (+)  
Ethylene production (-) and  
Membrane leakage (-). |

(+) indicates a significant increase (p<0.05) while - indicates a significant reduction (p<0.05).

### 6.5 Senescence

Cell membranes are the primary target of drought injury (Levitt, 1980). These membranes are dynamic systems with lipids arranged in a bilayer containing embedded proteins and others attached to the outer surface of...
membranes. An optimal lipid fluidity will allow protein function to continue during
drought, while rigidification of bulk membrane lipids has the opposite effect,
leading to membrane leakiness and loss of membrane function during
senescence (Thompson, 1988). The lipid to protein ratio changes (increases or
decreases) and sterol to lipid ratio decreases under simulated drought (Zwiazek
and Blake, 1990b). Membranes containing more lipids than proteins and those
with sterols remain more fluid, which provide a broader phase transition
temperature (Harwood, 1997; Guye, 1988).

Water stress accelerates key senescence processes in plants by
increasing ethylene production (Aharoni and Richmond, 1978; Dwivedi et al.,
1979), and is further increased by the formation of reactive oxygen intermediates
(Paulin et al., 1986; Sylvèstre and Paulin, 1987; Sylvèstre et al., 1989). These
changes increase membrane permeability, which initiates the process of
senescence (Dhindsa et al., 1981).

According to Nooden (1980) the critical questions regarding whole plant
senescence are (1) where does the degeneration start? and (2) which changes
are primary and central to the process. Because they are conspicuous, foliar
degeneration and pigment (chlorophyll) change have received most emphasis.
Visual symptoms of senescence were few in conifer seedlings which are rigid
and have a thick cuticle. Few visual symptoms were observed (mainly a small
degree of necrosis and flaccidity of needles) in present study. However, pigments
are slow to change when conifers are stressed (Innes, 1993). Membranes are
the earliest plant organelles to show symptoms of stress (Nilsen and Orcutt,
1996) and the increase in membrane permeability, as observed under drought in the present study, initiated the process of senescence (Dhindsa et al., 1981).

The ability of Ambiol and spermine to delay this increase in membrane permeability under drought in white pine seedlings, could result from the following:

i) Membrane stabilization:
Ambiol's structure is characterized by a saturated benzyl ring, five carbon side chain and one hydroxy -OH group which confers antioxidant properties (Santrucek and Krepelka, 1988). Antioxidants protect plants by scavenging chain carrying peroxyl radicals or by diminishing the formation of initiating lipid radicals. Ambiol and other antioxidants also reduce the level of endogenous lipoxygenase activity in senescing tissues (Siedow, 1991). Hence Ambiol may increase membrane stability by such an anti-senescence action.

Since Ambiol is a benzimidazole derivative, it could have the senescence delaying properties of a non-purine cytokinin (Fox, 1968). If it functions like other cytokinins, Ambiol will stimulate synthesis of polyamines in stressed plants which can scavenge free radicals, superoxide ions and stabilize membranes (Galston and Kaur-Sawhney, 1987; Drolet et al., 1986; Malabika et al., 1986).

Spermine scavenges free radicals which inhibit the superoxide-dependent conversion of ACC to ethylene (Drolet et al., 1986). This, in turn, will tend to preserve the structural integrity of proteins, including those in the thylakoid membranes (Tiburcio et al., 1994).
Figure 6.1. Schematic diagram showing the hypothetical sequence of changes that occur in preconditioned and unconditioned white pine seedlings under drought.
ii) inhibition of ethylene production:

Water stress increases ethylene production in plants (Aharoni, 1978; Guinn, 1976), which leads to premature senescence (Pell and Dann, 1991). Pretreatment with Ambiol and spermine inhibited drought-induced ethylene production in white pine seedlings. Since Ambiol is an antioxidant it will be likely to inhibit the formation of free radicals. This in turn will prevent the increase in ethylene synthesis which increases membrane permeability (Eskim and Grossman, 1977; Hipkins and Hillman, 1985) and cell disorganization that leads to senescence and cell death (Mayak and Halevy, 1980).

Ethylene and polyamines share the same biosynthetic pathway (see Chapter II) but their effects are opposite. S-adenosylmethionine (SAM) is the precursor for both ethylene and polyamines (Pell and Dann, 1991). In the presence of lipid hydroperoxides or superoxide ions, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) and then to ethylene (Drolet et al., 1986). However, polyamines inhibit this conversion of ACC to ethylene by reducing synthesis of ACC synthase and scavenging oxygen free radicals involved in catalytic conversion of ACC to ethylene (Pell and Dann, 1991).

Ambiol may prevent free radical formation by inhibiting oxidation of plant purines, which release superoxide and hydroxyl free radicals (Leshem, 1984). As an antioxidant, Ambiol may lower endogenous activity of lipoxygenases which generate free radicals under stress (Leshem, 1984).
6.6 Conclusions

Preconditioning with chemical compounds prevented turgor loss and inhibited several key senescence reactions in white pine seedlings. The most significant findings of this study are:

1. Seedlings treated with Ambiol and spermine showed significant osmotic adjustment during water-stress. This reduced the rate of decline in water potential in stressed seedlings, compared with the untreated unstressed controls.

2. Ambiol significantly reduced apoplastic water fraction and the resulting increase in symplastic water may have prevented the decline in water potential under drought. Its ability to maintaining turgor may be explained by the greater symplast volume in Ambiol treated seedlings.

3. Since Ambiol significantly reduced transpiration rate under drought, this would explain its turgor-enhancing properties under drought.

4. Drought decreased net photosynthesis in both preconditioned and unconditioned stressed seedlings. However, spermine-treated seedlings maintained significantly higher net photosynthesis, which increased water use efficiency under drought, compared to the untreated stressed seedlings.

5. Ambiol, AVG and spermine significantly reduced ethylene production under drought. This would explain how these treatments reduced membrane leakage in stressed plants. The reduction in membrane leakage was not the result of increased lipid stability, as shown by malondialdehyde (MDA) levels, which did not change significantly.
6. Spermine increased the maximum bulk modulus of elasticity ($\varepsilon_{\text{max}}$) under drought. The decline in elasticity (higher $\varepsilon_{\text{max}}$) in spermine-preconditioned seedlings may result from their higher turgor potentials, since elasticity decreases at higher turgor pressures.

Since electrolyte leakage and gas exchange measurements are highly correlated with plant field performance (Mattsson, 1997), increased stress tolerance (reduced electrolyte leakage and higher net photosynthesis) could enhance field performance in the preconditioned seedlings. Although xylem feedings was successful for preconditioning seedlings, root feeding method would be more convenient for large scale operations. Chemical treatments may provide an environmentally friendly, easy to apply, and inexpensive way of preconditioning seedlings. Further research should be conducted to test the long term effectiveness of chemical preconditioning in the field.
References


Blake, T. J. 1983. Transplanting shock in white spruce; effect of cold-storage and root pruning on water relations and stomatal conditioning. Physiol. Plant. 57: 210-216.


Fan, S., Blake, T.J. and Blumwald, E. 1994b. The relative contribution of elastic and osmotic adjustments to turgor maintenance of woody species. Physiol. Plant. 90: 408-413.


Wright, S. T. C. 1977. The relationship between leaf water potential ($\psi_{leaf}$) and the levels of abscisic acid and ethylene in excised wheat leaves. Planta 134: 183-189.


Zwiazek, J. J. and Blake, T. J. 1990b. Effects of pre-conditioning on electrolyte leakage and lipid composition in black spruce (Picea mariana) stressed with polyethylene glycol. Physiol. Plant. 79: 71-77.


Appendices

Appendix - A. Summary of the results of one way ANOVA.

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Factor</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter III</td>
<td>Spermine and membrane leakage</td>
<td>5.011</td>
<td>0.0091</td>
</tr>
<tr>
<td></td>
<td>AVG and membrane leakage</td>
<td>6.101</td>
<td>0.004</td>
</tr>
<tr>
<td>Chapter IV</td>
<td>Water potential</td>
<td>8.21</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>Relative water content at zero turgor</td>
<td>6.13</td>
<td>0.0384</td>
</tr>
<tr>
<td></td>
<td>Osmotic potential at zero turgor</td>
<td>8.78</td>
<td>0.0181</td>
</tr>
<tr>
<td></td>
<td>Osmotic potential at full turgor</td>
<td>15.57</td>
<td>0.0043</td>
</tr>
<tr>
<td></td>
<td>Apoplastic water fraction</td>
<td>15.64</td>
<td>0.0042</td>
</tr>
<tr>
<td></td>
<td>Bulk modulus of elasticity</td>
<td>7.68</td>
<td>0.0242</td>
</tr>
<tr>
<td></td>
<td>Photosynthesis</td>
<td>3.55</td>
<td>0.0240</td>
</tr>
<tr>
<td></td>
<td>Water use efficiency</td>
<td>3.44</td>
<td>0.0270</td>
</tr>
<tr>
<td>Chapter V</td>
<td>Water potential</td>
<td>13.8</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Ethylene</td>
<td>5.29</td>
<td>0.0150</td>
</tr>
<tr>
<td></td>
<td>Membrane leakage</td>
<td>5.29</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Photosynthesis</td>
<td>19.3</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Transpiration</td>
<td>7.66</td>
<td>0.0007</td>
</tr>
</tbody>
</table>
Appendix – B.

Needle pressure potentials, net photosynthesis, transpiration and water use efficiency of white pine seedlings after 11 days of drought. Each value is the mean of 5 independent measurements (different plant population) ± standard error of the means. Means followed by same letter do not differ significantly at p<0.05; as determined by one way ANOVA (pairwise multiple comparison procedure; Student-Newman-Keul method)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>$\Psi_{\text{needle}}$ (MPa)</th>
<th>Photosynthesis (µmol/m²/sec)</th>
<th>Transpiration (µmol/m²/sec)</th>
<th>Water Use Efficiency (µmol CO₂: mmol H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.86 ± 0.02a</td>
<td>0.25 ± 0.02a</td>
<td>0.14 ± 0.05a</td>
<td>2.11 ± 0.05a</td>
</tr>
<tr>
<td>ABA</td>
<td>-0.61 ± 0.04a</td>
<td>0.23 ± 0.009a</td>
<td>0.12 ± 0.02a</td>
<td>1.90 ± 0.13a</td>
</tr>
<tr>
<td>Ambiol</td>
<td>-0.68 ± 0.03a</td>
<td>0.24 ± 0.04a</td>
<td>0.10 ± 0.02a</td>
<td>2.37 ± 0.4a</td>
</tr>
<tr>
<td>AVG</td>
<td>-0.65 ± 0.01a</td>
<td>0.19 ± 0.05a</td>
<td>0.10 ± 0.03a</td>
<td>1.84 ± 0.35a</td>
</tr>
<tr>
<td>Spermine</td>
<td>-0.70 ± 0.04a</td>
<td>0.40 ± 0.06b</td>
<td>0.11 ± 0.03a</td>
<td>3.82 ± 0.5b</td>
</tr>
</tbody>
</table>