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New Plant Growth Regulators Protect Photosynthesis and Enhance Growth Under Drought of Jack Pine Seedlings

L. R. Rajasekaran[1]* and T. J. Blake[2]

[1]Department of Plant Science, Nova Scotia Agricultural College, PO Box 550, Truro, Nova Scotia, Canada B2N 5E3; and [2]Faculty of Forestry, Earth Sciences Centre, University of Toronto, 33 Wilcocks Street, Toronto, Canada M5S 3B3.

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Abstract. To determine whether natural plant growth regulators (PGRs) can enhance drought tolerance and the competitive ability of transplanted seedlings, 1.5-year-old jack pine (*Pinus banksana* Lamb.) seedlings were treated with homobrassinolide, salicylic acid, and two polyamines, spermine and spermidine, triacontanol, abscisic acid (ABA), and the synthetic antioxidant, Ambiol. PGRs were fed into the xylem for 7 days and plants were droughted by withholding water for 12 days. ABA, Ambiol, spermidine, and spermine at a concentration of 10 μg L^{-1} stimulated elongation growth under drought, whereas ABA, Ambiol, and spermidine maintained higher photosynthetic rates, higher water use efficiency, and lower Ci/Ca ratio under drought compared with control plants. The damaging effects of drought on membrane leakage was reversed by Ambiol, ABA, triacontanol, spermidine, and spermine. Because ABA, Ambiol, and both polyamines enhanced elongation growth and also reduced membrane damage in jack pine under drought, they show promise as treatments to harden seedlings against environmental stress. The protective action of these compounds on membrane integrity was associated with an inhibition of ethylene evolution, with a reduction in transpiration rate and an enhancement of photosynthesis, which together increased water use efficiency under drought. Although most of the tested compounds acted as antitranspirants, the inhibition in membrane leakage in ABA-, Ambiol-, and polyamine-treated plants appeared more closely related to the anti-ethylene action.

Key Words. ABA—Ambiol—Drought—Ethylene—Homobrassinolide—Membrane leakage—Photosynthesis—*Pinus banksiana*—Polyamines—Salicylic acid—Triacontanol

Conifer planting programs have expanded greatly over the past decade and almost a billion seedlings are planted each year in Canada. Low survival rates and slow seedling growth rates are reducing the long-term benefits from conifer plantations (Farnum et al. 1983). A lack of seedling vigor and poor regeneration success would explain why less than half of conifer stands in Ontario were successfully stocked to commercial conifers after cutting, whereas the remainder converted to hardwood stands after cutting (Hearnden et al. 1992). Because young, succulent seedlings dehydrate to the turgor loss point after transplantation to harsh boreal planting sites in northern Ontario (Blake and Sutton 1987, Grossnickle and Blake 1986), there is a need to harden seedlings against environmental stress.

Drought is the main environmental stress in boreal clear cuts. It slows growth (Kramer 1986), closes stomata (Hanson and Hitz 1982, Tan et al. 1992), and reduces net photosynthesis (Pn). Several lines of evidence suggest membranes are the primary sites of desiccation injury to cells and organelles. Loss of membrane integrity was suggested by the increase in electrolyte leakage in black spruce under drought (Fan and Blake 1994, Tan and Blake 1993). Severe dehydration causes a decline in sterols, lipids, and their ratio (Zwiazek and Blake 1990). Drought-induced membrane leakage may explain alteration in membrane constituents. Because ethylene production increases under environmental stress, it could also disturb membrane function in dehydrated seedlings (Blake and Reid 1981, Hipkins and Hillman 1985).

Plants can be hardened using a mild preconditioning

Abbreviations: ABA, abscisic acid; BR, brassinolide; Cs, stomatal conductance; EPT, ethanol pretreated; ER, elongation rate; HBR, homobrassinolide; NPT, without pretreatment; PA, polyamine; PGR, plant growth regulator; Pn, photosynthesis; SA, salicylic acid; Spd, spermidine; Spm, spermine; TI, transpiration; TRIA, triacontanol; WPT, water pretreated; WUE, water use efficiency.

*Author for correspondence: E-mail: rlada@cox.nsac.ns.ca

drought, heat, cold, or using applied chemicals (Kozlowski 1979). When repeated, drought accelerated stomatal closure, increased turgor potential, and delayed turgor loss when black spruce seedlings were subjected to a later, more severe, stress (Blake et al. 1991, Zwiazek and Blake 1989).

Chemical antitranspirants function by either metabolically closing stomata or by physically blocking stomatal pores. However, because most antitranspirants are phytotoxic, they inhibit growth (Kozlowski 1979). Abscisic acid (ABA) is an effective antitranspirant, and several synthetic analogs of ABA (e.g., the acetylenic alcohol) also increased net Pn and reduced transpiration (Tl) under drought, and thereby increased their ratio, called water use efficiency (WUE) (Blake et al. 1990a).

Several natural compounds (brassinolide, salicylic acid, polyamines, and triacontanol) promote growth and may now be considered to be plant growth regulators (PGRs). Brassinolide (BR) is the only naturally occurring steroidal plant hormone with a seven-membered fused lactone ring (Mandava 1988). BRs have been found in a wide range of plants, including dicots, monocots, gymnosperms, and algae (Kim 1991). BRs are found to promote or inhibit growth. For example, they promoted shoot elongation of mung bean hypocotyls (Kamuro and Inada 1991) and shoot elongation and dry matter production in jack pine seedlings (Rajasekaran and Blake 1998). However, they inhibited root growth and development in some species (Roddick and Guan 1991) and have many other sites of action. For example, they alter plasmalemma energization; assimilate uptake and transport; and enhance resistance to chilling, diseases, and salt stress (Cutler et al. 1991, Iwahori et al. 1990).

Salicylic acid (SA), a natural plant product in willow bark, combines growth enhancement and antisenescence properties (Raskin 1995). SA increases plant height growth, reverses ABA-induced stomatal closure and leaf abscission, and stimulates adventitious root initiation (Malamy and Klessig 1992). Polyamines (PAs) accumulate under nutrient and salt stresses, where they promote growth of unicellular organisms and cultured plant cells (Bagni and Pistocchi 1988). Spermine (Spm) enhanced both elongation growth and dry mass of jack pine seedlings (Rajasekaran and Blake 1998). Triacontanol (TRIA), a primary alcohol found in lucerne and tea also promoted growth and increased CO_2 assimilation in many agricultural species (Ries 1991). Although a number of newer PGRs have been found to promote growth of plants under favorable conditions, little information is available on their influence under unfavorable conditions. There have been no detailed studies on their effects on Pn and growth or their mechanism of action in drought-stressed plants.

Ambiol, a derivative of 5-hydroxybenzimidazole (2-methyl-4-dimethylaminomethyl-5-hydroxybenzimidazole dihydrochloride) was found to promote growth of both nonwoody species (Smirnov et al. 1983) and conifers (*Pinus sylvestris* L. and *Picea abies* L. Karst.) when added as a seed soak (Kuznetov et al. 1986, Vishnevetskaia et al. 1986). Ambiol has also promoted growth of canola and soybean seedlings under drought and showed antitranspirant properties (Darlington et al. 1996).

These newer PGRs may enhance survival and growth in seedlings challenged by drought. Natural and synthetic antioxidants, antitranspirants, and other compounds were tested to see whether they enhance drought tolerance by preventing membrane breakdown. It is possible that they reduce ethylene production with a consequent membrane-sparing action in drought-stressed plants.

Materials and Methods

Plant Material and Culture

Jack pine (*Pinus banksiana* L) seeds were germinated and grown for 18 months in a commercial potting mix (peat, vermiculite, sphagnum moss; Pro-mix, Premier Inc. Ontario, Canada) under natural photoperiod (Toronto latitude 43 degrees 80′N) and 22 ± 4°C temperature in an automated greenhouse. Seedlings were watered daily with an automated sprinkler system, fertilized twice weekly with a soluble fertilizer mixture (20:20:20 NPK; Plant Product Co. Ltd. Bramalea, Ontario, Canada). Thirteen groups of 25 seedlings were then left to acclimate for 10 days in a controlled environment growth room (PGW, Conviron Winnipeg, Manitoba, Canada) at 24/22°C day/night temperature, 220 μmol m^{-2} s^{-1} PAR and 16-h photoperiod. To minimize the diurnal variation in gas exchange and other parameters in the growth chamber, only five seedlings per treatment per replication could be measured in a 2-h period (8 AM–10 AM). On the eleventh day of acclimation the seedlings were treated with 25 mL of various PGRs dissolved in either 2 mL ethanol (HBR, TRIA, SA) or R.O. water (ABA, Ambiol, Spm, Spd). The compounds were HBR (5 ng L^{-1}), SA (10 μgL^{-1}), Spm (10 μg L^{-1}), Spd (10 μg L^{-1}), TRIA (10 μg L^{-1}), ABA (10 μg L^{-1}), or Ambiol (10 μg L^{-1}). Chemicals were introduced into the xylem using a cotton wick threaded through the stem with a needle. The needle was inserted in one side and passed out the other side of the stem. The excess thread was cut and sealed using paraffin wax. One end of the cotton thread was connected to the xylem and the other end was attached to a sealed reservoir. As liquid was absorbed by the xylem, it was replaced by capillary action from a 15-mL opaque plastic reservoir attached to the stem. Xylem feeding continued for 7 days, and plants also received soil irrigation daily. Appropriate controls without pretreatment (NPT), water pretreated (WPT), and ethanol (2 mL L^{-1}) pretreated (EPT) were run. Drought was then imposed by withholding water for 12 days. Plant location in the growth chamber was changed each day. There were five seedlings per treatment replicated five times.

Morphological and Physiological Measurements

New shoot growth elongation was measured each day and elongation rate (ER) was calculated and expressed as mm d^{-1}. Xylem pressure potential (ψ_x) was determined using a Scholander-type pressure chamber. Membrane leakage was determined by immersing needles in water and determining the increase in electrolyte leakage. Needles (10/plant located adjacent to those used for photosynthetic measurements) were separated, briefly washed, immersed in a glass vial containing 28 mL

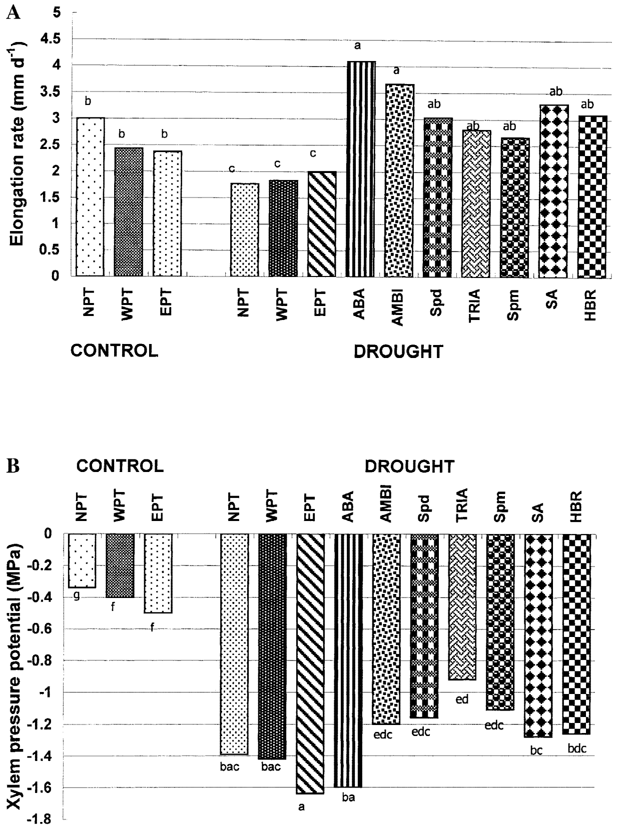

Fig. 1. Effects of xylem feeding of various plant growth regulators on daily stem elongation rate *(A)* and xylem pressure potential *(B)* of 18-month-old jack pine seedling exposed to natural drought. NPT, Non-pretreated; WPT, water pretreated; EPT, ethanol (2 mL L^{-1}) pretreated; ABA pretreated; AMBI, Ambiol pretreated; Spd, spermidine pretreated; TRIA, triacontanol pretreated; Spm, spermine pretreated; SA, salicylic acid pretreated; HBR, homobrassinolide pretreated. WPT served as control for ABA, AMBI, Spd, and Spm pretreatments, whereas EPT served as control for TRIA, SA, and HBR pretreatments.

of deionized water, and incubated at 20 ± 1°C for 24 h. Specific conductivity of the solutions was measured with a Hanna I conductivity meter (Hanna instruments, USA). After measurement, the solutions were returned to their vials, sealed, and then placed for 4 h in an hot air oven at 90 ± 2°C to kill the needle tissues. These samples were left to cool to room temperature and the measurement was repeated. Results were expressed as Membrane Injury Index, the % of total electrolytes (i.e., those that leaked from heat-killed needles). These techniques are described by Zwiazek and Blake (1990) and Odlum and Blake (1996).

Pn, stomatal conductance (Cs), transpiration (Tl), Ci/Ca, and WUE were measured with an LI-6200 Portable Photosynthetic System (Li-Cor-Inc., Lincoln USA). To avoid operator-induced carbon dioxide buildup, measurements were made immediately outside the growth chamber with a multivapor lamp (MVR 1000/U/VBU, GE, USA) under a PAR of 1,000 μmol m^{-2} sec^{-1}. WUE was calculated as the instantaneous ratio of Pn/Tl. Needle area was determined by water immersion as described previously by Johnson (1984).

Ethylene evolution was measured using the techniques of Blake and Reid (1981). Ten needles were sealed with rubber septum and incubated for 2 h at 20 ± 1°C in preweighed vials (5 mL). Two mL of the gas samples were then drawn from the vial with a syringe and injected into a Varian 3700 gas chromatograph (FID), using the following conditions: Poropak N (80/100) column (180 × 0.30 cm), carrier gas (nitrogen), and flow rate (20 mL min^{-1}), detector gases (hydrogen and air, 40 and 400 mL min^{-1}, respectively), injector and detector temperature (110°C) and the oven temperature (100°C). Ethylene was identified from its retention time (0.75 min) and concentration determined from peak areas.

The GLM procedure (SAS Institute Inc., Cary, NC) was used for ANOVA with the Duncan multiple range test for separation of differences between means for all the data.

Results

Stem Elongation Rate

ER declined by almost 42% after 12 days drought in non-pretreated control seedlings (Fig 1*A*). Elongation of ABA, Ambiol, SA, HBR, Spd, and Spm pretreated plants was significantly (p < 0.05) promoted under drought, compared with their own (specific) untreated controls. The stimulation under drought was the greatest with ABA (68%) and Ambiol (51%) compared with the unstressed controls. ER was significantly (p < 0.05) stimulated by Spd (25%) and Spm (10%), but to a lesser degree.

Xylem Pressure Potential

Drought lowered the xylem pressure potential (ψ_x) of the main stem significantly in non-pretreated (−0.34 MPa to −1.39 MPa), water pretreated (−0.4 MPa to −1.4 MPa), and ethanol pretreated (−0.5 MPa to −1.7 MPa) plants (Fig 1*B*). The decline in ψ_x under drought (309%) of the untreated seedlings was significantly (p < 0.05) greater than that of controls. Drought also caused a significant decline in ψ_x of the PGR-treated plants compared with their respective controls. Actual ψ_x values for the PGR-treated plants under drought were: −0.9 MPa (TRIA), −1.15 MPa (Spm), −1.1 MPa (Spd), −1.2 MPa (Ambiol), −1.3 (SA and HBR), and −1.6 MPa (ABA). However, the decline in ψ_x among the PGR-pretreated droughted plants was not statistically significant. Several treatments prevented the decline in ψ_x that occurred in the untreated controls. Ambiol, TRIA, Spd, and Spm treatments prevented this decline and there was no significant (p < 0.05) decline in ψ_x, relative to the untreated droughted controls. Treatment ψ_x values were in the range of −0.9 MPa (TRIA) to −1.2 MPa (Ambiol). In marked contrast to these compounds, ABA caused ψ_x to decline significantly (−1.6 MPa), relative to the untreated controls (−0.4 MPa).

Membrane Leakage

Index of injury values express the leakage of electrolytes from a stressed plant in terms of the total electrolytes, determined as leakage from killed tissue. Membrane In-

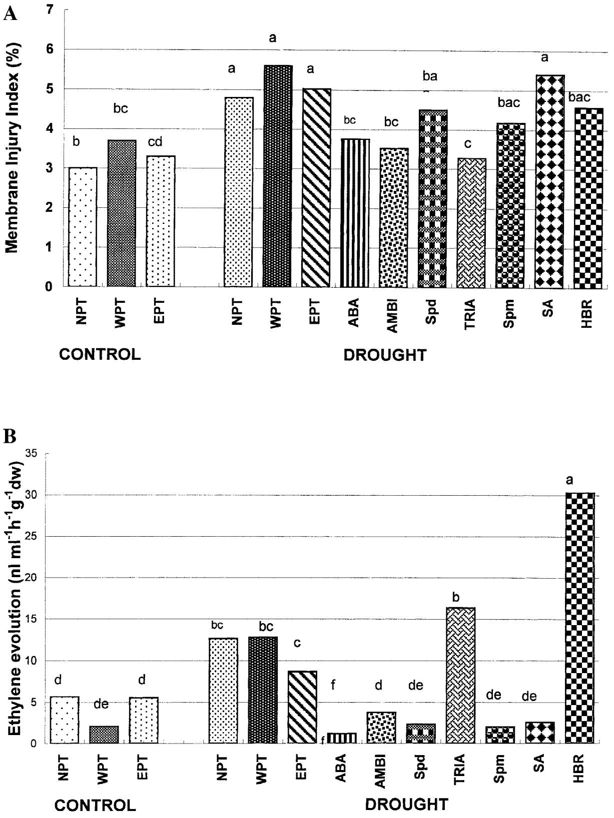

Fig. 2. Effects of a xylem feeding with various plant growth regulators on membrane leakage *(A)* and ethylene evolution *(B)* of jack pine seedlings under drought. Other details are as shown in the legend to Fig. 1.

jury Index of untreated jack pine increased significantly ($p < 0.05$) by 56–67% under drought, compared with the unstressed controls (Fig 2*A*). All compounds, except SA, reduced membrane leakage under drought, compared with the controls. However, the greatest reduction in leakage resulted from treatment with TRIA, Ambiol, and ABA, and the levels were all significantly less than that of the untreated, droughted plants. These compounds caused the index of injury to decline from 5.5 (drought, untreated) to 3.7% (ABA), 3.3 (Ambiol) to 3.2% (TRIA), which represents a reduction in injury of 33%, 40%, 42%, respectively. These differences were statistically significant at $p < 0.05$.

Ethylene Evolution

Ethylene evolution of the drought-stressed controls increased significantly under drought to levels that were 120–300% greater than the unstressed controls (Fig 2*B*). By contrast, ABA, Ambiol, Spd, Spm, and SA treatments all significantly reduced the drought-induced ethylene production compared with untreated stressed plants (12.5 nL mL $^{-1}$ h^{-1} g^{-1} dw). However, HBR stimulated ethylene evolution in droughted plants (30.1 nL mL^{-1} h^{-1} g^{-1} dw). Ethylene evolution was statistically similar under TRIA treatment to levels found in the untreated drought-stressed controls.

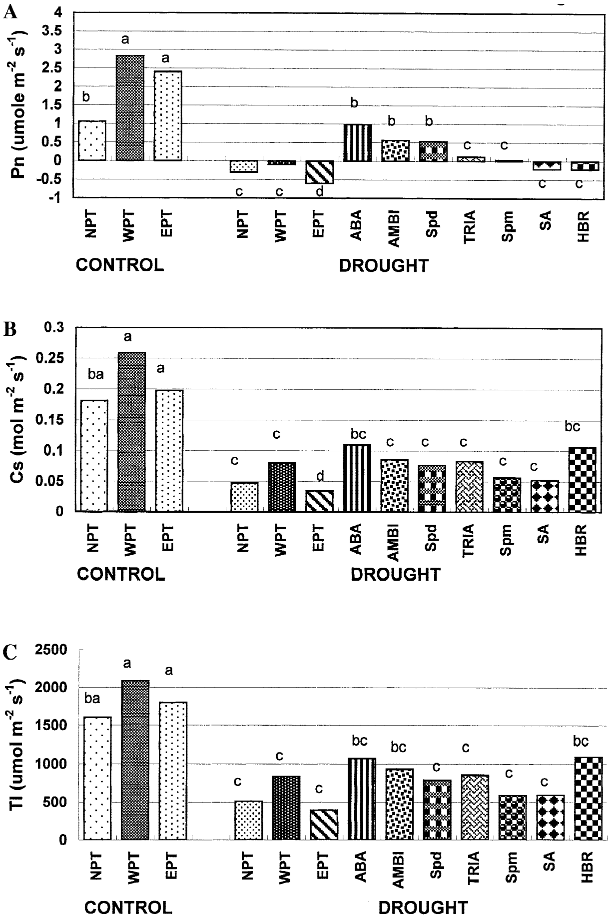

Fig. 3. Effects of a xylem feeding with plant growth regulators on net photosynthesis *(A)*, stomatal conductance *(B)*, and transpiration rate *(C)* of jack pine seedlings exposed to drought. Other details are as shown in the legend to Fig. 1.

Net Photosynthesis

Pn of untreated plants declined to about one third of prestress levels after 12 days of drought (i.e., from 1.05 μmol m^{-2} s^{-1} to -0.30 μmol m^{-2} s^{-1} (Fig. 3*A*). However, much of this reduction was prevented by treatment with ABA (0.98), Ambiol (0.56), and Spd (0.53 μmol m^{-2} s^{-1}). These treatments all had significantly higher Pn under stress than the untreated droughted plants. ABA, Ambiol, and Spd were equally effective in significantly ($p < 0.05$) preventing the drought-induced decline in gas

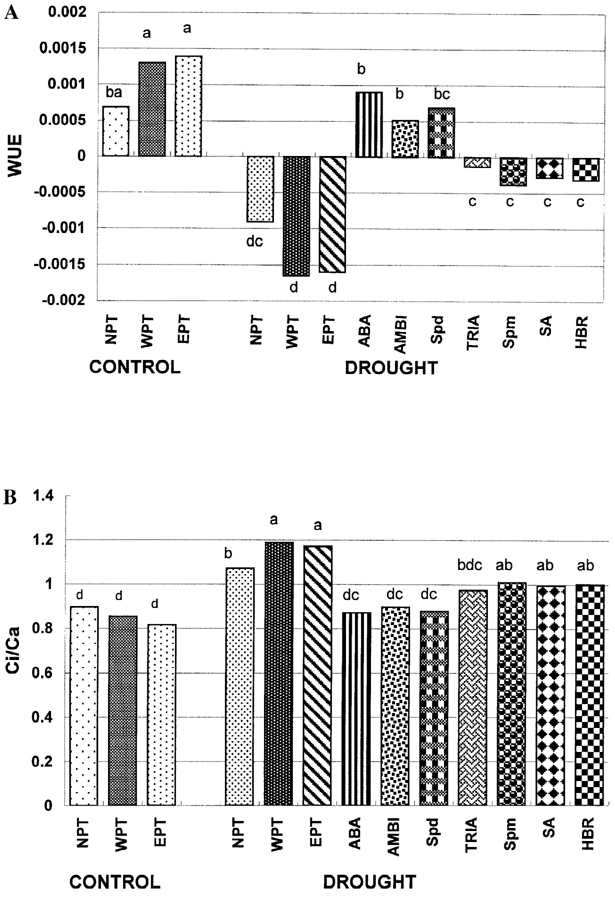

Fig. 4. Effects of a xylem feeding of various plant growth regulators on water use efficiency, WUE *(A)* and Ci/Ca ratio *(B)* of jack pine seedlings exposed to drought. Other details are as shown in the legend to Fig. 1.

exchange, and the levels observed in these treatments did not differ from those observed in untreated controls.

Stomatal Conductance

There was a significant ($p < 0.05$) decline in stomal conductance (Cs) after 12 d drought compared with untreated control plants. The decline in Cs under drought was about 80% (from 0.18 to 0.05 cm s^{-1}). Several PGR treatments (AMBI, Spd, TRIA, Spm, and SA) prevented this decline, and Cs did not differ significantly ($p < 0.05$) from values found in the untreated, stressed plants. The apparent decline in Cs of HBR- and ABA-treated plants under drought was also nonsignificant relative to the unstressed controls (Fig. 3*B*).

Leaf Transpiration Rate

The influence of PGRs on leaf Tl mirrored the Cs response to drought (Fig.3*C*). Drought caused a significant decline (1604–500 μmol m^{-2} s^{-1}) in Tl. Despite a large apparent decline in some PGR treatments (e.g., from 1604–588 μmol m^{-2} s^{-1} in the control and Spm treatment, respectively, the different PGR treatments did not differ significantly ($p < 0.05$).

Water Use Efficiency

Drought caused a significant decline in WUE (230%) in untreated seedlings, from 7×10^{-4} (in unstressed controls) to -9×10^{-4} (Fig. 4*A*). PGR pretreatment, especially, ABA, Ambiol, and Spd, caused a significant ($p < 0.05$) increase in WUE compared with droughted controls. The greatest increases in WUE were observed with ABA (9×10^{-4}), Spd (7×10^{-4}), and Ambiol (5×10^{-4}). The differences were not statistically significant ($p < 0.05$) compared with the unstressed controls.

Ci/Ca Ratio

Ci/Ca increased significantly ($p < 0.05$) in the untreated plants by 20% (from 0.85–1.2) under drought compared with unstressed control (Fig 4*B*). The increase in intercellular CO_2 concentration showed that drought reduced carboxylation. Several PGRs sustained carboxylation efficiency to varying degrees. Carboxylation efficiency was significantly ($p < 0.05$) greater as shown by the lower values obtained with ABA (0.87), Spd (0.88), and Ambiol (0.89) under stress compared with the untreated droughted plants. However, HBR, Spm, and SA were ineffective and showed an apparent, nonsignificant increase in Ci/Ca relative to the untreated stressed plants.

Discussion

The decline in ψ_x under drought (from −0.3 to −1.3 MPa) was accompanied by a significant inhibition of Pn (Fig 3*A*) and increased membrane leakage (Fig 2*A*). These changes under drought were associated with (1) a reduction in stem elongation, which declined by 45% (Fig 1*A*), (2); an increase in membrane damage, detected as an increase in membrane injury index (Fig.2*A*); and (3) higher rates of ethylene evolution (Fig 2*B*).

Along with the reduction in Cs (Fig. 3*B*), which limited CO_2 uptake, Pn declined (Fig. 3*A*). The decline in Pn was less than the (nonsignificant; $p < 0.05$) decline in transpiration rate (Fig. 3*C*), which increased WUE under drought (Fig. 4*A*). The increase in Ci/Ca ratio under drought also indicated that carboxylation was also disturbed by drought.

These Pn responses to drought were wholly or partially reversed by ABA and Ambiol, which also allowed stem elongation in jack pine to continue under drought.

The promotive effects of PGRs were not the result of a more favorable ψ_x because seedlings treated with HBR, SA, and TRIA failed to show any enhancement of stem elongation, despite their significantly ($p < 0.05$) higher xylem pressure potentials ($\psi_x = -0.9$ to -1.1 MPa). Hence, the growth-promoting action of Ambiol and ABA may have been triggered by other processes.

The primary effect of desiccation is on the membrane, and drought-induced inhibition of elongation growth in jack pine seedlings could be explained by a drought-induced increase in membrane leakage. Zwiazek and Blake (1990) observed that drought caused a reduction in sterols, phospholipids, and sterol/phospholipid ratio along with the increase in membrane leakage in dehydrating black spruce. A shift in phospholipid concentration could explain membrane damage because drought-induced peroxidation of lipids results from the formation of free radicals (O_2^-, H_2O_2, and/or -OH), which destabilize chloroplast, mitochondrial, and/or microsomal membranes.

PGR pretreatment helped to maintain membrane integrity under drought to differing degrees. Ambiol was one of the most effective compounds, reducing leakage by nearly 40% compared with the water pretreated–droughted plants (Fig 2*A*). Its low molecular weight, aromatic structure, and a single hydroxy group in the aromatic ring improve antioxidant properties (Burlakova and Kharpova 1985) and its benzimidazole structure suggests that Ambiol is a nonpurine cytokinin (Fox 1969). Ambiol is a structural analog of a cytokinin, zeatin, and α-tocopherol, and a membrane-stabilizing action may contribute to its antioxidant properties (Burlakova and Kharpova 1985). This suggests that in its antisenescence and membrane-preserving action, Ambiol may resemble a cytokinin.

Five of seven compounds tested showed a strong antiethylene action. This suggests a reduction in ethylene evolution may have prevented membrane damage under drought. Ethylene evolution in jack pine increased by almost 350% under drought. A buildup of ethylene would increase membrane leakage because ethylene alters membrane fluidity under drought (Mayak et al. 1977). An increase in ethylene production was rapidly followed by an increase in tonoplast permeability in carnations (Mayak et al. 1977). Permeability changes caused a massive loss of phospholipids in senescencing rose petals (Borochov et al. 1976). This suggests that the loss of membrane integrity under drought could result from an effect of ethylene on phospholipids. Many of the beneficial effects of Ambiol and ABA on membrane stability could result from the observed inhibition of ethylene synthesis (Fig 2*B*). Although Spm and Spd also reduced ethylene evolution, there was no significant reduction in membrane leakage. TRIA also reduced membrane injury without reducing ethylene evolution, which suggests a different mode of action. Taken together, this suggests that many mechanisms may protect membranes from drought damage in plants.

A decline in Cs and TI rate helped to conserve water in droughted plants (Fig 3*B,C*). Although a number of compounds lowered TI rates, treatment effects on membrane injury were variable. For instance, despite low values for Cs and Tl in SA-treated plants, membrane injury was greatest despite only a small decline in ψ_x. This suggests that membrane protection cannot be fully explained by their antitranspirant action.

PAs can counteract changes in membrane permeability induced by other PGRs. In particular, they affect the membrane localized proton-secreting system, one of the probable targets of auxin action (Galston 1995). A membrane-stabilizing effect under drought has not been previously reported for most of these tested compounds.

Despite excellent membrane protection, TRIA failed to promote growth under drought, which suggests that other factors are required to sustain growth under drought. The ability of TRIA-treated seedlings to continue to photosynthesize under drought could compensate for C losses in osmoregulation, respiration, and repair. However, for high rates of Pn to be sustained, stomata would have to remain open, and the resulting increase in TI rates could severely dehydrate plants. Hence, the ability to limit transpiration more than Pn (i.e., higher WUE) would allow both assimilation and growth to continue under drought.

Drought caused a significant reduction in Pn (Fig 3*A*). However, this was only partly a result of stomatal closure (Fig 3*B*) because carboxylation was also limited, as shown by the increased Ci/Ca ratio (Fig 4*B*). However, the decline in assimilation rates in ABA, Ambiol, and Spd pretreated plants was smaller compared with other treatments, which suggests that plants in these treatments were able to better use internal carbon dioxide. Such a protective influence of these compounds may explain the higher stomatal conductances, carboxylation efficiency, and WUE. The antitranspirant action observed in this study confirmed previous reports for ABA (Blake et al. 1990a, 1990b) and Ambiol (Darlington et al. 1996). However, Spd also proved to be an effective antitranspirant.

In conclusion, although not all compounds exhibited similar modes of action, the protective action of ABA, Ambiol, and Spd could be explained by the observed reduction in ethylene production and membrane leakage and a smaller reduction in Pn under drought. The ability of these compounds to sustain carbon assimilation under drought, lower TI rates, and maintain a higher WUE could also have protected membranes in plants treated with ABA, Ambiol, and Spd.

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