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Salt-related suppression of bud break in *Populus trichocarpa:* Cost of inclusion, ion-specific or osmotic effects?

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AND

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ABSTRACT

Salt-laden winter storms cause bud failure or delayed bud break of coastal deciduous trees. In five experiments detached shoots of different genotypes of trees and shrubs were set to break buds in solutions of variable composition and concentration containing NaCl, KCl, K_2SO_4, $CaCl_2$, Na_2SO_4, PEG and glycinebetaine to evaluate the effect of: 1) specific-ion stress, 2) osmotic stress and 3) osmotic adjustment on leaf expansion at bud break of black cottonwood (*Populus trichocarpa*). Ion-specific toxicity, osmotic effect or osmotic adjustments were not shown to affect leaf expansion growth. In solutions up to 80 mM neither sodium nor chloride was more detrimental than potassium or sulphate. Glycinebetaine did not alleviate salt stress but suppressed growth to the same degree as NaCl at 10 and 50 mM isoosmotic concentrations. Genotypes varied in vigour but all responded similarly to elevated concentrations of solutes. Leaf growth at bud break was found to decrease with rising solute concentration at constant osmotic potential of the external solution. The cost of inclusion of solute from the external solution was a likely explanation of the observed suppression of leaf growth.

Keywords: Leaf primordia, leaf growth, salt stress, water stress, polyethylene glycol, glycinebetaine

YFIRLIT

Hindrar salt laufgun alaskaaspar (Populus trichocarpa) *vegna kostnaðar frumnanna við inntöku saltsins, vegna eitrunar í frumunum eða mismunandi safaspennu innan og utan frumnanna?*

Í vetrarsæroki nærri opnu hafi sest salt á trjásprota og smýgur inn um börkinn og getur valdið því að brum á trjánum laufgast seint eða alls ekki. Í fimm tilraunum voru ársprotar trjá- og runnategunda af mismunandi klónum látnir laufgast í vatnslausnum með vaxandi styrk af NaCl, KCl, K_2SO_4, $CaCl_2$, Na_2SO_4, PEG og glycinebetaine til að prófa áhrif 1) eitrunar 2) vatnsspennu og 3) aðlögunar að breyttri vatnsspennu á laufgun og laufvöxt alaskaaspar (*Populus trichocarpa*). Þessir þrír þættir skýrðu ekki laufvöxt og laufgun. Natríum og klórið hömluðu ekki laufvexti umfram kalíum og súlfat í lausnum að 80 mM styrk. Glycinebetaine dró ekki úr saltálagi en hindraði laufvöxt jafnmikið og NaCl við 10 og 50 mM styrk en óbreytta vatnsspennu. Tegundir og klónar laufguðust mis hratt en brugðust svipað við vaxandi styrk allra efnalausna. Laufvöxtur við laufgun minnkaði með vaxandi lausnarstyrk við sömu vatnsspennu. Kostnaður frumnanna af upptöku efnanna gat best skýrt niðurstöðurnar.

INTRODUCTION

Deciduous trees exposed to saline aerosols during the dormant season absorb salt into their shoots that may inhibit or delay bud break the following spring (Barrick & Davidson 1980, Buschbom 1968, Sucoff & Hong 1976). Jonsson (2006) showed that salt-related bud failure or delayed bud break in black cottonwood (*Populus trichocarpa* Torr. & Gray) exposed to salt-laden winter storms was a consequence of salt surge into buds at dormancy release and thus inhibiting subsequent leaf growth. Actively growing plants, even on highly saline substrates, maintain [Na^+] and [Cl^-] at low levels in shoot apices and expanding leaves (Munns 1993). Salt surge into buds at dormancy release and subsequent suppression of leaf growth is a special case where immature apical tissues may be exposed to high concentrations of Na^+ and Cl^-. Saline solutions impose both ionic and osmotic stresses on plants (Tester & Davenport 2003). Ion-specific stress is ascribed to sodium or chloride ions interfering with biochemical processes in the cytoplasm (Munns 2002, Tester & Davenport 2003). Osmotic stress is the result of dehydration of the cell arising from differences in water potential between the cell and the external solution. Expanding plant cells exposed to external osmotic potential below that of the cell content will immediately suspend growth (Sutcliffe 1979, Munns 2002). Plant cells may adjust to the external osmotic potential by inclusion of the external solute or by synthesis of organic solutes internally. The questions are whether salt-related suppression of bud break is 1) an ionic effect or 2) osmotic effect, or 3) whether salt-affected buds adjust to salt stress. Setting detached dormant shoots to break buds in solutions of different solutes and variable concentrations is a simple experimental method that might imitate conditions in salt-affected buds following dormancy release. In five experiments this method was used to evaluate the above questions. The specific objectives were to: a) compare early leaf expansion growth in solutions of Na^+ and Cl^- to those of K^+, Ca^{2+} and SO_4^{2-} in order to assess ion-specific stress as a plausible cause of suppressed leaf growth at bud break; b) determine NaCl concentration limit of 1) bud break, 2) root formation, 3) immediate leaf growth suspension and 4) bud mortality of black cottonwood; and c) elucidate the effect of osmotic adjustment by comparing leaf expansion growth at bud break of several genotypes of deciduous trees and shrubs in solutions of NaCl and glycinebetaine.

MATERIALS AND METHODS

Experiment A - Ion-specific stress

Experiments A, B and D used the same collection of visually dormant black cottonwood long shoots from 15 trees of five clones (Table 1). Visually dormant shoots of black cottonwood from three trees (two clones; Table 1) were randomly assigned to solutions of five salts: sodium chloride (NaCl), sodium sulphate (Na_2SO_4), potassium chloride (KCl), potassium sulphate (K_2SO_4) and calcium chloride ($CaCl_2$), at four concentrations: 20, 40, 60 and 80 mM as well as deionised water. Each treatment was replicated in five beakers, one cutting of each tree per beaker. The shoots were allowed to break buds for 17 days (23 April to 10 May) at room temperature (18-21 °C). On the termination of the experiment bud scales were removed from the terminal buds and the maximum length of leaf and shoot growth measured with a digital calliper (± 0.01 mm) from the bud base to the most distal leaf tip. The presence of any necrotic leaves in the terminal buds was also recorded.

Experiment B - Salt tolerance of poplar clones

A bunch of cuttings approximately 80-120 mm long from the 15 black cottonwood trees of five clones (Table 1) with visibly dormant terminal buds were randomly assigned to nine treatment solutions of [NaCl]: 0, 25, 50, 75, 100, 250, 500, 750, 1000 mM. There were four, five, nine, five and four cuttings per bunch from trees one, nine, ten, twelve and fifteen, respectively, but ten cuttings per bunch from the remaining ten trees. The cuttings were set to break buds in the treatment solutions for

Table 1. Provenances including latitude, longitude and elevation above sea level, trade name, Icelandic Forestry Research cone identity number (IFR No.) and sex of named study clones from Alaska, USA (Sævarsdóttir & Óskarsson 1990).

Species	Provenance	Trade name (Clone no.)	Sex	Experiment
Black cottonwood (*Populus trichocarpa* Torr. & Gray)	Copper River Delta, Alaska, USA 60° 20' N, 145° 00' W, 20 m elevation	'Brekkan' (6310001)	♂	A, B, D, E
		'Iðunn' (6310002)	♀	B, D, E
		'Keisari' (6310005)	♂	A, B, D
	Harlequin Lake, Alaska, USA 59° 24' N, 138° 59' W, 30 m elevation	'Depill' (6313002)	♂	B
	Yakutat, Alaska, USA ' 59° 32' N, 139° 45' W, 20 m elevation	Salka' (6314004)	♀	B
Feltleaf willow (*Salix alaxensis* (Anderss.) Cov.) Alaska, USA	Ptarmigan Creek, Alaska, USA	'Olavía'	♀	E
	Alaska, USA	'Mjölnir'	♂	E
		Töggur'	♂	E
Hooker willow (*Salix hookeriana* Barratt.)	Alaska, USA	'Katla'	♀	E
	Alaska, USA	'Taða'	♀	E
Dark-leaved willow (*Salix myrsinifolia* Salisb.)	North Norway	'Mógilsá'	n.a.	E
		'Dökk'	n.a.	E
Tealeaf willow (*Salix phylicifolia* L.)	Hrauneyjar, Iceland (indigenous)	n.a.	n.a.	E
Downy birch (*Betula pubescens* Ehrh.)	Iceland (indigenous)	None	♀♂	E
	Iceland (indigenous)	None	♀♂	E

10 days (24 April to 4 May) at a temperature of 21-22 °C. By the end of the treatment period the length from the base of the terminal bud to the tip of the longest leaf was measured. Bud scales were removed from non-breaking terminal buds and the length of the leaf primordia measured. Presence of callus or roots was recorded on all cuttings and root numbers were recorded on cuttings in deionised water.

Experiment C - Salt tolerance of willow and poplar

Four visually dormant cuttings (an internode with an auxiliary bud) of a single black cottonwood clone (unconfirmed identity) and four willow cuttings of a single clone (*Salix* sp. 'brekkuvíðir', provenance: Iceland, sex: ♀) composed of several internodes were randomly assigned to 14 treatment solutions of increasing [NaCl]: 0, 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 250, 500, 750 and 1000 mM. A baseline sample of twelve randomly selected poplar cuttings was also measured. The cuttings were set to break buds at room temperature (21-23 °C). Length as well as diameters at the upper and lower ends of all poplar cuttings was measured at the start of the experiment (24 April) with a digital calliper. At the same time bud scales were removed from the baseline sample and the length of the longest leaf primordia recorded. On day seven the cuttings were scored for bud break, defined as green leaf tips visible at the bud apex, and for presence of callus or necrosis at the lower end of the cutting. Bud scales were removed and the length of the longest leaf primordia or the leaf including the petiole recorded. The leaves or primordia were scored as either green or necrotic. Numbers and lengths of roots were also recorded. The poplar cuttings were re-measured on days 14 and 19. Willow cuttings were only measured on day 7 recording cutting length and mid-diameter, length of the topmost bud and largest leaf after removal of the bud scale. The cuttings were also scored for bud break, presence of roots and leaf necrosis (present or absent).

Experiment D - Osmotic adjustment of poplar clones

Visually dormant black cottonwood shoots from three trees (three clones; Table 1) were randomly assigned to treatment solutions of sterilised deionised water, 145 mM polyethylene glycol (PEG), and five concentrations of glycinebetaine (GB): 1, 5, 10, 25 and 50 mM either with or without 50 mM NaCl. The treatment solutions, except for an isolated control solution of deionised water, were adjusted to a common osmotic potential of –0.35 MPa with PEG. Each treatment was replicated in five beakers, one cutting of each clone per beaker. The shoots were set to break buds for 17 days (23 April to 10 May) at room temperature (18-21 °C). Bud scales were removed by the end of the experiment and the maximum length of leaf and shoot growth was measured from the bud base to most distal leaf tip. Any presence of necrotic leaves in the terminal buds was recorded.

Experiment E - Osmotic adjustment of different species

Cuttings of dormant long shoots of twelve clones from six species (Table 1) were assigned to 26 treatment solutions, deionised water (isolated control) and all combinations of GB at five concentrations (0, 1, 5, 10 and 50 mM) and NaCl at five concentrations (0, 10, 20, 30 and 40 mM). The 25 treatment combinations of GB and NaCl were adjusted with PEG to a common solution osmotic potential of -0.31 MPa. Ten shoots of each clone were inserted in a plastic mat floated in a tray of treatment solution. Due to lack of material from a birch clone 2 only 5-9 cuttings of that genotype were assigned to each treatment tray. Treatments were replicated in two trays, a total of 6159 cuttings. The cuttings were set to break buds at room temperature and scored on days 10 and 18 from the onset of the experiment. Cuttings were scored as breaking if green leaf tips were visible at the bud apex.

Data analysis

The osmotic potential of NaCl, Na_2SO_4, KCl, K_2SO_4 and $CaCl_2$ solutions was estimated by equations 1, 2, 3, 4 and 5, respectively.

$$-\psi_{NaCl} = 4.6 \cdot 10^{-3} M_{NaCl} - 2 \cdot 10^{-9} M_{NaCl}^2 \quad (1)$$

$$-\psi_{Na_2SO_4} = 6.1 \cdot 10^{-3} M_{Na_2SO_4} - 3 \cdot 10^{-6} M_{Na_2SO_4}^2 \quad (2)$$

$$-\psi_{KCl} = 4.5 \cdot 10^{-3} M_{KCl} - 2 \cdot 10^{-7} M_{KCl}^2 \quad (3)$$

$$-\psi_{K_2SO_4} = 6.3 \cdot 10^{-3} M_{K_2SO_4} - 6 \cdot 10^{-6} M_{K_2SO_4}^2 + 8 \cdot 10^{-9} M_{K_2SO_4}^3 \quad (4)$$

$$-\psi_{CaCl_2} = 6.3 \cdot 10^{-3} M_{CaCl_2} - 6 \cdot 10^{-7} M_{CaCl_2}^2 + 2 \cdot 10^{-9} M_{CaCl_2}^3 \quad (5)$$

where, ψ is the osmotic potential of the electrolyte solution in megapascals (MPa) and M is the concentration of the corresponding salt in millimoles per litre (mM). Equations 1-5 were derived from tabulated values in the Handbook of Chemistry and Physics (Weast 1971). The osmotic potential of the non-electrolyte solutions GB and PEG was estimated by equation 6 (e.g. Zumdahl 1998).

$$-\psi = MRT \quad (6)$$

where, ψ is the osmotic potential (MPa), M is the molarity of the solute (mol L^{-1}), R is the gas law constant (0.008315), and T is the absolute temperature (K = 273 + °C). Adding the component osmotic potentials of the solutes derived the osmotic potential of mixed solutions.

Leaf growth in buds (g) was estimated by equation 7 (Jonsson 2006):

$$g = l - ah \qquad (7)$$

where g is the leaf growth (mm), l is the length from bud base to the tip of the longest leaf (mm), h is bud height (mm) and a is the ratio of leaf primordia length to bud height at the onset of the experiment estimated in a baseline sample (Experiment C). Buds were scored as having suspended growth immediately if $g \leq 0$ in equation 7. The solution concentration inducing immediate growth suspension was estimated from that score with a logit-model (cf. Lindén et al. 1996). Similarly, the concentration limit of root formation (Experiment B), bud break and leaf necrosis (Experiment C) were estimated with a logit-model from scores for these attributes. Probability limits of 50% and 95% incidence were derived by equation 8:

$$M_p = \frac{\ln\left(\frac{p}{1-p}\right) - \alpha}{\beta} \qquad (8)$$

where M_p is the concentration limit value (mM) at probability p and α and β are coefficients of the logit-model.

Statistical analysis

Analysis of variance was used to compare treatment means. In Experiment C repeated measures analysis of variance was used to compare leaf growth by observation dates (1, 8 and 13 May) and [NaCl] treatments of 50% survival by the end of the experiment (non-necrotic leaf primordia). Analysis of covariance of internode diameter, initial bud height and bud diameter was also included. Contrast analyses and the Tukey Post Hoc test were used to compare specific treatment combinations and linear trends. Correlation statistics and non-linear regression analysis were used to estimate relationships between factors. The data were analysed with the STATISTICA software, Kernel release 5.5 A (StatSoft, Inc., Tulsa, OK).

RESULTS

Experiment A - Ion-specific stress

Leaf growth from terminal buds of black cottonwood varied significantly by solution osmotic potential (Covariate: $R^2 = 0.366$, $F_{1,298} = 172.0$, $P < 0.001$) and adjusted for ψ leaf growth did not vary between deionised water and salt treatments (Contrast: $F_{1,298} = 2.0$, $P = 0.156$). Leaf growth varied between salts ($F_{4,298} = 39.0$, $P < 0.001$). Even so, leaf growth did not vary significantly between solutions of NaCl and KCl (Contrast: $F_{1,298} = 2.8$, $P = 0.093$) or NaCl and $CaCl_2$ (Contrast: $F_{1,298} = 0.7$, $P = 0.416$). There was thus no evidence of suppression of leaf growth due to sodium compared to potassium or calcium. There was significantly more leaf growth in solutions of NaCl and KCl as compared to the respective sulphates (Contrast: $F_{1,298} = 71.1$, $P < 0.001$). Leaf growth in KCl was also significantly greater than in K_2SO_4 (Contrast: $F_{1,298} = 6.5$, $P = 0.011$). Thus, sulphates suppressed leaf growth compared to the chlorides. Leaf growth was significantly suppressed in solutions of

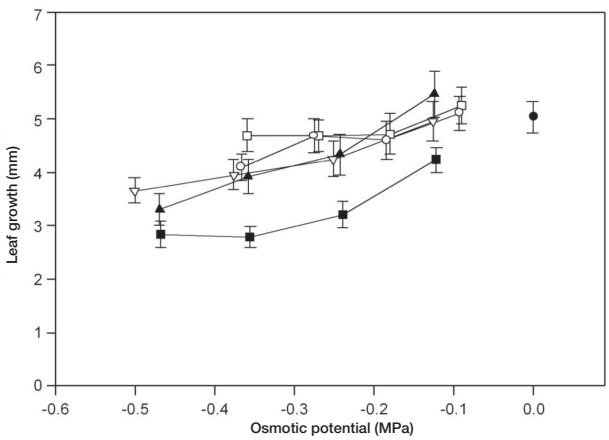

Figure 1. Leaf growth (mean ± SE) from terminal buds of black cottonwood in deionised water (●) and solutions of NaCl (○), Na_2SO_4 (■), KCl (□), K_2SO_4 (▲) and $CaCl_2$ (▽) at 20, 40, 60 and 80 mM concentrations plotted by solutions osmotic potential.

Na_2SO_4 compared to solutions of K_2SO_4 (Contrast: $F_{1,298}$ = 77.4, P < 0.001). Adjusted for osmotic potential NaCl, KCl, K_2SO_4 and $CaCl_2$ were all equally detrimental to leaf growth, but Na_2SO_4 suppressed leaf growth more than the other salts tested (Figure 1). The present results were not consistent with ion-specific stress attributable to [Na^+] or [Cl^-] as the cause of salt-related suppression of leaf expansion growth in black cottonwood.

Experiment B - Salt tolerance of poplar clones

All cuttings formed roots in deionised water and in deionised water roots per cutting varied significantly between the five clones (One way ANOVA: $F_{4,122}$ = 3.06, P= 0.019). The mean solution ψ at which 50% and 95% of the cuttings failed to form roots were -0.55 ± 0.17 (120 mM) and -0.86 ± 0.15 MPa (188 mM), respectively, and did not vary between the five tested clones (One way ANOVA: 50% limit: $F_{4,10}$ = 1.419, P=0.297; 95% limit: $F_{4,10}$ = 1.361, P = 0.314). Leaf necrosis was not systematically scored in the present trial, but most of the leaves were dead in the high concentration treatments (> 250 mM) by the end of the study. Leaf lengths at the end of the experiment decreased significantly with increasing molarity (Linear trend: $F_{32,1098}$ = 7.5, P < 0.001). Leaf lengths varied significantly by clones ($F_{4,1098}$ = 61.6, P < 0.001) and a significant interaction was observed between clones and solution molarity ($F_{32,1098}$ = 7.5, P < 0.001, Figure 2a).

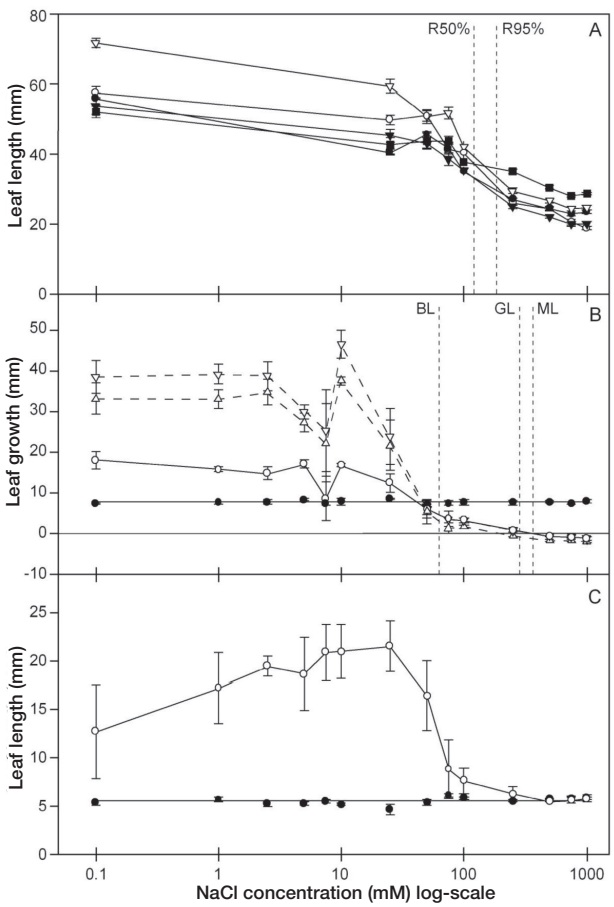

Figure 2. (A) Leaf length (mean ± SE) of black cottonwood clones 'Salka' (●) 'Brekkan' (○), 'Depill' (▲), 'Iðunn' (▽) and 'Keisari' (■) by NaCl concentrations. Vertical broken lines indicate NaCl concentration limits for 50% (R50%) and 95% (R95%) of cuttings failing to form roots. (B) Leaf growth (mean ± SE) of black cottonwood by day 7 (○), 14 (△) and 19 (▽) and leaf growth necessary for bud break (●). Vertical broken lines indicate NaCl concentration limits (50% incidence) for bud break (BL), immediate growth stagnation (GL) and necrosis of leaf primordia (ML). (C) Leaf length (○) and bud height (●) (mean ± SE) of *Salix* sp. 'Brekkuvíðir' by NaCl concentrations.

Experiment C - Salt tolerance of willow and poplar

By day seven the [NaCl] thresholds of bud break, immediate growth suspension and leaf necrosis of black cottonwood were 63, 282 and 355 mM, respectively (Figure 2b). At the start of the experiment the mean height of

buds and length of leaf primordia were 17.4 ± 0.5 and 10.0 ± 0.3 mm (mean ± SE, N = 12), respectively. The mean ratio of leaf primordia to bud height was 0.57 ± 0.01 (coefficient a of Equation 7). The necrosis [NaCl] thresholds estimated for buds by days 14 and 19 were 63 and 60 mM, respectively. Analysis of leaf growth (mm day^{-1}) between observation days was, therefore, limited to treatments ≤ 50 mM (-0.23 MPa). At the beginning of the experiment the mean lengths of leaf primordia were 10.4 ± 1.2 (N = 56). On day seven the mean leaf lengths in deionised water were 28.0 ± 4.1 (N = 4) mm. Leaf growth ([NaCl] ≤ 50 mM) was not significantly explained by 1) cutting length, 2) upper or 3) lower diameters of the shoot segment, or 4) cutting volume calculated from length and diameter (R^2 = 0.262, $F_{4,14}$ = 1.2, P=0.337). Leaf growth varied significantly between treatments ($F_{7,14}$ = 3.4, P = 0.025) and dates ($F_{2,36}$ = 39.6, P < 0.001), but a significant (P < 0.05) interaction was not observed between observation date and treatment ($F_{14,36}$ = 1.7, P=0.101). Leaf growth did not change significantly with treatments ≤ 10 mM (-0.05 MPa) (Linear trend: $F_{1,14}$ = 0.1, P=0.731) but was significantly depressed (Contrast: $F_{1,14}$ = 9.7, P = 0.008) in solutions of 25 and 50 mM. Mean leaf growth in treatments ≤ 10 mM (-0.05 MPa) was 2.37 ± 0.36, 2.42 ± 0.75 and 1.02 ± 0.74 mm day^{-1} by day 7, 14 and 19, respectively. The rate of leaf growth did not change between days 7 and 14 (Linear contrast: $F_{1,18}$ = 0.1, P=0.713) but had significantly decreased by day 19 (Linear contrast: $F_{1,18}$ = 275.3, P< 0.001). Similarly the rate of leaf growth did not change significantly between days 7 and 14 in treatment solutions of 25-50 mM (-0.12 ≥ ψ ≥ -0.23 MPa) (Linear contrast: $F_{1,18}$ = 2.9, P=0.106). Leaf length by day 7 was significantly different between willow and poplar ($F_{1,84}$ = 35.3, P < 0.001) and [NaCl] ($F_{13,84}$ = 17.2, P < 0.001) but an interaction of species and [NaCl] was not significant ($F_{13,84}$ = 1.8, P=0.061). Even so, the willow broke buds in solutions of higher [NaCl] than the poplar, apparently due to less leaf growth required to break buds (Figure 2b and 2c). The present data do not support a difference in the response of the tested poplar and willow clones to increasing salt concentration in the external solution.

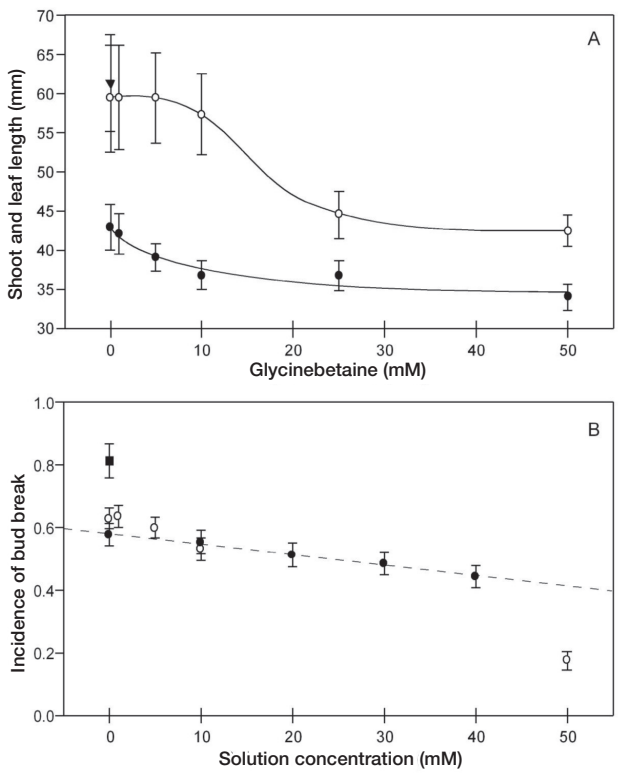

Figure 3 (A) Shoot and leaf length (mean ± SE) of black cottonwood by glycinebetaine concentrations with (●) and without (○) 50 mM [NaCl] adjusted to a solution osmotic potential of –0.36 MPa. Filled triangle (▲) denotes leaf and shoot growth in deionised water. (B) Incidence of bud break (treatment mean ± SE) for all genotypes (Table 1) combined in deionised water (■) and by solution concentrations of glycinebetaine (○) and NaCl (●) adjusted to a solution osmotic potential of –0.31 MPa.

Experiment D - Osmotic adjustment of poplar clones

Leaf growth in deionised water (isolated control) and 145 mM (-0.36 MPa) PEG was 45.7 ± 6.0 and 43.7 ± 6.6 mm (mean ± SE, N = 15), respectively, and the difference was not significant (Contrast: $F_{1,158}$ = 0.4, P=0.540). Thus, solution ψ of -0.36 MPa did not significantly affect poplar leaf expansion. Leaf growth in solutions adjusted to ψ of -0.36 MPa was significantly suppressed by NaCl ($F_{1,158}$ = 129.9, P< 0.001) and GB ($F_{5,158}$ = 11.3, P < 0.001) and there was a significant interaction effect on leaf growth between the two solutes ($F_{5,158}$ = 3.0, P = 0.012). Leaf growth decreased with increasing GB concentrations (Linear trend: $F_{1,158}$ = 50.9, P< 0.001). Leaf growth in 50 mM NaCl was invariably lower with inclusion of GB and decreased with increasing GB concentrations (Linear trend: $F_{1,158}$ = 10.7, P= 0.001, Figure 3a). The response to GB was more complacent in 50 mM [NaCl] as compared to GB alone (Figure 2b), hence, the significant interaction effect. Leaf growth in 50 mM [NaCl] and 50 mM GB was 27.3 ± 2.7 and 26.9 ± 1.8 mm, respectively, and the difference between the respective solutions was not significant (Contrast: $F_{1,158}$ = 0.015, P= 0.902). However, leaf growth in 50 mM [NaCl] and 50 mM GB was significantly less than without these chemicals, i.e. compared to 145 mM PEG (Contrast: $F_{1,158}$ = 35.0 P < 0.001). Leaf growth in combined solutions of 50 mM [NaCl] and 50 mM GB was 18.4 ± 1.3 mm and significantly less than in 50 mM solutions of these solutes separately applied (Contrast: $F_{1,158}$ = 9.5 P = 0.002). Thus, GB and sodium chloride both suppressed growth and to the same degree at 50 mM.

Experiment E - Osmotic adjustment of different species

Bud break varied by treatments ($F_{25,312}$ = 66.3, P<0.001) and clones ($F_{11,312}$ = 395.5, P< 0.001), and clones responded differently to treatment ($F_{275,312}$ = 4.0, P < 0.001). Bud break was higher in deionised water than in treatments solutions with ψ of -0.31 MPa (Contrast: $F_{1,312}$ = 154.5, P < 0.001). The incidence of bud break averaged for all genotypes in deionised water and PEG was 81 ± 26% (N = 24) and 67 ± 35% (N = 24), respectively, and the difference was highly significant (Contrast: $F_{1,312}$ = 19.4, P<0.001). Thus, PEG alone (-0.31 MPa) suppressed bud break by 17% overall, but bud break of black cottonwood was not affected by a solution ψ of -0.31 MPa (Table 2). In isoosmotic solutions (-0.31 MPa) bud break varied with GB concentrations ($F_{4,300}$ = 327.8, P<0.001), [NaCl] ($F_{4,300}$ = 24.7, P<0.001) and the interaction of GB and NaCl was also significant ($F_{16,300}$ = 3.0, P<0.001). In solutions adjusted to -0.31 MPa the incidence of bud break decreased (Figure 3b) with both rising concentrations of GB (Linear trend: $F_{1,300}$ = 892.8, P < 0.001) and NaCl (Linear trend: $F_{1,300}$ = 98.4, P<0.001). Mean incidence of bud break across all genotypes decreased linearly in NaCl concentrations to 40 mM (Equation 9) and the linear function was highly significant (Adj.R^2 = 0.992, $F_{1,3}$ = 508.0, P< 0.001, Figure 3b):

$$I = 0.5816 - 0.0033M \qquad (9)$$

where I is incidence of bud break and M is [NaCl] in mM. The mean levels of bud break in 10 mM solutions of GB and NaCl were 70.3 ± 7.1 and 61.4 ± 7.4%, respectively, and the difference was not significant (Tukey HSD test: P = 0.590). Thus, at that concentration GB and sodium chloride were equally detrimental to bud break. Bud break varied significantly by clones in deionised water ($F_{11,12}$ = 48.4, P< 0.001) and PEG ($F_{11,12}$ = 104.0, P < 0.001) and the two measures were highly correlated (R = 0.922, N = 12). A linear regression model of bud break by concentration of GB and NaCl combined up to 45 mM with separate coefficients for individual clones, but a common slope coefficient (-0.0042, t_{443} = -9.188, P< 0.001) explained 87% of the observed variation in bud break (Table 2). The coefficients for the individual clones were all highly significant (Table 2). Introduction of separate slopes for the different clones added little to the relationships (explained 89% of the varia-

Table 2. Mean incidence of bud break by genotypes in deionised water and polyethylene glycol (PEG, ψ = –0.31 MPa), as well as intercept with *t*-value (SE = 0.024, df = 443) and *P*-value for regression of incidence of bud break with different intercepts by genotypes but common slope of –0.0042 (Adj. R^2 = 0.992, SE = 0.00046, t_{443} = -9.188, P<0.001).

Species	Clone	Deionised water	PEG	Intercept	*t*-value	*P*-level
Populus trichocarpa	'Brekkan'	1.00	1.00	1.09	44.90	$P < 0.001$
	'Iðunn'	1.00	1.00	1.09	45.01	$P < 0.001$
Salix alaxensis	'Ólavía'	1.00	0.65	0.77	31.79	$P < 0.001$
	'Mjölnir'	1.00	1.00	1.01	41.43	$P < 0.001$
	'Töggur'	0.95	0.75	0.69	28.43	$P < 0.001$
Salix hookeriana	'Katla'	0.40	0.05	0.146	6.00	$P < 0.001$
	'Taða'	0.95	0.65	0.775	31.89	$P < 0.001$
Salix myrsinifolia	'Mógilsá'	0.50	0.40	0.32	13.04	$P < 0.001$
	'Dökk'	0.95	1.00	1.04	42.73	$P < 0.001$
Salix phylicifolia	'Hrauneyjar'	0.30	0.00	0.12	4.92	$P < 0.001$
Betula pubescens	1	0.70	0.65	0.56	23.01	$P < 0.001$
	2	1.00	0.84	0.84	34.55	$P < 0.001$
Overall mean		0.81	0.67			

tion). Thus, genetic variability in suppression of bud break by GB and NaCl up to 45 mM was not convincingly shown. We can conclude that both sodium chloride and glycinebetaine suppress bud break at ψ of -0.31 MPa.

DISCUSSION

Ion-specific stress

There is a consensus in the literature that excessive concentrations of salts in the cytoplasm are damaging to the functioning of plant cells (Garcia et al. 1997). High concentrations of chloride or sodium, or high Na^+: K^+ ratios can disrupt enzyme processes *in vitro* (Tester & Davenport 2003, Munns 1993, Marschner 1995). Munns (1993) proposed that cell mortality occurs as salt concentrations exceed the vacuole capacity. It is however still unknown if cells fail due to ion-specific toxicity in the cytoplasm or because of rising concentrations in the apoplast, desiccating the cell (Munns 2002). The presently estimated necrosis limits (Experiment C) of leaf primordia by day seven (355 mM) were a little lower than those estimated by Jonsson (2006) for black cottonwood exposed to salt-laden winter storms (410 mM). Both estimates are of the expected order of magnitude for the salt-related mortality limit proposed by Munns (1993).

Active growth processes and notably protein synthesis are sensitive to [Na^+] (Tester & Davenport 2003, Kozlowski 1997). Shoot apices and immature leaves have cells with small vacuoles and limited capacity for sequestration of Na^+ or Cl^-. Protection of shoot apices and immature leaves from high [Na^+] has been proposed as a crucial feature necessary for salt tolerance (Tester & Davenport 2003). Chloride is generally much more toxic to plant growth than sulphate salinity (Marschner 1995). Potassium is a key nutrient in plants – essential for enzyme activation, protein synthesis and photosynthesis, and it mediates osmoregulation during cell expansion, stomatal movements and tropisms (Mäser et al. 2002). Therefore, leaf expansion growth at bud breaking should be more suppressed by sodium or chloride than potassium or sulphate. However, the present results were not consistent with that hypothesis. Based on our results ion-specific effects might explain leaf mortality at high solute concentrations but seem an unlikely explanation of the observed inhibition of bud break.

Osmotic stress

Mature plant cells have a turgor pressure of about 0.5 to 0.7 MPa and immature cells have considerably higher turgor pressures (Sutcliffe 1979, Munns 2002). Presently, leaf growth of black cottonwood immediately ceased at -1.3 MPa (282 mM NaCl), possibly reflecting the turgor pressure of immature leaf cells. Jonsson (2006) estimated leaf growth stagnation in black cottonwood buds exposed to salt-laden winter storms at -0.93 MPa. The growth stagnation limits presently estimated in laboratory experiments were, thus, similar to those estimated in the field.

Jonsson (2006) estimated the [Cl^-] limit to bud break by mid-May in black cottonwood exposed to salt-laden wind at 3.3 mg g^{-1} leaf tissue water. Assuming equal molar ratios of Na and Cl, this concentration would correspond to 92 mM NaCl or an osmotic potential of – 0.42 MPa. The presently estimated bud break limit (Experiment C) of 63 mM (– 29 MPa) is lower than that estimated in the field. The discrepancy might partly be explained by transpiration from the cut upper end of the cutting, causing higher concentrations in the bud than in the external solution.

Polyethylene glycol (PEG) is a large molecule that does not enter plant cells and induces only an external osmotic effect (Sutcliffe 1979). Leaf growth or bud break of black cottonwood was not affected by PEG solutions of -0.31 and -0.36 MPa (Experiments D and E). Therefore, failure of black cottonwood to break buds would not indicate an osmotic effect per se.

Osmotic adjustment

Growth response to altered external osmotic potential is immediate, but osmotic adjustment is a slower process that may enable growth to resume (Munns 2002). Plant cells readily take up sodium and chloride but maintain low [Na^+] and [Cl-] in the cytoplasm by pumping these ions into the vacuoles or back to the apoplast (Tester & Davenport 2003). As salt concentrations rise in the vacuole, plant cells avoid internal osmotic stress by synthesis of organic solutes (compatible solute) in the cytoplasm (Munns 2002, Tester & Davenport 2003). Glycinebetaine is a highly effective compatible solute that a number of species synthesize in response to salinity and drought (Marschner 1995, Blunden et al. 2005). It is non-toxic to species that naturally accumulate this solute, even at high cytoplasmic concentrations (Marschner 1995, Martìnez et al. 2004). Moreover, GB has been shown to stabilize protein structure and enzyme function in vivo (osmoprotectant) under salt stress (Sakamoto & Murata 2002). Compatibility of GB in non-accumulator species has been questioned (Gibon et al.1997, Sulpice et al. 1998). Hincha (2006) showed that GB at high concentrations destabilizes lipid membranes. Even so, a number of studies have shown that exogenous application of GB up to a 10 mM concentration alleviates salt, water and chilling stress in non-accumulator species (e.g. Yang & Lu 2005, Xing & Rajashekar 1999, Chen et al. 2000). Exogenous application of GB up to a 10 mM concentration also increased photosynthesis in maize plants without salt stress (Yang & Lu 2006). Chen et al. (2000) showed that loading cells with GB in solutions up to 10 mM increased chilling tolerance of maize cells. Yang & Lu (2006) showed a detrimental effect of GB application to maize above 10 mM concentration (leaf concentration 8-10 μmol g^{-1} FM). Similarly, Chen et al. (2000) showed that GB solutions up to 10 mM did not affect growth or viability of maize cells, but both cell growth and viability decreased linearly at higher concentrations. Our results are consistent with their observation, i.e. 1) no detrimental effect on leaf expansion growth in GB solutions to 10 mM (Figure 3a) but 2) linear decrease in leaf growth rate above that concentration (Figure 3b).

Synthesis of organic osmotica is costly to the cell (Munns 2002) and might thus adversely affect growth. Osmotic adjustment by inclusion of the external solute is much less costly to the cell than internal synthesis of compatible solutes (Munns 2002, Tester & Davenport 2003). Osmotic adjustment of plant cells to

solutions of PEG alone must be by internal synthesis of organic solutes only. Replacing PEG by GB ≤ 10 mM should reduce the water potential difference between the cell and the external solution without risking cellular toxicity. However, in the present study both NaCl and GB were more detrimental to leaf growth than PEG at isoosmotic concentrations. The GB used was of high purity derived from sugar beets. Hence, toxic impurities are an unlikely explanation. Addition of glycinebetaine did not improve leaf growth in NaCl solutions at any concentration tested up to 50 mM (Figure 3a and b). Accordingly, our results do not support the conclusion that osmotic adjustment or protection by GB has an important effect on leaf expansion under conditions of salinity or osmotic stress.

An important difference between mature and growing cells is that the latter have a decreasing surface area to volume ratio. Also, cytoplasmic volume changes much less than vacuole volume during cell expansion. Thus, sequestration of sodium or chloride in the expanding vacuole would only result in a rising concentration and a lower water potential if the sequestration rate exceeds the volumetric swelling rate of the vacuole. Hence, the need for costly expenditure on compatible solutes in the cytoplasm may be relatively small for an expanding cell exposed to salinity.

Cost of inclusion?

Leaf cells readily absorb Na_2SO_4, K_2SO_4, NaCl, KCl and $CaCl_2$ from the apoplast solution (Smith & Robinson 1971, Macklon & Armstrong 1994). GB also enters plant cells at a fast rate (Gibon et al. 1997). Movement of solutes into plant cells and vacuoles is through diverse specialized ports on the plasma membrane and tonoplasts, facilitating both passive and active influxes and effluxes (Tester & Davenport 2003, White & Broadley 2001, Gibon et al. 1997). The rate of influx is proportional to the concentration in the external solution (Smith & Robinson 1971). Net accumulation of solutes in plant cells is a balance between influx and efflux (Tester & Davenport 2003). Tester and Davenport (2003) suggested that the passive influx rate of Na^+ is exceedingly high and would within a few minutes raise the [Na^+] in the cytoplasm to that of the external solution. These high rates of unidirectional influx of Na^+ do not result in rapid accumulation of Na^+ due to the substantial and energetically expensive efflux of Na^+ across the plasma membrane (Tester and Davenport 2003). Similar control mechanisms probably exist for other solutes. Gibon et al. (1997) suggested that the cost of net uptake of GB into plant cells might partly explain the detrimental effect of that solute at high external concentrations. Perturbations due to high GB concentrations in the cell would also be involved (Gibon et al.1997, Sulpice et al. 1998, Hincha 2006).

Zero bud break of black cottonwood according to equation 9 (intercept = 1.00, Table 2) would occur at 303 mM [NaCl] (-1.38 MPa), assuming no osmotic effect and that the linear relationship is valid beyond 40 mM. That is approximately at the immediate growth suspension limit determined in Experiment C (282 mM [NaCl], -1.30 MPa).

We suggest that leaf growth at bud break is suppressed primarily by disturbance to cellular growth processes induced by excessive influx of an external solute. The perturbations might result from: 1) the cost of controlling solute influx, 2) activation of processes to stabilize cellular function in response to rising internal concentrations, as well as 3) direct interference of the solute with cellular processes. The present experiments are insufficient to evaluate the specific processes that might be involved. Nevertheless, solute-specific toxicities seem to play a minor role in this effect. The primary evidence for that interpretation is: 1) allegedly toxic as well as more benign solutes that are absorbed by cells suppressed leaf growth to a similar degree, 2) PEG that is not absorbed by plant cells did not affect leaf growth and bud break, and 3) unexpectedly, NaCl and GB were equally detrimental to leaf growth at 10 mM, whereas a protective effect of GB was expected. Ion-specific stress, osmotic effect or osmotic adjustment were not shown to affect

leaf expansion growth of black cottonwood or other species presently used for comparison. Our results were based on leaf growth over several days at the time of bud break. Munns (2002) proposed that plant growth in response to salt varies over time and occurs in three phases: 1) transient initial osmotic effect that recovers within hours of exposure, 2) a reduced but steady growth rate at elevated salinity that may continue for days and weeks or until, 3) accumulation of salt in mature leaves to toxic levels causes loss of leaf area and thus reduced total plant production. Munns (2002) cited several studies showing that the phase 2 response is not an ion-specific effect and that different solutes, both electrolytes and non-electrolytes, induce a similar growth response. Our results are apparently analogous to the phase 2 growth response.

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