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# Salinity tolerance ecophysiology of *Equisetum giganteum* in South America: a study of 11 sites providing a natural gradient of salinity stress

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## Abstract

### Background and aims

The basic set of adaptations necessary for salinity tolerance in vascular plants remains unknown. Although much has been published on salinity stress, almost all studies deal with spermatophytes. Studies of salinity tolerance in pteridophytes are relatively rare but hold promise for revealing the fundamental adaptations that all salt-tolerant vascular plants may share. The most basal pteridophytes to exhibit salinity tolerance are members of the genus *Equisetum*, including the giant horsetail, *Equisetum giganteum*, the only pteridophyte to occur in salinity-affected regions of the Atacama Desert valleys of northern Chile. Here it can constitute a significant vegetation component, forming dense stands of shoots >4 m high.

### Methodology

Physiological parameters (stomatal conductances; efficiency of photosystem II; sap osmotic potential) were measured in *E. giganteum* populations in northern Chile across a range of groundwater salinities at 11 sites. In addition, Na, K, electrical conductivity and total plant water potential were measured in the plants and groundwater from each site.

### Principal results

*Equisetum giganteum* exhibits similar stomatal conductances and photochemical efficiencies of photosystem II across a wide range of groundwater salinities. It lowers cell sap osmotic potential with increasing salinity and produces positive root pressure, as evidenced by guttation, at the full range of salinities experienced in the Atacama Desert. *Equisetum giganteum* maintains low Na concentrations in its xylem fluid and cell sap when soil water Na is high. It also maintains high K/Na ratios in xylem fluid and cell sap when soil water has low K/Na ratios.

### Conclusions

*Equisetum giganteum* is well adapted to salinity stress. Efficient K uptake and Na exclusion are important adaptations and closely similar to those of the facultative halophyte fern *Acrostichum aureum*.

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## Introduction

Although there is much research on plant responses to salinity stress, almost all such studies have dealt with spermatophytes (especially angiosperms) because of their pre-eminent economic and ecological importance. Few studies have addressed salinity tolerance in pteridophytes, partly because few pteridophytes exhibit salinity tolerance (Yensen 1999). Until now, pteridophyte studies have exclusively involved the ferns (class Pteropsida) and usually only the gametophyte stage. The current study helps to redress the paucity of knowledge of pteridophyte salinity tolerance by investigating adaptations to this stress in *Equisetum giganteum*, a species belonging to a distinct pteridophyte class, the Sphenopsida.

*Equisetum* is a small (15 species) but widespread genus. It is the only surviving genus in a class that is morphologically and physiologically unique among living plants. Rothwell (1996) considers *Equisetum* the ‘most successful genus of all living vascular plants’ because of its wide distribution in physical and ecological space. However, the ecophysiology of *Equisetum* is poorly understood, especially in species outside the north temperate zone. The current study explores the comparative ecophysiology of *E. giganteum* populations growing in salt-affected river valleys of the extreme north of Chile, as well as freshwater areas of central Chile and northwest Argentina.

*Equisetum giganteum* is a widespread species associated with wetlands and other moist habitats at elevations typically above 500 m in the American tropics and extending to temperate South America (Hauke 1963). Coastal northern Chile ( $\sim 18\text{--}20^\circ\text{ S}$  latitude) appears to be one of the few localities where this species grows in saline soil. Coastal Chile also includes some of the few low-elevation (3–90 m) populations known for this species. *Equisetum giganteum* probably arrived in the northern Chilean valleys when the region was wetter. During the past 1000 years it would have adapted to increasing salinity in the valleys as the Atacama region became highly desertified (Graf 1994).

Inland areas of high salinity usually occur in arid regions where evaporation is greater than precipitation and little leaching occurs (Fitter and Hay 2002), often combined with the presence of rocks high in sodium salts (Chapman 1975). Such conditions prevail in the extreme north of Chile. In the coastal Atacama Desert region of the far north, mean annual precipitation is  $<2.5\text{ mm}$  (González 1986) and the geology is rich in saline minerals (Erickson 1981 cited in Goudie et al. 2002). *Equisetum giganteum* is also found in the coastal area of this region, which has saline river and

irrigation waters containing high  $\text{Na}^+$ ,  $\text{Cl}^-$  and B concentrations (Ferreira et al. 1997).

## Previous studies of salinity tolerance in pteridophytes

Salinity tolerance occurs widely in the plant kingdom but only a very few non-flowering plants and a handful of pteridophytes are known to be salinity tolerant (Yensen 1999, 2006). Until now, most studies of pteridophyte salinity tolerance have dealt with the gametophyte generation (Lloyd and Buckley 1986; Warne et al. 1996, 1999; Li and Ong 1997, 1998; Wendell et al. 1999). Auge et al. (1989) found that a salinity-tolerant mutant sporophyte of the aquatic fern *Ceratopteris richardii* grew better than the wild type under high salinity but more poorly under low salinity. A few laboratory studies have been published on salinity tolerance of the sporophytes of floating aquatic ferns in the genera *Azolla* and *Salvinia* (Haller et al. 1974; Divakaran et al. 1980). A study of *Azolla pinnata* showed that increased tolerance of higher salinity levels can be induced by first acclimating plants at intermediate salinity levels (Rai and Rai 1999). Medina et al. (1990) undertook a field study of salinity tolerance of sporophytes of the mangrove fern *Acrostichum aureum* and found that leaf cell sap osmotic concentration increased substantially with increasing soil salinity. The K/Na and Cl/Na ratios of *A. aureum* were markedly higher than those found for typical (angiosperm) mangroves.

Page (1997) discussed isolated colonies of the hybrid horsetail, *Equisetum*  $\times$  *moorei*, on the southeast coast of Ireland growing on dunes where they grow close to the high tide line, suggesting considerable exposure to saline soil water and salt spray. Page (1997) noted an unpublished experiment with this hybrid that involved floating cut stem pieces in seawater for various lengths of time and evaluating their ability to re-sprout. Remarkably, immersion for up to 10 d in seawater did not reduce the ability of stems to sprout roots and form new plants.

## Objectives of this study

- (i) To measure the range of groundwater salinities and groundwater Na and K concentrations in which *E. giganteum* grows in northern and central Chile, and northwest Argentina.
- (ii) To determine whether across the salinity gradients, *E. giganteum* is physiologically stressed, as measured by stomatal conductance and photosynthetic efficiency of photosystem II.
- (iii) To establish whether *E. giganteum* osmotically adjusts to changes in the water potential of groundwater.

- (iv) To assess the extent to which xylem fluid and cell sap composition of *E. giganteum* change with differences in groundwater salinity.
- (v) To establish the extent to which Na and K concentrations of shoot cell sap change in response to differences in groundwater salinity.

## Materials and methods

### Field sites

This study consists of fieldwork carried out in northern and central Chile, and northwest Argentina, from March to May 2004 and from December 2005 to February 2006 (Fig. 1). The Atacama Desert, which extends along the western part of Arica Province and Tarapacá Region, is the 'driest terrestrial region on this planet' (Ehleringer *et al.* 1998). The only sources of water in the area are surface water and groundwater from high Andean precipitation (Margaritz *et al.* 1990), and coastal fog from the Pacific Ocean (Goudie *et al.* 2002). The Lluta River, which has highly acidic water, and the Camarones River, which has alkaline water, are the major river systems in the area (Barr *et al.* 1993). River water from the Andes becomes increasingly saline as it flows west towards the Pacific Ocean (Margaritz *et al.* 1990). This is also the case for the Lluta River (Escobar and Morales 1994) and presumably the Camarones River. In the Lluta River valley, groundwater salinity reaches almost half that of ocean water near the mouth of the river (see Results, Objective 1).

The environment in which these *E. giganteum* populations are found in northern Chile appears quite unusual since this is, essentially, a wetland species (Hauke 1963). There is almost no precipitation and little natural vegetation in the region (Cortes 1976), although the major rivers provide adequate moisture for the few crops growing in the irrigated alluvial soils of the river valleys (Ferreyra *et al.* 1997). However, soils and irrigation water of the Lluta are highly saline with high boron concentrations. This limits agriculture to a few specially adapted local cultivars of corn, onions, tomatoes and alfalfa (Sotomayor *et al.* 1994). The water of the Camarones and Tarapacá rivers, where *E. giganteum* populations are also found, are even more saline (J. Delatorre, UNAP, Iquique, Chile, unpubl. res.; González 1986). In contrast, the environments in which *E. giganteum* grows in northwestern Argentina and in central Chile are much milder. Rainfall in these latter areas is sufficient to support lush vegetation and prevent salinity build-up.

The *E. giganteum* field sites for this study are as follows (Fig. 1).

### **Northern Chile (with sites ranging from low to high salinity)**

- (i) Freshwater river in the town of Guañacagua in the Codpa Valley, Arica Province.
- (ii) Floodplain of the Lluta River, 0.5 km from the Pacific shore, Arica Province.
- (iii) Upstream sites in the Lluta Valley: Puente Santa Lucia and Molinos, Arica Province.
- (iv) Marshy streambed in the Chiza Valley, Arica Province.
- (v) Small saline stream near the town of Mamiña, Tarapacá Region.
- (vi) Small stream near Guasquiña, Tarapacá Region.
- (vii) Streams near the village of San Lorenzo de Tarapacá, Tarapacá Region.
- (viii) Wetlands in the Tana Valley near the Panamerican Highway bridge and further upstream at Calatambo, Arica Province.

### **Central Chile (low-salinity sites only)**

- (ix) Elqui Valley below the dam on the river.

### **Argentina (low-salinity sites only)**

- (x) Along the Lules River, Tucumán Province.
- (xi) Marshy areas near the town of Aguas Calientes, Salta Province.

### Selection of shoots

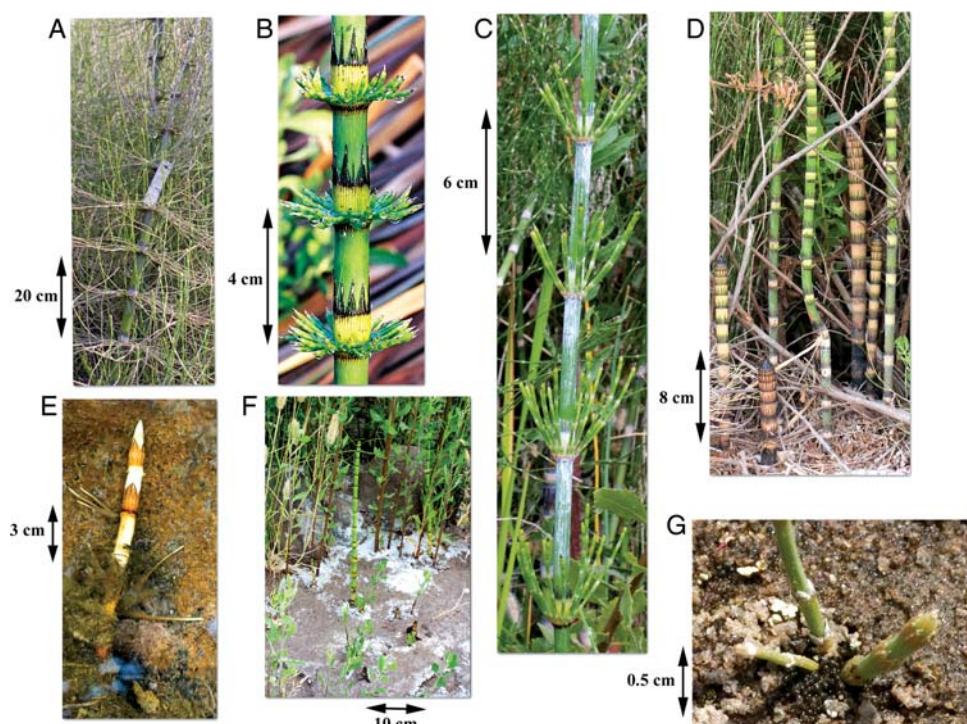
Shoots were selected for sampling based on their health and maturity, as described below. Although it is impossible to determine which shoots may be part of the same clone and which are different in the field without genetic characterization of each shoot, it is likely that shoot maturity and location of measurement on the shoots have more effect on physiological variables than genetic differences among clones. In addition, the high density of many stands of shoots necessitated measurements along the edges of the colonies, which minimized damage that would have had physiological effects. Shoots were chosen where water sources accessed by the colonies could be readily identified by locating roots or rhizomes growing in the water or saturated mud. Shoots were also chosen to represent young but mature and healthy shoots in areas of varying water salinity levels. The goal of the study was to assess the effects of salinity, so random sampling of colonies to assess the overall physiological status of each colony was not appropriate. Rather, emphasis was placed on measuring physiological parameters of shoots at similar stages of maturity at sites in different parts of the valleys with differing salinity levels. Given the clonal nature of *Equisetum*, the shoots measured at each site may have been linked by rhizomes and thus do not necessarily represent completely independent samples. However, measurements of diurnal stomatal conductance patterns and shoot sensitivity to shade while controlling for time of day (data not shown) indicated that adjacent shoots within a few centimetres of each other can have highly divergent physiological status. This



**Fig. 1** Field sites where *E. giganteum* was studied in Chile and Argentina. The sites represented a variety of different groundwater salinities. In northern and central Chile, *E. giganteum* grows in desert areas, but also in valleys that receive abundant groundwater from Andean precipitation. Sites in northwest Argentina were mesic and with abundant groundwater.

indicates that even nearby shoots are behaving independently. Since roots emerging from the base of each stem of each shoot are presumed to be the major source of water and nutrients for that shoot, it is not surprising that shoot physiology is sensitive to local conditions, rather than being highly integrated as a clone. In this study, the shoots we measured

were usually 1 m or more apart. It was, therefore, appropriate to treat them statistically as independent replicates for response to salinity level at each site, thus avoiding pseudoreplication (Hurlbert 1984). Lateral branches were used for chlorophyll fluorescence measurements and main shoots were selected for estimating stomatal conductance.



**Fig. 2** *Equisetum giganteum* in northern Chile. (A) An ~2-year-old shoot, first labelled as a young mature shoot for stomatal conductance measurement in spring 2004 and relocated in January 2006, Chiza Valley, Arica Province. (B) Guttation of shoots at a high-salinity site near the mouth of the Rio Lluta (groundwater EC  $\sim 25 \text{ mS cm}^{-1}$ ). (C) Deposits of guttation fluid solutes on young shoots, Tarapacá Valley, Tarapacá Region, northern Chile. (D) Developing shoots, Lluta Valley, Arica Province. (E) Rhizome growing in surface stream, Tarapacá Valley near the town of Tarapacá. (F) Expanding shoots growing amid surface salt encrustations, Tarapacá Valley, Tarapacá Region. (G) Gametophyte and young sporophyte with salt encrustations, near Molinos, Lluta Valley, Tarapacá Region.

## Overall salinity

Salinity was measured using a Con 100 temperature-compensating electrical conductivity (EC) meter (Oakton Instruments, Vernon Hills, IL, USA) to measure groundwater and river water salinity. A Cardy Twin Conductivity Meter B-173 (Horiba Ltd, Tokyo, Japan), with automatic temperature compensation, was used for measuring guttation fluid EC. Output was converted to p.p.m. (w/v) using the approximate conversion factor of  $700^{\circ}\text{EC}$  (Bunt 1988). This conversion to total dissolved solids (TDS) accounts for the high proportion of sulphate ions in many north Chilean waters (Escobar and Morales 1994; Sotomayor et al. 1994).

## Na and K

Sodium ion concentrations were measured using a Cardy Na Compact Ion Meter C-122 (Horiba Ltd). Potassium ion concentrations were measured using a Cardy K Compact Ion Meter C-131 (Horiba Ltd). These meters are designed to measure accurately the ion concentrations of small samples of solution, including shoot sap, although at

the highest concentrations, values may be underestimated under some circumstances (Dunn et al. 2004; Taber and Lawson 2007).

## Water and osmotic potential

Water potentials were measured using an HR-33T Dew Point Microvoltmeter (Wescor Inc., Logan, UT, USA). C-30 chambers were used for total shoot water potential measurements and a C-52 chamber was used for most sap osmotic potential measurements. Sap was expressed using either a Markhart Leaf Press (Wescor Inc.) (Markhart 1985) for lateral branches or a heavy-duty garlic press for young main shoots. For larger samples of groundwater and expressed young shoot sap, an Advanced Instruments Model 3300 Micro Osmometer (Advanced Instruments, Norwood, MA, USA), which is based on the freezing point method, was used.

## Chlorophyll fluorescence

Maximum photochemical efficiency of photosystem II ( $F_v/F_m$ ) was measured using an Opti-Sciences OS-1 Modulated

Fluorometer (Opti-Sciences, Inc., Hudson, NH, USA) with dark adaptation clips.  $F_v/F_m$  was measured on lateral branches of young fertile *E. giganteum* shoots that were of mature size, but had green cones. Selecting this particular kind of shoot allowed standardization of the technique among sites and minimized the confounding effect of differences in age of shoots and lateral branches. This is because cones mature within a few months. Therefore, when cones are green the branches on which they are borne are relatively young but mature. Lateral branches appear to senesce with age, even when the central shoot remains green (Fig. 2A). Coning branches in the middle portion of each shoot were measured. Measurements of  $F_v/F_m$  were made on two or more lateral branches gathered together within the dark adaptation clip to ensure sufficient fluorescence for a reliable reading.

### Stomatal conductance

Maximum stomatal conductances of water vapour ( $g_w$ ) and transpiration of main shoots of giant horsetails were measured using a Li-Cor LI-1600 steady-state porometer (Li-Cor Biosciences, Lincoln, NE, USA) by sealing the chamber vertically against an internode in the mid-section of young but mature and healthy shoots with green branches at each site. The health of lateral branches was used as a major criterion of main shoot health because these branches senesce as shoots age (Fig. 2A). Shoots were selected such that the sheaths were turning brown (a sign of stem maturity in *E. giganteum*; Hauke 1963) and the branches had extended to a greater length than the internodes, but did not exhibit signs of senescence (such as browning of the branch tips). The central shoots were selected to be 2–3 cm in diameter so that the stem curvature was small enough to allow formation of a good seal. A narrow chamber head designed for measurement of grass blades was used to create the necessary seal when positioned parallel to the shoot axis. Stomatal conductances were measured on stem tissue near the middle of the main vertical portion, since there was detectable variation in  $g_w$  between the middle and top portions of the shoot (C. Husby, unpubl. res.). Stomatal conductance of a population of shoots was measured over the diurnal period (08:30 to 20:00 h), revealing that the maximum stomatal conductance occurred, on average, between 10:00 and 12:00 h for shoots exposed to full sun (C. Husby, unpubl. res.). Therefore,  $g_w$  was measured between those times. Also, by mid-morning, guttation fluid and dew had evaporated, leaving the shoots dry, a necessary prerequisite for porometry. At that time of year, sunrise was at 06:44 h and sunset was at 18:39 h, although the actual time that

sunlight reached plants in the valley was generally later due to the shadows cast by the valley walls.

### Statistical analyses

Least squares regression analyses were used to assess the type of relationships among pairs of measured variables as well as the significance of these relationships. Curve fitting was carried out using JMP (Version 8; SAS Institute Inc., Cary, NC, USA) and FindGraph (Version 2.262, UNIPHIZ Lab, Tver, Russian Federation). Adjusted  $r^2$  ( $r_{adj}^2$ ) was used as the primary selection criterion to determine the best-fitting models, along with the biological relevance of models with similar  $r_{adj}^2$  values. When heteroscedasticity was apparent, appropriate transformations were applied to assess the statistical significance of the relationships more accurately.

In addition, analysis of variance (ANOVA) models were used to assess the effects of groundwater Na and K concentrations on cell sap and guttation fluid Na and K concentrations. These were implemented in JMP (Version 8). Plots of the proposed explanatory variables were used to determine which were correlated with other variables and thus should be excluded from the models. Residual plots were examined to assess whether the data adequately met the underlying assumptions of the model regarding homogeneity of variances and normality.

Because this is an observational study, rather than experimental, the ANOVA cannot distinguish all possible confounding factors that may co-occur to some extent with differences in salinity, including genetic differences among populations. However, since the variables measured are generally quite sensitive to salinity differences in other plants, especially sap elemental composition, it seems reasonable to assume that salinity is the main driving force of any observed co-variation between the physiological parameters being measured and groundwater composition. If other environmental or genetic differences dominate, significant differences in the ANOVA would not be expected unless other environmental or clonal variables consistently vary in the same direction as salinity in terms of their effects on physiology. Since there were high- and low-elevation high-salinity sites, and since elevation is likely to be the most significant factor affecting environmental variables, the results are not likely to be confounded by this factor. Since cloud cover did not occur during the day at any site and the plants always grow with abundant ground moisture, salinity seems likely to be the driving factor for differences among sites.

### Specific methods addressing objectives

**Overall salinity** Populations of *E. giganteum* in Chile and Argentina were explored during three expeditions, in

December 2002, March–May 2004 and December 2005–February 2006. Sampling focused on three areas where this species grows extensively: the river valleys of the Arica Province and Tarapacá Region in extreme northern Chile (Lluta, Chiza, Tana, Guasquiña and Tarapacá valleys, in addition to a small saline marshy site near Mamiña), where surface water and groundwater become increasingly saline as they flow west towards the Pacific Ocean (Margaritz et al. 1990), thus providing a natural gradient of salinity for the study of *E. giganteum* physiological tolerance. In addition, groundwater was sampled in *E. giganteum* colonies in two areas not affected by salinity: the Elqui Valley in central Chile (a population below the large dam on the river) and mesic portions of Salta (near the town of Aguas Calientes) and Tucumán (near the Lules River) provinces in northwest Argentina.

**Physiological stress** Because reduction in the efficiency of the photosynthetic apparatus and reduction in stomatal conductance are typical reactions to salinity stress (Larcher 2003),  $F_v/F_m$  and maximum stomatal conductance were measured in healthy mature shoots across a range of sites with differing groundwater salinities in northern Chile and northwest Argentina from March to May 2004.

**Osmotic adjustment** Total and osmotic water potentials of branches ( $\psi_t$ ), as well as osmotic potential ( $\psi_s$ ) of young shoots, were measured across a range of sites varying in groundwater salinity in northern and central Chile. Measurements of pre-dawn water potential allow inference about the water potentials of the water sources that *E. giganteum* is utilizing in northern Chile in comparison to measured water potentials of groundwater and surface water sources (Berger and Heurteaux 1985). Branches were sampled in the pre-dawn hours and sealed in C-30 chambers in the field. Chambers were placed in a cooler (to prevent tissue degradation during equilibration) with ice for vapour pressure equilibration and total water potential was measured in a room of stable temperature 4–6 h later. After  $\psi_t$  was measured, branches were removed from the chambers, sap expressed to saturate a filter paper disk with a Markhart Leaf Press and osmotic potential measured with a C-52 chamber.

**Xylem fluid composition:** Healthy young *Equisetum* shoots readily guttate under conditions of high humidity and negligible transpiration, for example at night or early morning (Johnson 1936; Nobel 1999). *Equisetum* species guttate through hydathodes (Johnson 1936) that serve as exits for xylem water when there is positive hydrostatic pressure (called root

pressure) in the xylem (Nobel 1999). The exit of this xylem water, termed guttation fluid, results in the formation of small droplets in the vicinity of the hydathodes (Fig. 2B). These droplets provide a non-disruptive way to measure xylem fluid properties in the field. Because solutes in xylem fluid accumulate over time on young shoots (Fig. 2C), it is necessary to rinse these deposits off before guttation occurs to avoid contamination of the xylem fluid with extra solutes. Thus, in the late afternoon before measurement, shoots to be sampled were tagged and rinsed with distilled water to remove surface deposits. The tops of shoots were then enclosed with plastic garbage bags to prevent evaporation of guttation fluid. Guttation fluid was then sampled in the pre-dawn hours (03:00–05:00 h) and measured for EC, Na concentration and K concentration. Also, at the Tarapacá Valley site, white guttation fluid deposits (Fig. 2C) were sampled by wiping water-saturated Cardy meter sampling sheets across them and measuring the Na and K concentrations to obtain a time-integrated measure of guttation fluid composition.

**Shoot cell sap composition** The tops of young developing main shoots (Fig. 2D) were sampled at sites of varying groundwater salinity. These succulent new shoots are less physiologically sensitive to local environmental factors, such as light and temperature, than older mature shoots (data not shown), and thus provided a more consistent comparison of differences in cell sap composition and osmotic potential among sites. The shoots sampled across sites were broken off, sealed in plastic bags to prevent desiccation and transported back to the laboratory in a cooler box filled with ice. Shoots were crushed in a sturdy garlic press and the expressed sap was measured for EC, Na, K and osmotic potential. Furthermore, groundwater samples from each site were measured for the same parameters. The contribution of cell sap K to osmotic adjustment was calculated using a temperature-adjusted model of the chemical potential of KCl solutions of various concentrations (Rawlins and Campbell 1986).

## Results

### Qualitative observations

*Equisetum giganteum* was always found growing in valley bottoms in areas of a high water table, not deeper than 0.5 m. In areas where groundwater reached the surface, stem bases were often immersed in the water. Rhizomes and associated roots typically extended down and into the water table, as is normally the case for *Equisetum* (Hauke 1963). In areas of slowly

flowing surface water (streams or flooded areas), rhizomes and roots were often growing submerged. Rhizomes were observed in surface streams in the Tarapacá Valley (Fig. 2E), the Camiña Valley and the Lluta Valley. Furthermore, there was clear evidence of surface salt accumulation in areas where *E. giganteum* was growing (Fig. 2F), suggesting that soil salt concentrations were higher than those in groundwater or surface water. In two valleys gametophytes and young sporophytes were found, indicating that sexual reproduction is occurring (Fig. 2G), although this species, like all others in the genus, reproduces vegetatively by extensive rhizome growth. Since the gametophytes and young sporophytes must access water near the soil surface, these must be able to cope with the high salt levels there. Guttation was observed in the pre-dawn hours at all sites on developing young shoots (Fig. 2B), but not on mature shoots. Also, guttation was less frequent at the highest-salinity sites than in others. Typical vegetation growing in association with *E. giganteum*, especially in the most saline sites, such as the mouth of the Lluta River, included the halophyte *Distichlis spicata* (Zhao et al. 1989), *Tessaria absinthioides*, *Typha angustifolia* and *Scirpus* spp.

### Objective 1

*Equisetum giganteum* was observed growing in habitats with groundwater ECs ranging from  $0.44 \text{ mS cm}^{-1}$  (in northwest Argentina) to  $25.7 \text{ mS cm}^{-1}$  (near the mouth of the Lluta River, Chile, in 2004). The EC range corresponds to concentrations of  $315\text{--}17\,990 \text{ mg L}^{-1}$  TDS. In comparison, full-strength seawater has a salinity of  $\sim 35\,000 \text{ mg L}^{-1}$  (EC  $\sim 44\text{--}50 \text{ mS cm}^{-1}$ ). At all sites, groundwater EC was measured within or immediately adjacent to an *E. giganteum* stand and the water table was never more than  $\sim 0.5 \text{ m}$  below the surface. The highest EC was measured near the coast north of the city of Arica, where the Lluta River reaches the Pacific. In 2006, the groundwater EC at this site was considerably less, only  $9.6 \text{ mS cm}^{-1}$ , whereas EC at a much further inland site, along the road to Mamiña, was  $14.5 \text{ mS cm}^{-1}$ , the highest measured that year. Clearly there is variability in groundwater salinity. This is likely to be the outcome of variation in the amount of precipitation in the Andes.

At some sites, groundwater reached the surface and was measured where *Equisetum* shoots were growing out of the surface water. Since *Equisetum* generally draws water from saturated soil near the water table (Hauke 1963), it seems likely that these salinities represent those of the actual water sources utilized by the plants. Furthermore, the soil above the water table in the Atacama valley receives its moisture exclusively

from the water table (via capillarity) and there is no rain to leach salts away. Hence, evaporation should cause the soil water above the water table to be even more saline than the groundwater (Fig. 2F and G).

Groundwater Na concentration was highly correlated with EC (Fig. 3A), whereas K was not significantly correlated with EC (Fig. 3B). Although there was a significant linear association between Na and K concentrations, the correlation was weak (Fig. 3C).

### Objective 2

There was no significant effect of groundwater salinity on stomatal conductance ( $g_w$ ) (Fig. 4A). There was a significant negative association between increasing EC and  $F_v/F_m$ , but the correlation was very weak (Fig. 4B). Also, some of the highest  $F_v/F_m$  values were measured at a site of relatively high EC. Average  $F_v/F_m$  and average stomatal conductance showed no significant association (Fig. 4C).

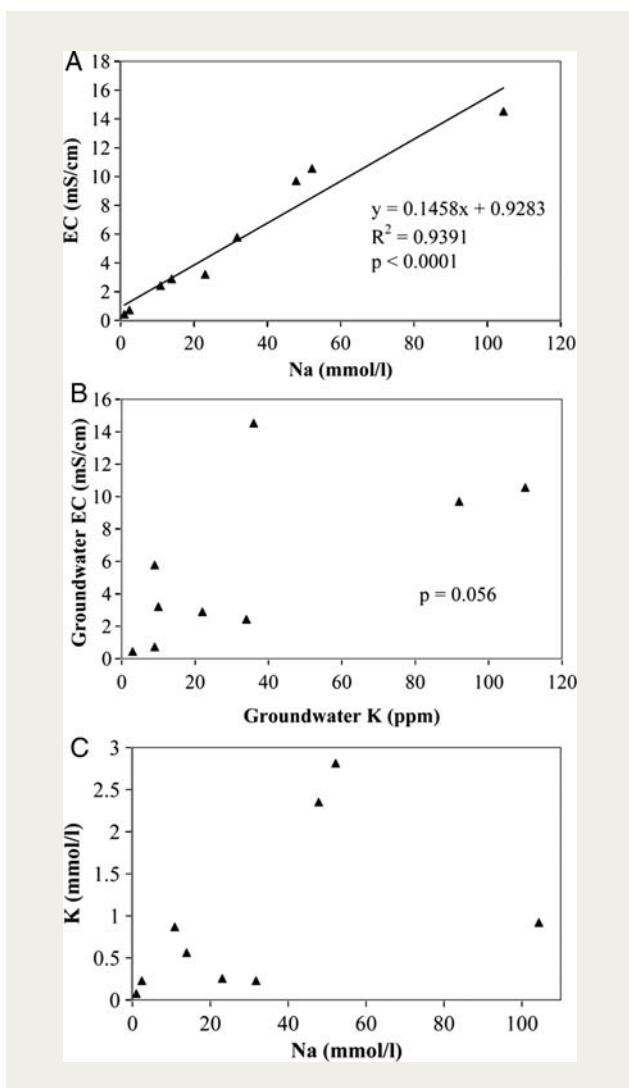
### Objective 3

Both  $\psi_t$  and  $\psi_s$  of lateral branches were consistently lower than groundwater potential at all sites, reaching a minimum at the sites with highest groundwater EC (Fig. 5).

### Objective 4

Guttation fluid EC was highly positively correlated with groundwater EC (Fig. 6A). There was a weak but significant correlation between groundwater Na and guttation fluid Na, but the significance of this relationship disappeared when a natural log transformation was used to reduce variance heteroscedasticity (Fig. 6B). A stronger and more robust correlation was found between groundwater K and guttation fluid K (Fig. 6C), which remains significant when log transformed to ameliorate heteroscedasticity. The K/Na ratio in guttation fluid showed no significant correlation with this ratio in groundwater (Fig. 6D). Guttation fluid K was significantly correlated with guttation fluid Na, with K concentration increasing 2.4-fold for every unit increase in Na (Fig. 7A). The white deposits had a high mean proportion of K to Na (17.42 on a molar basis, s.e. = 8.61, n = 12), as did the fresh guttation fluid (4.42, s.e. = 0.481, n = 30), and there was a significant exponential increase in K concentration with increasing Na concentration (Fig. 7B). In contrast, the mean K/Na ratio of groundwater was two orders of magnitude lower (0.0471, s.e. = 0.0111, n = 9).

When an ANOVA model is used to assess the combined effects of groundwater Na and K on guttation fluid Na, both of these factors and their interaction were significant (Table 1), and this model accounts for 49 % of the variability in the response variable

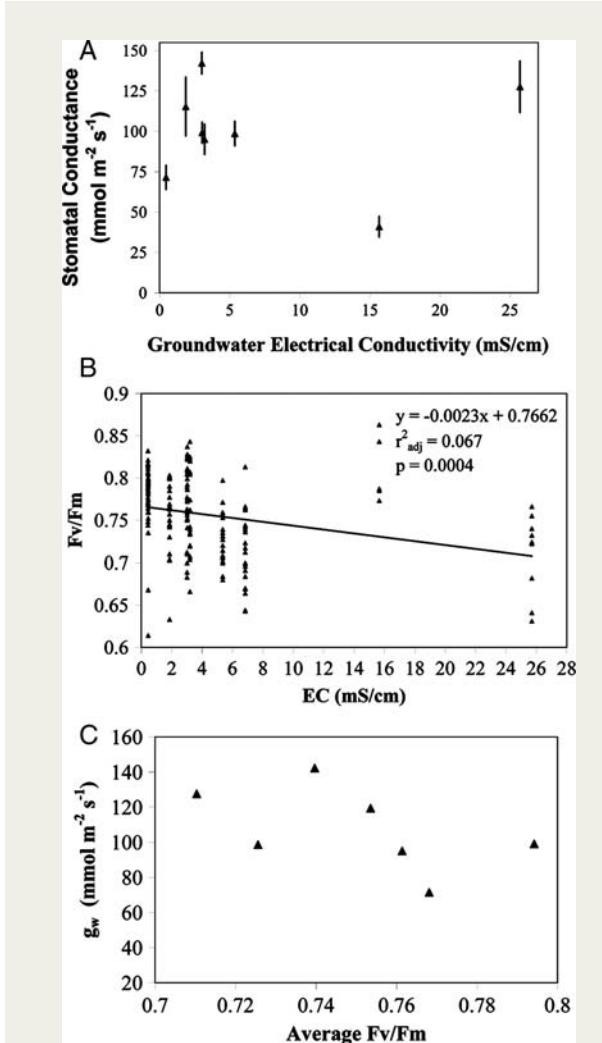


**Fig. 3** *Equisetum giganteum*: populations in river valleys of Arica Province and Tarapacá Region, northern Chile, and the Elqui Valley, central Chile ( $n = 9$ ). (A) Groundwater Na concentrations, (B) K concentrations vs electrical conductivity, (C) relationship between groundwater K concentration and Na concentration.

(measured by  $r_{\text{adj}}^2$ ). For guttation fluid K, all factors are once again significant (Table 1), and the model accounts for 66 % of the variability in the response variable (measured by  $r_{\text{adj}}^2$ ).

### Objective 5

Although shoot sap Na concentration increased initially with increasing groundwater Na, at the highest groundwater Na, the increase had stopped (Fig. 8A). A similar pattern was observed for K (Fig. 8B). There was a weak linear association between groundwater K/Na and shoot sap K/Na (Fig. 8C). Shoot sap EC exhibited a non-



**Fig. 4** *Equisetum giganteum*: groundwater EC and physiological stress indicators. (A) Relationship between groundwater EC and main shoot stomatal conductance across sites in northern and central Chile. No significant correlation was detectable ( $P = 0.651$ ,  $n = 68$ ); error bars are standard errors of the mean. (B) Relationship between groundwater EC and branch chlorophyll fluorescence across sites in northern Chile and northwest Argentina ( $n = 168$ ). (C) Relationship between average chlorophyll fluorescence and average stomatal conductance of shoots across sites in northern Chile and northwest Argentina. No significant correlation was detectable ( $P = 0.233$ ,  $n = 7$ ).

linear association with groundwater osmotic potential, showing an initial decrease and then an increase at the highest osmotic potentials (Fig. 9). The pattern approximately paralleled that of shoot sap K, suggesting K as a major component of shoot sap EC, as was corroborated by the very strong linear correlation between shoot sap K and shoot sap EC (Fig. 10A). In contrast,

the association of shoot sap Na and shoot sap EC, although significant, was considerably weaker and showed a rapid levelling off (Fig. 10B). Shoot sap K increased initially with shoot sap Na, but then levelled off (Fig. 10C). Shoot sap EC and shoot sap osmotic

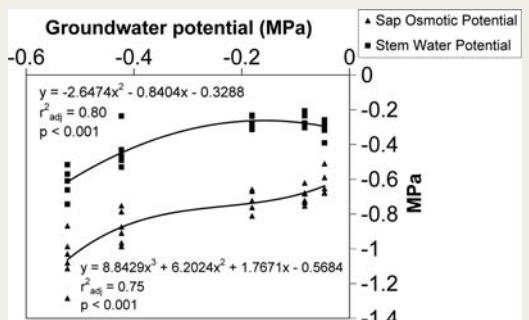
potential were highly correlated (Fig. 11A). There was a significant linear relationship between shoot sap K and osmotic potential (Fig. 11B) and the mean contribution of K (as KCl) to shoot sap osmotic potential was 68 % (s.e. = 1.028,  $n = 30$ ), with the contribution levelling off at more negative sap osmotic potentials (Fig. 11C).

When an ANOVA model is used to assess the combined effects of groundwater Na and K on shoot sap Na, both these factors and their interaction are significant (Table 1), and this model accounts for 57 % of the variability in the response (measured by  $r^2_{\text{adj}}$ ). For shoot sap K, none of the individual factors is significant (Table 1), although the overall model is significant and accounts for 36 % of the variability in the response variable (measured by  $r^2_{\text{adj}}$ ).

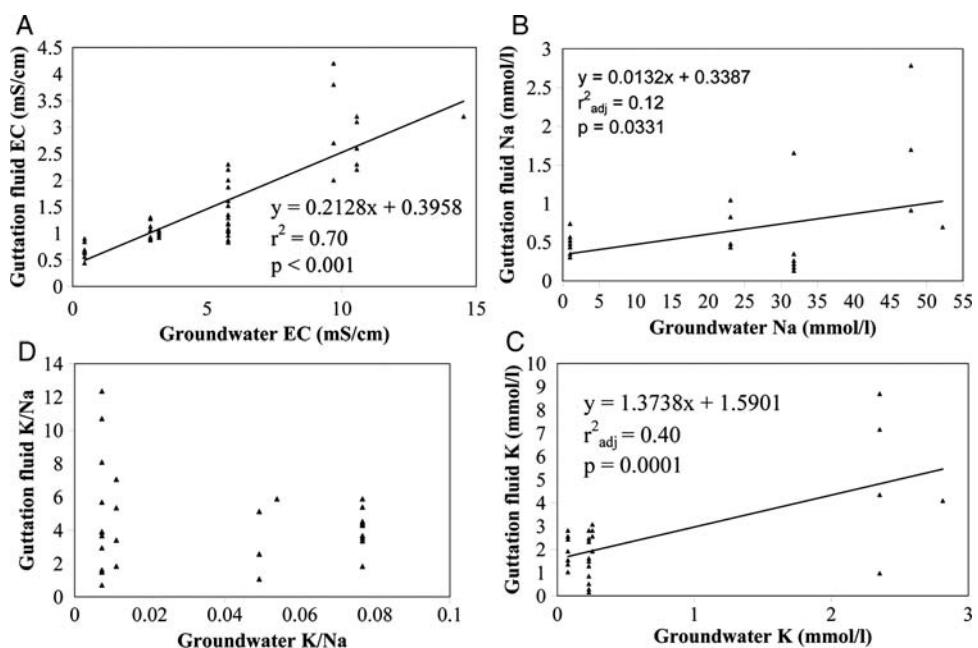
## Discussion

### Objectives 1 and 2

The results indicate that *E. giganteum* maintains a high degree of homeostasis in stomatal conductance, photosynthetic physiology and root function over a broad



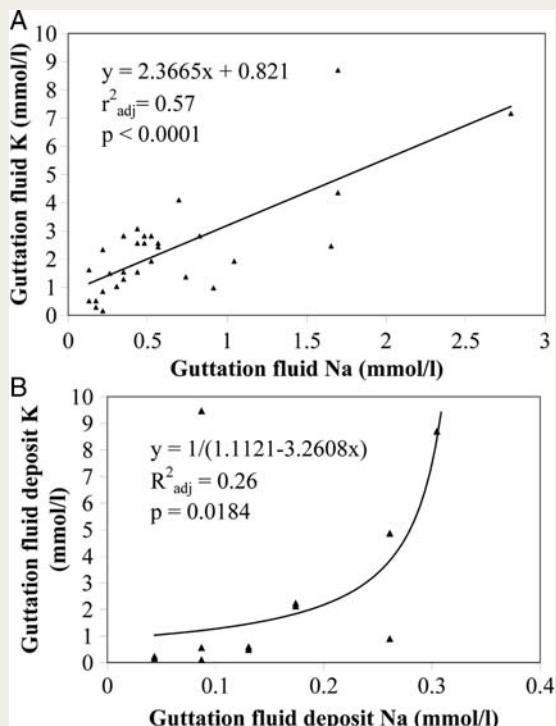
**Fig. 5** *Equisetum giganteum*: total water potential and sap osmotic potential for young mature shoots. These are pre-dawn measurements made in Chile ( $n = 29$ ).



**Fig. 6** *Equisetum giganteum*: relationships between groundwater composition and guttation fluid composition. Measurements were taken of populations in northern and central Chile. (A) Relationship between groundwater EC and guttation fluid EC ( $n = 52$ ). (B) Relationship between groundwater Na concentration and guttation fluid Na concentration ( $n = 30$ ). After natural log transformation to reduce heteroscedasticity, the correlation is no longer significant ( $P = 0.3341$ ). (C) Relationship between groundwater K concentration and guttation fluid K concentration ( $n = 30$ ). When log transformed to reduce variance heteroscedasticity,  $r^2 = 0.18$  and  $P = 0.020$ . (D) Relationship between groundwater Na/K concentration ratio and guttation fluid Na/K concentration ratio. No significant correlation is detectable either in the original units ( $P = 0.49$ ,  $n = 30$ ) or when a square root transformation is applied to the y-axis to reduce variance heteroscedasticity ( $P = 0.70$ ).

range of groundwater salinities in the Atacama Desert and northwestern Argentina (Objectives 1 and 2). Since root pressure ‘develops only when root systems are healthy’ (Kramer and Boyer 1995), the observation that *E. giganteum* generates root pressure, even under highly saline conditions, suggests that its roots are coping well with groundwater salinity. These findings

suggest that *E. giganteum* is best classified as a facultative halophyte, like *A. aureum*, because it adapts well to moderate salinities and maintains a low Na/K ratio in its cell sap (Medina et al. 1995). In contrast, if *E. giganteum* were a typical glycophyte exhibiting salinity stress, as is the case for most pteridophytes (Yensen 1999), evidence of water stress (reduced  $g_w$ ) and photosynthetic stress (reduced  $F_v/F_m$ ) would have been found at these salinities (Netondo et al. 2004). A saturated soil paste EC of  $4 \text{ mS cm}^{-1}$  is considered saline, meaning that many of the soils where *E. giganteum* grows in northern Chile contain amounts of salts well in excess of levels that cause substantial yield reductions in crops (Muñns and Tester 2008). That *Equisetum* grows in such saline conditions is especially surprising because horsetail species are seldom associated with saline habitats and the highest salinities experienced by *E. giganteum* in northern Chile are in the range of those tolerated only by halophytes (Brady and Weil 1996). These unexpected findings suggest that *E. giganteum* has very effective mechanisms for mitigating salinity stress.



**Fig. 7** *Equisetum giganteum*: guttation fluid composition. (A) Relationship between Na and K concentrations in guttation fluid. Measurements were taken of populations in northern and central Chile ( $n = 30$ ). (B) Shoot surface deposit Na and K composition ( $n = 7$ ) northern Chile.

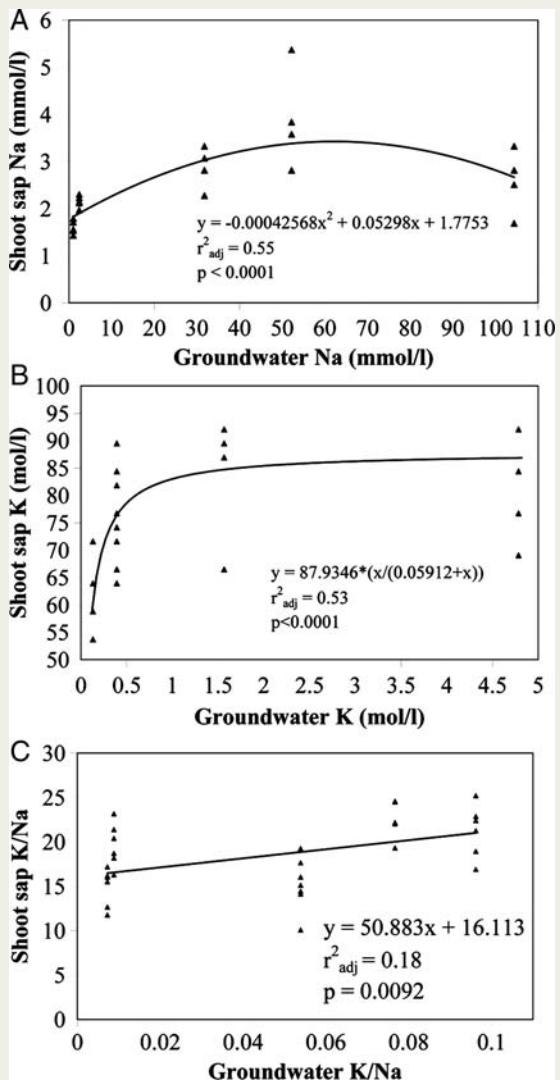
### Objective 3

The preferential accumulation of K by *E. giganteum* is not surprising given the high K requirement exhibited by the related species *E. arvense* (Andersson 1999). Shoot sap EC and shoot sap osmotic potential were highly correlated (Fig. 11A), suggesting that ionic solutes such as K are the major contributors to osmotic adjustment in *E. giganteum*, rather than non-polar osmolytes such as cytoplasmically compatible sugars. The fact that the contribution of K to shoot sap  $\psi_s$  decreases with more negative  $\psi_s$  (Fig. 11C), coupled with the lack of levelling off of shoot sap osmotic potential with increasing EC (Fig. 11A), suggests that polar solutes besides K contribute increasingly to  $\psi_s$  as osmotic adjustment becomes greater.

**Table 1** P-values and  $r^2_{\text{adj}}$  for influence of groundwater Na and K concentrations on cell sap and guttation fluid concentrations, based on ANOVA for *E. giganteum* guttation fluid ( $n = 30$ ) and shoot sap ( $n = 31$ ).

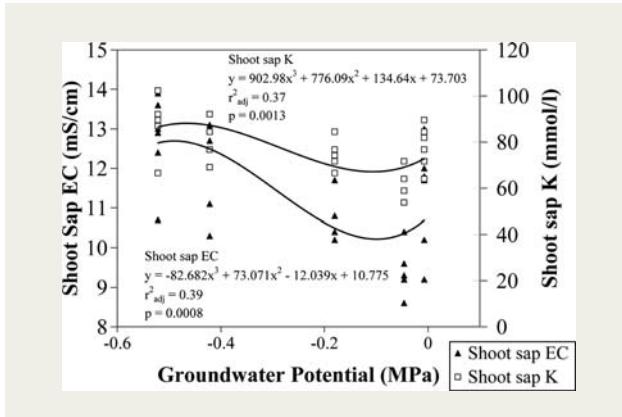
| Effect                                | Guttation fluid Na | Guttation fluid K | Shoot sap Na | Shoot sap K |
|---------------------------------------|--------------------|-------------------|--------------|-------------|
| Groundwater Na                        | 0.0027*            | 0.0013*           | 0.0138*      | 0.5901      |
| Groundwater K                         | 0.0011*            | 0.0005*           | <0.0001*     | 0.0658      |
| Groundwater Na $\times$ K interaction | 0.0026*            | 0.0025*           | 0.0007*      | 0.3786      |
| Overall model                         | 0.0001*            | <0.0001*          | <0.0001*     | 0.0016*     |
| Model $r^2_{\text{adj}}$              | 0.4906             | 0.6611            | 0.5743       | 0.3623      |

\*Denotes statistical significance of model effects at the  $\alpha = 0.05$  level.



**Fig. 8** *Equisetum giganteum*: groundwater and cell sap composition in northern and central Chile ( $n = 31$ ). (A) Relationship between groundwater Na concentration and cell sap concentration. (B) Relationship between groundwater K concentration and cell sap K concentration. (C) Relationship between K/Na ratios in groundwater and cell sap.

Recent studies have shown that increasing concentrations of polar solutes can enhance the hydraulic conductivity of angiosperm vessels by affecting the properties of pectins in vessel pit membranes (Gascó *et al.* 2008; Trifilo *et al.* 2008). Potassium has been shown to be particularly effective in this regard (Zwieniecki *et al.* 2001; Gascó *et al.* 2008), and there is evidence of possible active regulation of xylem conductivity through interactions with the phloem

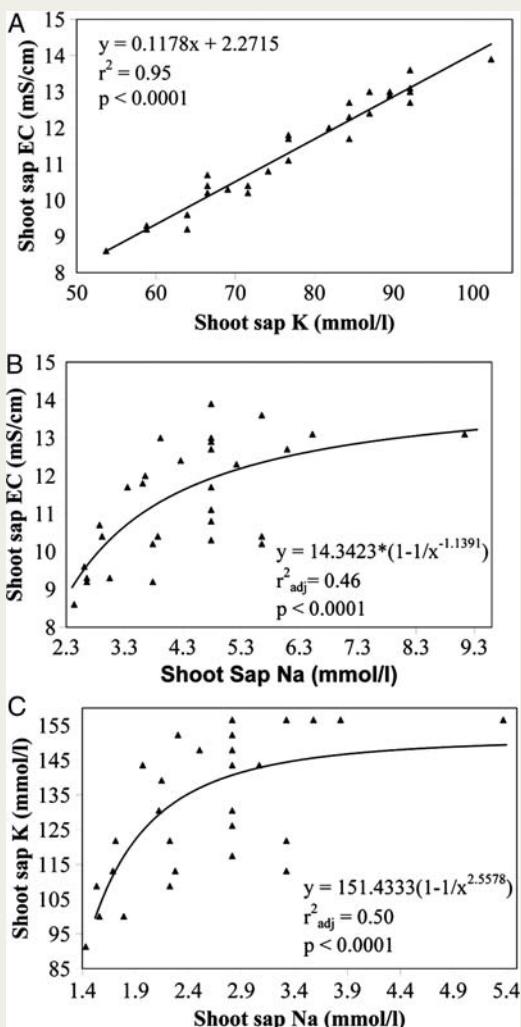


**Fig. 9** *Equisetum giganteum*. Relationships among groundwater osmotic potential and shoot sap EC and K concentrations across sites in northern and central Chile ( $n = 30$ ).

(Zwieniecki *et al.* 2004). Sodium chloride may play a role in regulating xylem conductivity in mangroves (López-Portillo *et al.* 2005). Although this phenomenon has not yet been studied in pteridophytes or gymnosperms, it is possible that preferential accumulation of K by *E. giganteum* under saline conditions facilitates water uptake by enhancing hydraulic conductivity of the xylem, thus requiring less osmotic adjustment to maintain tissue hydration. However, important questions remain regarding the extent to which this phenomenon may function *in planta* (van Leperen 2007).

#### Objectives 4 and 5

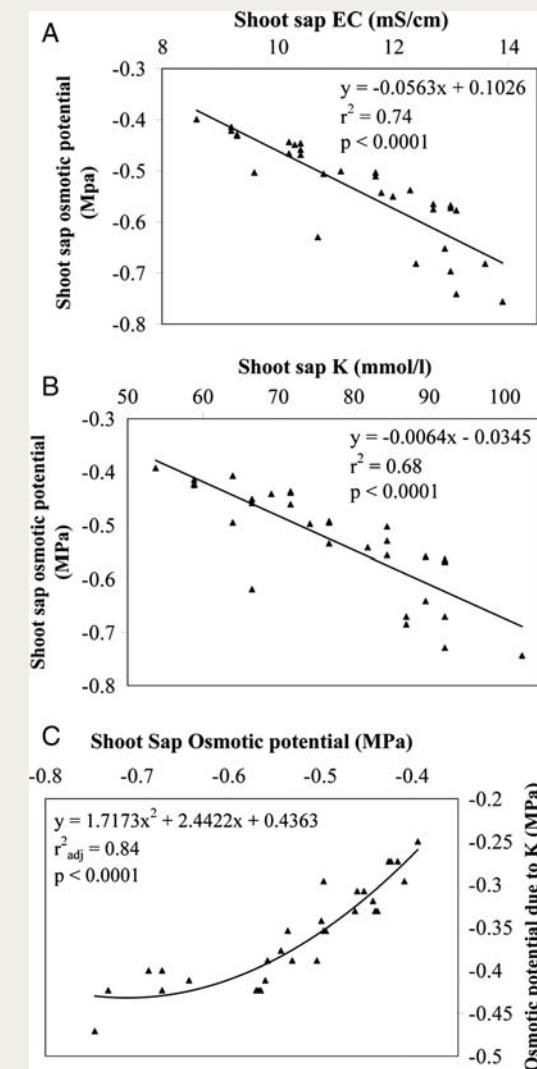
Plants exhibit three types of salinity tolerance: (i) initial osmotic stress tolerance at the onset of salinity stress, involving osmotic adjustment and increased leaf area and stomatal conductance under greater stress to produce evaporative power to extract soil water; (ii) exclusion of Na at the roots; and (iii) tolerance of plant tissues to Na accumulation (Munns and Tester 2008). The osmotic response tends to happen initially and later the response to toxic ions becomes manifest (Munns and Tester 2008). The fact that Na does not accumulate in either the xylem fluid of mature shoots or the cell sap of young shoots suggests that the roots of *E. giganteum* effectively exclude this ion. In addition, the fact that older main shoots remain green and capable of producing new lateral branches for at least 2 years (Fig. 2A) suggests that *E. giganteum* does not tolerate salinity by allowing build-up of Na in older shoots. The fact that lateral branches have a shorter lifespan than central shoots allows for the possibility that Na may accumulate in the branches, leading to their senescence. However, Na contents of old and young branches were not measured in this study. Also, because the Na



**Fig. 10** *Equisetum giganteum*: shoot sap Na and K composition in northern and central Chile ( $n = 31$ ). (A) Relationship between shoot sap K concentration and shoot sap EC. (B) Relationship between shoot sap Na concentration and shoot sap EC. (C) Relationship between Na and K concentrations in shoot sap.

content of roots was not measured in this study, it is not possible to rule out Na accumulation in roots, as occurs, for example, in a corn variety selected to grow in high-salinity areas of the Atacama River valleys (Bastias *et al.* 2004).

Other measurements made during this research indicated that  $g_w$  of mature shoots is more sensitive to environmental factors (light and other site differences) than is  $g_w$  of young developing shoots (C. Husby, unpubl. res.). Along with the guttation frequently observed in developing shoots (Fig. 2B), these observations suggest that *E. giganteum* has



**Fig. 11** *Equisetum giganteum*: relationships between cell sap composition and osmotic potential in northern and central Chile. (A) Relationship between shoot sap EC and shoot sap osmotic potential ( $n = 31$ ). (B) Relationship between cell sap K concentration and sap osmotic potential for young shoots ( $n = 31$ ). (C) Relationship between cell sap K concentration and sap osmotic potential ( $n = 30$ ).

more effective mechanisms for maintaining higher water potentials in developing shoots than in mature shoots. Guttation of developing shoots clearly shows that *E. giganteum* is able to generate substantial root pressure, even when its water source is moderately saline, and can selectively channel this pressure to developing shoots (Fig. 2B). Such a strategy would allow young shoots to elongate even when salinity substantially lowers the osmotic potential of the groundwater.

Theoretical and experimental studies of root pressure by Pickard (2003a, b) provide compelling arguments that active osmolyte uptake mechanisms in root symplast play a crucial role in generating positive xylem pressures during conditions of low transpiration. Because *E. giganteum*, like other *Equisetum* species (Schaffner 1933; Johnson 1936), exhibits extensive guttation, it would appear to be an excellent subject for testing the generality of Pickard's (2003a) root pressure model and exploring its implications. Equiseta have the advantage of consistently guttating over a limited and well-defined area without requiring detopping for easy removal and measurement of xylem exudate, as is necessary for herbaceous plants, such as tomatoes, commonly used for such studies (Barthes et al. 1996; Pickard 2003b). Use of unbranched intact *Equisetum* shoots (e.g. shoots of *Equisetum hyemale* or developing shoots of *E. giganteum* before branch emergence) would allow for likely greater accuracy in studying whole-plant water relationships without the need for extreme destructive manipulation to measure exudation rates.

Further experimental studies are necessary to elucidate the nature of salinity tolerance in *E. giganteum*. Unfortunately, plants of northern Chilean provenance of this species have proven difficult to cultivate in a variety of settings in the USA. By contrast, plants of the northwest Argentinian provenance, which grow in non-saline areas, adapt much more readily to cultivation. Experimental studies of the Argentinian provenances may provide insight into how much of the salinity tolerance of the Atacama form of *E. giganteum* is attributable to local selection and how much is simply due to physiological mechanisms already present in this species of very ancient lineage. The occurrence of salinity tolerance in a sterile *Equisetum* hybrid in Ireland (Page 1997) suggests that a significant level of 'pre-adaptation' to this stress may be present in the genus. In any case, the fact that a plant from such an ancient fern lineage (Hauke 1963; Stewart and Rothwell 1993; Qiu et al. 2007) exhibits the ability to thrive in an environment where levels of salinity, irradiance and vapour pressure deficit are high is unexpected. Since low humidity tends to exacerbate salinity stress in plants that exhibit little salinity tolerance (An et al. 2001), the fact that *E. giganteum* grows vigorously in the Atacama valleys appears all the more remarkable, and provides further evidence that the Atacama form of *E. giganteum* exhibits a considerable degree of stress tolerance. As Rothwell (1996) observed, the remarkable ecological range occupied by horsetails makes it 'extremely difficult to regard *Equisetum* as a relict'. Its ability to adjust physiologically to Na concentrations  $>100 \text{ mmol L}^{-1}$

without showing appreciable physiological stress suggests that its adaptive ability is similar to that of a coastal angiosperm such as *Crambe maritima* (de Vos et al. 2010). This comparison is bolstered by the observation that *E. × moorei* adapts successfully to coastal conditions in Ireland (Page 1997).

## Conclusions and forward look

*Equisetum* appears to be the most basal vascular lineage exhibiting salinity tolerance. Thus, its ability to exclude Na and osmotically adjust by accumulating K (and other osmolytes), which it shares with the fern *A. aureum*, are likely to be the most fundamental salinity tolerance mechanisms among vascular plants. Future experimental studies of salinity tolerance in *Equisetum* taxa will probably provide insights into the detailed mechanisms involved and the full ranges of tolerance across taxa.

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## Contributions by the authors

C.H. largely designed the study, performed most of the field measurements, analysed the data and wrote the manuscript. J.D.-H. assisted with provision and use of laboratory equipment and logistics of fieldwork in Chile. V.O. assisted with laboratory measurements. S.O. contributed to conceptual development of the study, and assisted with provision and use of measurement equipment and manuscript editing. D.P. performed some of the field measurements. L.N. and A.G. assisted with location of field populations and other crucial logistics of fieldwork in Argentina.

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## Conflict of interest statement

None declared.

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