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Growth responses of *Melilotus siculus* accessions to combined salinity and root-zone hypoxia are correlated with differences in tissue ion concentrations and not differences in root aeration

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ABSTRACT

Soil salinity and root-zone hypoxia often occur together in saline landscapes. For many plants, this combination of stresses causes greater increases in Na^+ and Cl^- in shoots, and decreases in K^+, than from salinity alone. These changes in ion concentrations from combined salinity and hypoxia can have more adverse consequences for growth than from salinity alone. The herbaceous forage legume *Melilotus siculus* naturally occurs in saline soils prone to waterlogging; however, accessions differ in their tolerances, although all form high levels of aerenchyma. We hypothesised that tolerance to combined salinity and hypoxia would be associated with either greater aerenchyma formation in roots or the innate ability of the accessions to regulate tissue ion concentrations. Fifteen accessions of *M. siculus* were grown in nutrient solution with two salinities (0 or 200 mM NaCl) and two aeration treatments (aerated or hypoxic) for 21 days. Dry mass (shoot and root), root porosity and ion concentrations (Cl^-, Na^+, K^+) in shoots and roots were assessed. In the *M. siculus* accessions variation in the shoot dry mass under saline–hypoxic conditions was negatively correlated with shoot Cl^- and Na^+, and positively correlated with the shoot K^+. Shoot ion concentrations under saline–hypoxic conditions were related to concentrations under saline–aerated conditions, but not to the porosity of the main root, which was relatively high (~18 to 25%). Differences in the tolerance of *M. siculus* accessions to combined salinity and root-zone hypoxia were mediated by variation in the plants' ability to regulate ions, and were not related to variation in root porosity, which was relatively high in all accessions. The interaction between salinity and hypoxia was not detrimental to *M. siculus*, a waterlogging tolerant species.

1. Introduction

Soil salinity and waterlogging (hypoxia) are major stress factors that can occur together in saline landscapes (Barrett-Lennard, 2003; Bennett et al., 2009; Barrett-Lennard and Shabala, 2013). For many plants, this combination of stresses causes greater increases in Na^+ and Cl^- concentrations in the shoots than from salinity alone and greater decreases in K^+ concentrations in the shoots than from salinity alone (reviewed by Barrett-Lennard, 2003; Colmer and Flowers, 2008; Barrett-Lennard and Shabala, 2013). Furthermore, these changed ion concentrations under combined salinity and hypoxia can have more adverse consequences for growth than salinity alone. For example with wheat, plants exposed for 33 days to salinity under aerated conditions or nonsaline conditions with hypoxia remained alive, but plants exposed to 30 or 60 mM NaCl with hypoxia died (Barrett-Lennard et al., 1999). These deaths with combined salinity and hypoxia were associated with 4–6 fold increases in concentrations of Na^+ and Cl^-, and 40% decreases in the concentration of K^+, in the shoots compared with plants grown at the same salinities under aerated conditions (Barrett-Lennard et al., 1999).

At a mechanistic level, salinity and hypoxia can have a variety of effects on plant metabolism. With salinity, there are immediate

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adverse osmotic effects on growth (Munns, 1993). However, in the longer-term there can be increases in Na^+ uptake and decreases in K^+ uptake, which decrease the ratio of K^+/Na^+ in the cytosol. The physicochemical similarities of these ions mean that Na^+ can compete with K^+ threatening a wide range of key cytoplasmic processes – enzymatic reactions, protein synthesis and ribosome functions (reviewed by Shabala and Cuin, 2007). In addition, salinity can increase the production of reactive oxygen species (ROS), which may cause lipid peroxidation in cellular membranes and also damage to other cellular constituents (reviewed by Bose et al., 2014). Intriguingly, there may be antagonistic feedback between ROS production under saline conditions and K^+ homeostasis. Several types of K^+-permeable channels are activated by ROS, providing a pathway for K^+ to leak from cells (reviewed by Anschütz et al., 2014). Hypoxia in plant tissues decreases the production of ATP. This has many adverse effects including impacts on the H^+-ATPases responsible for membrane function; for example membrane depolarisation (e.g. Buwalda et al., 1988; Teakle et al., 2013) can change the control of Na^+ exclusion and K^+ uptake (Barrett-Lennard and Shabala, 2013).

Many marshland species are better adapted to the combination of salinity and waterlogging than dryland species (Colmer and Flowers, 2008; Jenkins et al., 2010; Barrett-Lennard and Shabala, 2013). Species or accessions with tolerance to this combination of stresses appear better able to regulate Na^+ and Cl^- concentrations and maintain K^+ concentrations in shoots than less tolerant species or accessions (Barrett-Lennard and Shabala, 2013). However, it is not known which mechanisms enable this better ion regulation in tolerant species or accessions. Possibilities include: (i) an innately superior ability to regulate shoot ion concentrations under saline conditions (irrespective of the level of root-aeration), (ii) physiological adaptations that improve internal root aeration (such as high porosity via the development of aerenchyma and perhaps also a barrier to radial oxygen loss—Colmer, 2003), which ensure that the roots can maintain energy production, thereby maintaining ion regulation and growth, or (iii) combinations of both of the above.

Until now, the mechanism(s) enabling better ion relations in shoots of adapted genotypes under saline plus hypoxic conditions have not been resolved. In a study of 17 accessions of *Hordeum marinum*, faster growth in saline–hypoxic solution was associated with lower Na^+ concentrations and higher K^+ concentrations in the expanding leaves (Malik et al., 2009; Barrett-Lennard and Shabala, 2013). However, it was not possible to rule in (or out) the role that better root aeration had in these responses because there was inadequate root material in this study to make replicated measurements of root porosity (Malik et al., 2009).

The present work considers these issues in a study of the tolerance of accessions of the forage annual legume *Melilotus siculus* (Turra) Vitman ex B. D. Jacks (syn. *Melilotus messanensis*) to the combined stresses of salinity and hypoxia. Amongst pasture legumes, this species is reputed to have exceptional tolerance to these stresses, based on comparisons of *M. siculus* and other pasture legume species to combinations of salinity and hypoxia (Teakle et al., 2012) and to these stresses individually (Rogers et al., 2011). Under nonsaline–hypoxic conditions, a single accession of *M. siculus* at least partly maintained internal root aeration because of the development of a secondary aerenchyma in a highly porous tissue called phellem that grew on the upper portions of the main root axis (tap root) and on lateral roots, as well as the hypocotyl (Teakle et al., 2011; Verboven et al., 2012). In addition, under saline–hypoxic conditions, this species had a superior ability (compared with two others—*Trifolium michelianum* and *Medicago polymorpha*) to regulate ion concentrations (Cl^-, Na^+ and K^+) in shoots (Teakle et al., 2012). We build on this understanding here by examining the variation amongst 15 accessions of *M. siculus* in growth, ion relations and root aeration characteristics to the combined effects of root-zone salinity and hypoxia. Three-week old plants were subjected to combinations of treatments of salinity (0 or 200 mM NaCl) and root-zone aeration (aerated or hypoxic nutrient solution) for 21 days. In addition to the *M. siculus* accessions, a single accession of *T. michelianum* Savi (common name "balansa clover") was included for comparison; this species is widely grown on slightly saline soils prone to waterlogging (Nichols et al., 2008; Boschma et al., 2008; Rogers et al., 2008). *T. michelianum* is sensitive to salinity (Rogers et al., 2008; Teakle et al., 2012) so it was expected to have a contrasting response to *M. siculus* under conditions of salinity with hypoxia.

One way in which to evaluate the effects of combinations of stresses on plant growth is to compare the impacts of the stresses separately and when combined; this approach has been widely used in studies of heavy metals (see theoretical approach of Berry and Wallace, 1981; Wallace and Berry, 1983). Adapting this approach, stress responses can be assessed by multiplying the effects of each stress individually, and comparing the result to plant growth with the combination of stresses. If the multiplied result is the same as with the combination of stresses the interaction is defined as "additive"; if the multiplied result is greater than with the combination of stresses the interaction is defined as "synergistic". When the multiplied result is less than with the combination of stresses the interaction is defined as "antagonistic" (Berry and Wallace, 1981; Wallace and Berry, 1983). To illustrate: in the example of wheat referred to earlier (Barrett-Lennard et al., 1999), the individual effects of the salinity and hypoxia were each non-lethal, but the combination of stresses was lethal and therefore clearly antagonistic. The present paper uses this framework to assess the interaction between salinity and hypoxia on the growth and ion relations of 15 *M. siculus* accessions compared with *T. michelianum*.

Not surprisingly, we found that the variation in shoot dry mass (DM) amongst accessions of *M. siculus* was associated with variation in shoot ion concentrations, i.e. accessions with high shoot DM had low Na^+ and Cl^-, and high K^+. There seemed to be two possibilities for the underlying mechanism(s). The first possibility (Hypothesis 1) was that differences in the ability of the plants to internally aerate their roots influenced their ion regulation. The second possibility (Hypothesis 2) was that the differences in ion regulation of the accessions were due to variation in the innate ability of the accessions to regulate their shoot ions in aerated saline conditions. Hypothesis 1 would be supported if the concentrations of Na^+, Cl^- and K^+ in the shoots under saline–hypoxic conditions were correlated with traits associated with root aeration (e.g. porosity of the main root). Hypothesis 2 would be supported if the concentrations of Na^+, Cl^- and K^+ in shoots under saline–hypoxic conditions were proportional to the concentrations of those ions under saline–aerated conditions. Finally, if accessions had a range of levels of root aeration, we might expect the interactions between salinity and hypoxia on shoot ion relations and growth to be additive with high internal root aeration (i.e. high root porosity), but such interactions might become increasingly antagonistic as root porosity decreased.

2. Materials and methods

2.1. Experimental material and growth conditions

Fifteen accessions of *M. siculus* were selected based on their differential responses to the individual effects of either saline or stagnant deoxygenated nutrient solution, in previous experiments (Rogers et al., 2008, 2011). In addition, one accession of *T. michelianum* ("balansa clover" cv. Paradana), an annual pasture legume, was included for comparison (Boschma et al., 2008; Rogers et al., 2008).

Seeds of all accessions were scarified, surface-sterilised with 0.04% (w/v) sodium hypochlorite, rinsed thoroughly in deionised water, and imbibed in aerated 0.5 mM $CaSO_4$ in darkness for 3 h. Afterwards, seeds were transferred to mesh over aerated 10%-strength nutrient solution (composition given below). Three days later, seedlings were transferred to aerated 25% strength nutrient solution and exposed to light. Seven days after imbibition, seedlings were transplanted into pots containing 4.5 L of aerated 50% strength nutrient solution. Each pot contained 4 plants individually held by polyethylene foam. Pot bases were wrapped in black plastic and pot lids in aluminium foil. Pots were kept in root cooling tanks set at 15 °C in a glasshouse with natural irradiance (May to August, Perth, Western Australia). Average glasshouse relative humidity was 60% and the average glasshouse air temperature was 25/15 °C (day/night). Seven days after transplanting, solutions were changed to aerated full-strength nutrient solution (Rogers et al., 2008), consisting of: 0.5 mM KH_2PO_4, 3 mM KNO_3, 4 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 37.5 μM FeEDTA (ethylenediaminetetraacetic acid iron(III) sodium salt), 23 μM H_3BO_3, 4.5 μM $MnCl_2$, 4 μM $ZnSO_4$, 1.5 μM $CuSO_4$ and 0.05 μM MoO_3. The solution was buffered with 2.5 mM MES (2-[*N*-morpholino]ethanesulfonic acid) adjusted to pH 6.3 by the addition of KOH. Nutrient solutions were aerated, renewed weekly and topped-up with deionised water as required.

2.2. Saline and hypoxic treatments

Twenty-one days after seeds were imbibed, four treatments were imposed: nonsaline–aerated, saline–aerated, nonsaline–hypoxic, and saline–hypoxic. In the appropriate pots, salinity was imposed by the daily addition of 50 mM NaCl to the nutrient solution over four consecutive days to a final concentration of 200 mM NaCl. Hypoxia commenced one day after the final NaCl concentration was reached. The nutrient solution was first bubbled with N_2 until the dissolved O_2 concentration had declined to ~10% of that in air-equilibrated solution. The pots were left under this hypoxic pre-treatment for 24 h to avoid anoxic shock (Gibbs and Greenway, 2003), and then the solution was replaced with deoxygenated stagnant agar nutrient solution (0.1% w/v dissolved agar added to the nutrient solution and bubbled over-night with N_2 to purge the O_2). The 0.1% agar prevents convective movements in the nutrient solution, simulating the decrease of O_2 and the accumulation of plant-generated ethylene as occurs in waterlogged soils (Wiengweera et al., 1997).

Due to the large number of accessions, the experiment was split so that the plants were grown in two batches, back-to-back, over a three-month period. Two replicate pots of all accessions with all treatments were grown in each batch. Each replicate consisted of one pot with four plants; having four plants per replicate reduced the influence of any plant-to-plant variability within accessions. Data are expressed per plant for each replicate. Within treatments, the final shoot dry mass (DM) of plants did not vary between the batches ($P > 0.4$), so the 4 replicates (2 from each batch) were therefore used in a single analysis without the need to maintain these as blocks.

2.3. Root porosity, maximum main root diameter and cross sectional area of phellem

Root porosity (proportion of gas volume per unit root volume) was measured in the main roots using the 'buoyancy method' of Raskin (1983) with the equations as modified by Thomson et al. (1990). Maximum main root diameter was measured with a digital caliper. Root phellem (i.e. secondary aerenchyma) – the spongy white layer formed externally to the phellogen – was then removed using a scalpel and the diameter was re-measured. Diameters, with and without phellem, were used to calculate the cross sectional area of phellem at the position of maximal diameter in the main root (assuming the root to be a cylinder); this aerenchymatous tissue enables the internal transport of O_2 from shoots into, and along the basal portions of, roots (Teakle et al., 2011; Verboven et al., 2012).

2.4. Dry mass responses

Plants were harvested 21 days after the initiation of NaCl treatments (i.e. 42 days after imbibition). Plants were separated into shoots and roots. Within shoots, the youngest fully-expanded leaf was separated for ion analyses (described below). Roots were washed in 4 mM $CaSO_4$ solution containing an iso-osmotic concentration of mannitol (for the saline treated plants) and were separated into main and lateral roots for ion analyses. All plant material was weighed after oven drying at 70 °C for 3 days.

2.5. Tissue ion concentrations

Concentrations of Cl^-, Na^+ and K^+ were measured in whole shoots, the youngest fully-expanded leaf and in the two root fractions. Dried tissue samples of each plant component were ground to a fine powder and 100 mg was extracted with 10 mL of 0.5 M HNO_3 by shaking for 2 days in darkness at 30 °C, following the procedure of Munns et al. (2010). The diluted extracts were used for Na^+ and K^+ determinations with a flame photometer (Jenway Ltd, model PFP7, Essex, UK) and Cl^- with a chloridometer (Buchler-Cotlove Chloridometer 662201, Fort-Lee, New Jersey, USA). The reliability of these analyses was confirmed by taking a reference plant sample (ASPAC no. 85) with known ionic composition through the same procedures. The ratio of K^+/Na^+ (on a molar basis) for shoots and for roots was also calculated.

2.6. Statistical analyses

Data were evaluated by three-way ANOVAs with 'salinity treatment', 'hypoxic treatment' and 'accession' as main factors. Normality and the homogeneity of variances satisfied ANOVA assumptions. When significant interactions were detected, subsequent Fisher's protected least significant difference (LSD) tests were applied to determine the effect of the treatments among accessions. To test the hypotheses, relationships were explored between DM (shoot and root), ion concentrations (Cl^-, Na^+, K^+ and K^+/Na^+ ratio), and main root porosity (Table 2). To determine the type of interaction (antagonistic, additive or synergistic) between root-zone salinity and hypoxia, we calculated the relative dry mass (DM) of plants in the saline–hypoxic treatment ($DM_{saline–hypoxic}/DM_{nonsaline–aerated}$) and the product of the relative DM of each factor when applied individually (i.e. $DM_{saline–aerated}/DM_{nonsaline–aerated} \times DM_{nonsaline–hypoxic}/DM_{nonsaline–aerated}$; see Section 1 for explanation). Similar calculations were made of the relative Cl^- and Na^+ concentrations in tissues of plants in the saline–hypoxic treatment with the product of the relative tissue ion concentrations between each factor applied individually. Interactions were additive if the relative DM (or ion concentration) under saline–hypoxic conditions was approximately equal to the product of the relative DM of each stress when applied individually. Interactions were antagonistic if the relative DM (or ion concentration) under saline–hypoxic conductions was substantially less than the product of the relative DM of each stress when applied individually.

3. Results

This section will focus on the dry mass (DM) of shoots and roots, ion concentrations in the shoots and the aeration characteristics of the roots. Table 1 summarises the average effects of 21 days exposure to the different treatments (nonsaline–aerated, saline–aerated, nonsaline–hypoxic, or saline–hypoxic) on shoot

Table 1

Average values for shoot and root dry mass (DM), ion concentrations in tissues (Cl^-, Na^+ and K^+, and ratio of K^+/Na^+) and root aeration traits in: (A) 15 accessions of *Melilotus siculus*, and (B) one accession of *Trifolium michelianum* ('Balansa'). Plants were grown for 21 days with nonsaline–aerated, saline–aerated, nonsaline–hypoxic or saline–hypoxic treatments in nutrient solution.

Parameters	Treatments			
	Nonsaline–aerated	Saline–aerated	Nonsaline–hypoxic	Saline–hypoxic
A. *Melilotus siculus*[1]: Dry mass (g per plant)				
Shoot	0.711^{a}	0.435^{b}	0.755^{a}	0.497^{b}
Root	0.709^{a}	0.413^{b}	0.756^{a}	0.463^{b}
Ion concentrations (μmol g^{-1} DM)				
Whole shoot				
Cl^-	188^{b}	1287^{a}	185^{b}	1407^{a}
Na^+	215^{b}	1378^{a}	228^{b}	1597^{a}
K^+	1567^{a}	865^{b}	1627^{a}	623^{c}
K^+/Na^+ ratio	7.4^{a}	0.65^{b}	7.2^{a}	0.41^{b}
Young leaf				
Cl^-	180^{c}	890^{b}	189^{c}	1176^{a}
Na^+	213^{b}	928^{a}	203^{b}	1235^{a}
K^+	1609^{a}	1096^{b}	1556^{a}	914^{b}
K^+/Na^+ ratio	7.9^{a}	1.29^{b}	7.9^{a}	0.83^{b}
Main root				
Cl^-	284^{c}	1233^{b}	284^{c}	1733^{a}
Na^+	338^{c}	1287^{b}	357^{c}	1863^{a}
K^+	1735^{a}	785^{b}	1725^{a}	593^{c}
K^+/Na^+ ratio	5.3^{a}	0.61^{b}	4.9^{a}	0.32^{c}
Lateral roots				
Cl^-	319^{b}	1469^{a}	303^{b}	1593^{a}
Na^+	355^{c}	1759^{a}	441^{b}	1908^{a}
K^+	2345^{b}	1687^{c}	2730^{a}	1818^{c}
K^+/Na^+ ratio	6.6^{a}	1.00^{b}	6.2^{a}	0.96^{b}
Root aeration traits				
Main root porosity (%)	20.1^{bc}	17.6^{c}	25.0^{a}	22.4^{ab}
Maximum diameter (mm)	5.16^{b}	3.82^{c}	6.34^{a}	4.72^{b}
Phellem area (mm^2)	15.63^{b}	10.74^{c}	26.39^{a}	17.94^{b}
B. *Trifolium michelianum* ('Balansa')[2]: Dry mass (g per plant)				
Shoot	1.088^{a}	0.246^{b}	0.915^{a}	0.303^{b}
Root	0.452^{a}	0.180^{b}	0.572^{a}	0.268^{b}
Ion concentrations (μmol g^{-1} DM)				
Whole shoot				
Cl^-	189^{c}	1199^{b}	188^{c}	2025^{a}
Na^+	207^{c}	1507^{b}	230^{c}	2278^{a}
K^+	1874^{a}	551^{b}	1699^{a}	402^{b}
K^+/Na^+ ratio	9.3^{a}	0.38^{b}	7.4^{a}	0.18^{c}
Young leaf				
Cl^-	164^{c}	1023^{b}	220^{c}	1579^{a}
Na^+	218^{c}	1257^{b}	198^{c}	1752^{a}
K^+	1435^{a}	797^{b}	1438^{a}	688^{b}
K^+/Na^+ ratio	7.0^{a}	0.61^{b}	7.9^{a}	0.47^{b}
Main root				
Cl^-	260^{c}	1300^{b}	244^{c}	1887^{a}
Na^+	327^{b}	1277^{a}	319^{b}	1467^{a}
K^+	1620^{a}	466^{b}	1808^{a}	312^{b}
K^+/Na^+ ratio	5.1^{a}	0.38^{b}	5.7^{a}	0.21^{c}
Lateral roots				
Cl^-	396^{c}	1436^{b}	361^{c}	1942^{a}
Na^+	313^{c}	1749^{b}	417^{c}	2208^{a}
K^+	1916^{b}	1263^{c}	2887^{a}	1162^{c}
K^+/Na^+ ratio	6.2^{a}	0.74^{b}	6.9^{a}	0.54^{c}
Root aeration				
Main root porosity (%)	18.9^{a}	15.2^{a}	18.8^{a}	19.3^{a}

[1] Values are the means of 15 accessions. Different superscript letters within rows indicate significant differences between treatments based on the $LSD_{0.05}$.

[2] Values are the mean of a single accession. Different superscript letters within rows indicate significant differences between treatments based on the $LSD_{0.05}$.

and root DM, concentrations of Cl^-, Na^+ and K^+ in all tissues, and the traits associated with root aeration. The statistical significance of the salinity and hypoxic treatments, accessions and their interactions for these parameters are summarised in the Supplementary materials—Table S1.

3.1. Shoot and root dry mass

For shoot and root DM of *M. siculus* there were significant main effects of the saline and aeration treatments and of accession, with most of the interactions being significant for the shoots (Table S1). For shoots, the nonsaline–hypoxic treatment did not affect average DM compared to the nonsaline–aerated controls (Table 1A), and it even promoted the growth of three accessions with low vigour under control conditions (SA41637, SA41642 and SA41645 in Fig. 1a). In contrast the saline–aerated treatment decreased average shoot DM by ~40% (compared with the nonsaline–aerated treatment), but the imposition of the saline–hypoxic treatment caused slight increases in average shoot DM (~10% compared with the saline–aerated treatment—Table 1A).

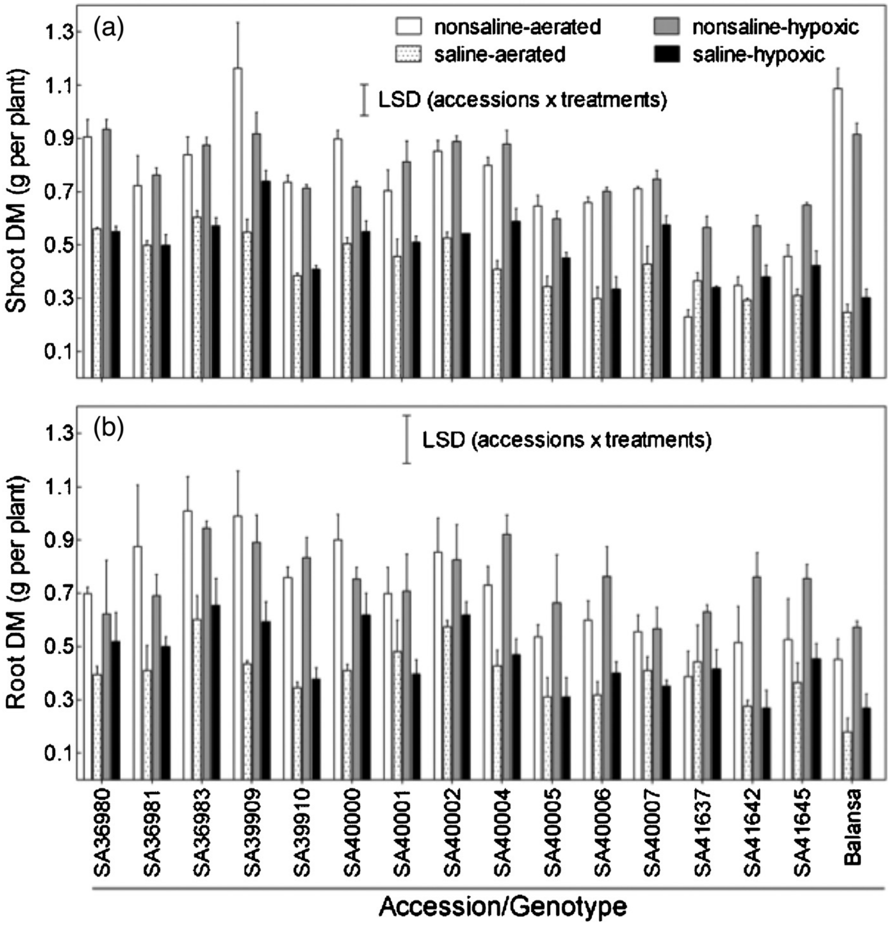

Fig. 1. Shoot (a) and root (b) dry mass (DM) of fifteen accessions of *Melilotus siculus* grown for 21 days with nonsaline–aerated, saline–aerated, nonsaline–hypoxic or saline–hypoxic treatments in nutrient solution. Data for *Trifolium michelianum* ('Balansa') are also shown for comparison. Plants were 21 days old when treatments were imposed. Values are means per plant of four replicate pots with four plants per pot. The error bar is the SEM. The LSD is the Fisher's protected least significant difference at $P = 0.05$ (for comparisons of means across accessions and treatments).

In general, the average trends in root DM were similar; there was no adverse effect of nonsaline–hypoxic treatment on root DM, but the saline–aerated treatment reduced average root DM by ~40%, and the saline–hypoxic treatment increased average root DM by ~10% relative to the saline–aerated treatment (Table 1A).

For *T. michelianum*, the plants in nonsaline-hypoxia had shoot and root DM similar to the control (nonsaline–aerated) plants, but shoot DM decreased by 72–77% in the saline–aerated and saline–hypoxic treatments ($P < 0.01$; Table 1B, Fig. 1a) and root DM decreased by 41–60% in the saline–aerated and saline–hypoxic treatments ($P < 0.01$; Table 1B, Fig. 1b). These responses of *T. michelianum* are consistent with its reputation of being waterlogging tolerant but salt-sensitive (Rogers et al., 2008, 2011).

3.2. Ion concentrations in the tissues

Effects of treatment on concentrations of Cl^-, Na^+, K^+ and the K^+/Na^+ ratio for each of the tissues measured (shoots, youngest fully-expanded leaf, main root and lateral roots) across all measured accessions are given in Table S2. Data averaged across all *M. siculus* accessions and for *T. michelianum* are given in Table 1A and B, respectively.

For *M. siculus*, in the shoots, there were significant main effects on shoot ion concentrations (Cl^-, Na^+, K^+) for salinity, hypoxia and accession, and significant interactions between salinity and hypoxia, salinity and accession, and hypoxia and accession (Table S1). Averaged across all *M. siculus* accessions, shoot concentrations of Cl^- were ~5.8-fold higher, concentrations of Na^+ were ~5.4-fold higher, and concentrations of K^+ were ~45% lower under saline–aerated compared with nonsaline–aerated (control) conditions (calculated from means in Table 1A). Compared with saline–aerated plants, the imposition of saline–hypoxic conditions slightly increased average shoot concentrations of Cl^- and Na^+ (by ~9 and 16%, respectively), and decreased average concentrations of K^+ by 28% (Table 1A). In comparison, *T. michelianum* in the saline–aerated treatment also had increased shoot concentrations of Cl^- and Na^+ (by 5.3- and 6.3-fold, respectively) and decreased shoot concentrations of K^+ (by 71%) compared with the control (Table 1B). However the major difference with *T. michelianum* was in the scale of the changes in shoots with the imposition of the saline–hypoxic treatment; there were 69% and 51% increases in Cl^- and Na^+ concentrations, respectively, and a 21% decrease in K^+, compared with the saline–aerated treatment (Table 1B). Hypoxia under saline conditions clearly had more adverse effects on ion regulation in *T. michelianum* than in *M. siculus*.

In the main root of *M. siculus*, there were significant main effects of salinity and hypoxia on the ion concentrations (Cl^-, Na^+, K^+), and significant interactions between salinity and hypoxia, salinity and accession, and hypoxia and accession. The lateral roots had similar effects except for non-significance in the main effect of the hypoxic treatment on Cl^- and the interaction between salinity and hypoxia on Na^+ (Table S1). Averaged across all *M. siculus* accessions, in the main root under saline–aerated conditions the concentrations of Cl^- and Na^+ increased by 3.3- and 2.8-fold, respectively, and concentrations of K^+ decreased by 55% compared with nonsaline–aerated control (see average values in Table 1A). Similar trends occurred with the lateral roots (Table 1A).

Interestingly, there were significant differences between root types in their responses to hypoxia under saline conditions. Averaged across all *M. siculus* accessions with the saline–hypoxic treatment, the main root had 41% higher Cl^-, 45% higher Na^+ and 24% lower K^+ than in the saline-aerated treatment, but lateral roots had insignificant differences in Na^+, Cl^- and K^+ between the two treatments. As a consequence, under saline–hypoxic conditions, the average ratio of K^+/Na^+ was 60–200% higher in laterals than in the main root (see average values in Table 1). In contrast, roots of *T. michelianum* exposed to the saline–aerated treatment had increased concentrations of Cl^- and Na^+ (by 2.6- to 4.6-fold) and decreased concentrations of K^+ (by 34–71%) compared with the control (Table 1B). Moreover, the saline–hypoxic treatment further increased Na^+ and Cl^- by 15–45% and decreased K^+ by 8–33% compared with the saline–aerated treatment (Table 1B).

3.3. Relationships between ion concentrations and shoot dry mass

Across all *M. siculus* accessions, shoot DM was related to ion concentrations in the tissues under both saline–aerated and saline–hypoxic conditions (Fig. 2a–h). Under saline–aerated conditions, shoot DM was negatively correlated with concentrations of Cl^- and Na^+ in the shoots ($P = 0.003$ and 0.004, respectively; Fig. 2a and b) and was positively correlated with concentrations of K^+ and the ratio of K^+/Na^+ in the shoots ($P < 0.001$ in each case; Fig. 2c and d). Similarly, under saline–hypoxic conditions shoot DM was negatively correlated with Cl^- and Na^+ concentrations in whole shoots ($P < 0.001$ and 0.006, respectively; Fig. 2e and f), and positively correlated with shoot K^+ concentration and the ratio of K^+/Na^+ ($P = 0.007$ and < 0.001, respectively; Fig. 2g and h). Under these saline conditions, both aerated or hypoxic, the two accessions with greatest shoot DM were SA36983 and SA39909, respectively; in each case, these two accessions had amongst the lowest concentrations of Cl^- and Na^+ and highest K^+ in the shoots, and as a result, a high ratio of K^+/Na^+ (Fig. 2e–h).

Some of the strongest evidence linking shoot ion (Cl^-, Na^+, K^+) regulation in *M. siculus* under saline–hypoxic conditions to the innate capacity of the various accessions to control the entry of these ions, comes from a range of cross correlations. For Cl^-, Na^+ and K^+ in the shoots, concentrations under saline–hypoxic conditions were positively correlated with concentrations under saline–aerated conditions ($P = 0.014$, 0.020 and 0.042, respectively; Table 2). Furthermore, the shoot DM under saline–hypoxic conditions was negatively correlated with Cl^- and Na^+ concentrations and positively correlated with K^+ concentrations and the ratio K^+/Na^+ in the shoots of plants under saline–aerated conditions ($P = 0.003$, 0.010, 0.005 and < 0.001, respectively; data not shown).

Ion concentrations in the youngest fully-expanded leaves of *M. siculus* in the saline–aerated and saline–hypoxic treatments were also correlated with shoot DM (data not shown). There were no significant correlations between shoot DM and root concentrations of Cl^-, Na^+, K^+ or K^+/Na^+ ratio, in either the main root or lateral roots ($P > 0.10$ for Na^+, $P = 0.4$ to 0.7 for K^+ and Cl^-, and $P = 0.4$ to 0.8 for K^+/Na^+ ratio; data not shown).

3.4. Root aeration

We used three measurements as indices of the potential for internal root aeration: (a) the porosity of the main root, (b) maximum cross sectional area of the phellem, and (c) maximum root diameter. Statistical analysis showed strong main effects of hypoxia and salinity ($P < 0.001$) and weaker main effects of accession ($P < 0.05$) on root porosity, maximum root diameter and phellem cross sectional area (Table S1). Across all treatments/replicates, the porosity of the main root was correlated with phellum cross sectional area and main root diameter, so the data for root porosity will be assumed to be indicative of potential root aeration (Fig. 3).

Averaged across all accessions of *M. siculus*, under nonsaline–aerated conditions the main root porosity was ~20% (v/v) (Table 1A). The imposition of hypoxia under nonsaline conditions increased this average by ~5% (v/v) and the imposition of salinity under aerated conditions decreased this average by ~2.5% (v/v). Under saline–hypoxic conditions the porosities of the main root of individual accessions varied between ~18 and 25% (v/v) (Fig. 3) with an average value for the species of ~22%

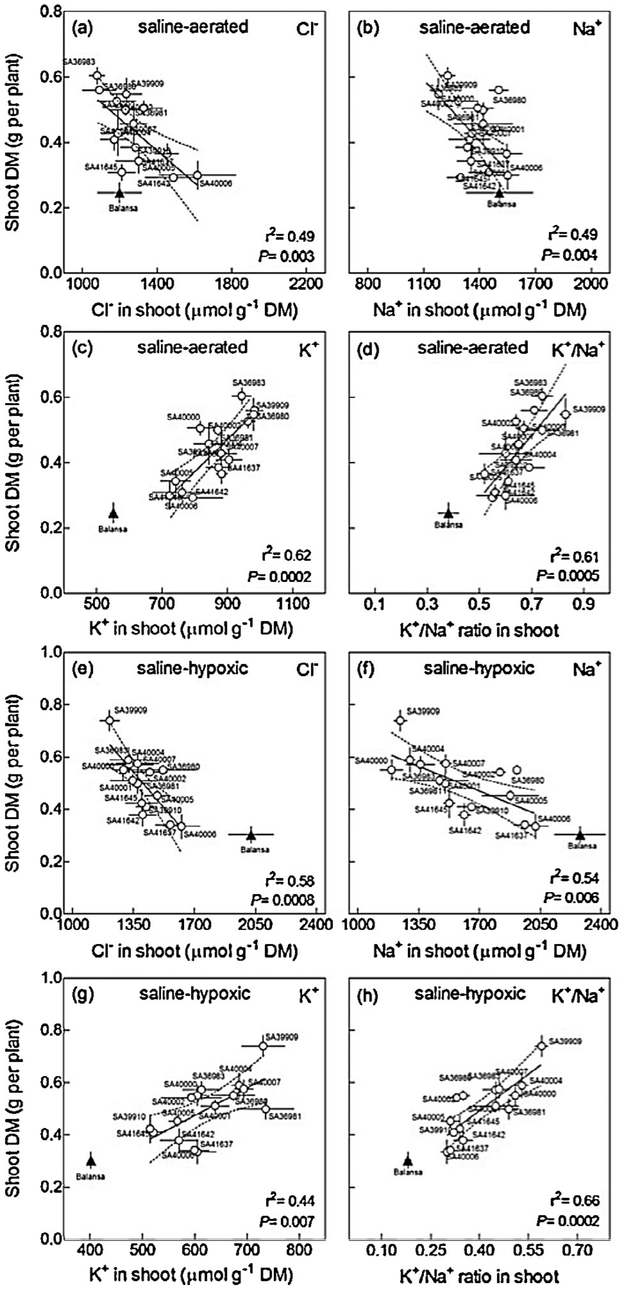

Fig. 2. Relationships between shoot dry mass (DM) and ion concentrations (Cl^-, Na^+, K^+ and K^+/Na^+ ratio) in whole shoots of fifteen accessions of *Melilotus siculus* subjected for 21 days to saline–aerated and saline–hypoxic treatments in nutrient solution (data taken from Fig. 1a and Table S2). *Trifolium michelianum* ('Balansa') is shown with a black triangle for comparison (but was not included in the regressions). Data are means of four replicate pots (four plants per pot) ± SEM.

Table 2
Significance of relationships between ion concentrations and shoot DM, and relationships testing Hypotheses 1 and 2.

Assessed parameters	Variance accounted for	*P*-value	+ or − trend	Source
Ion concentrations in shoot under saline–hypoxic conditions vs shoot DM under saline–hypoxic conditions				
Cl^-	0.56	<0.001	–	Fig. 2e–f
Na^+	0.41	0.006	–	
K^+	0.41	0.006	+	
K^+/Na^+	0.65	<0.001	+	
H1. Porosity of main root under saline–hypoxic conditions vs shoot ion concentrations under saline–hypoxic conditions				
Cl^-	<0.10	NS		Fig. S1[1]
Na^+	<0.10	NS		
K^+	0.14	NS		
K^+/Na^+	<0.02	NS		
H1. Porosity of main root under saline–hypoxic conditions vs plant DM under saline–hypoxic conditions				
Shoot DM	<0.10	NS		Fig. S2
Root DM	<0.10	NS		
H2. Ion concentrations under saline–aerated conditions vs ion concentrations under saline–hypoxic conditions				
Cl^-	0.34	0.014	+	Fig. S3
Na^+	0.30	0.020	+	
K^+	0.23	0.042	+	
K^+/Na^+	0.35	0.011	+	
H2. Shoot DM under saline–aerated conditions vs shoot DM under saline–hypoxic conditions				
	0.56	<0.001	+	Fig. S4

[1] See Supplementary materials.

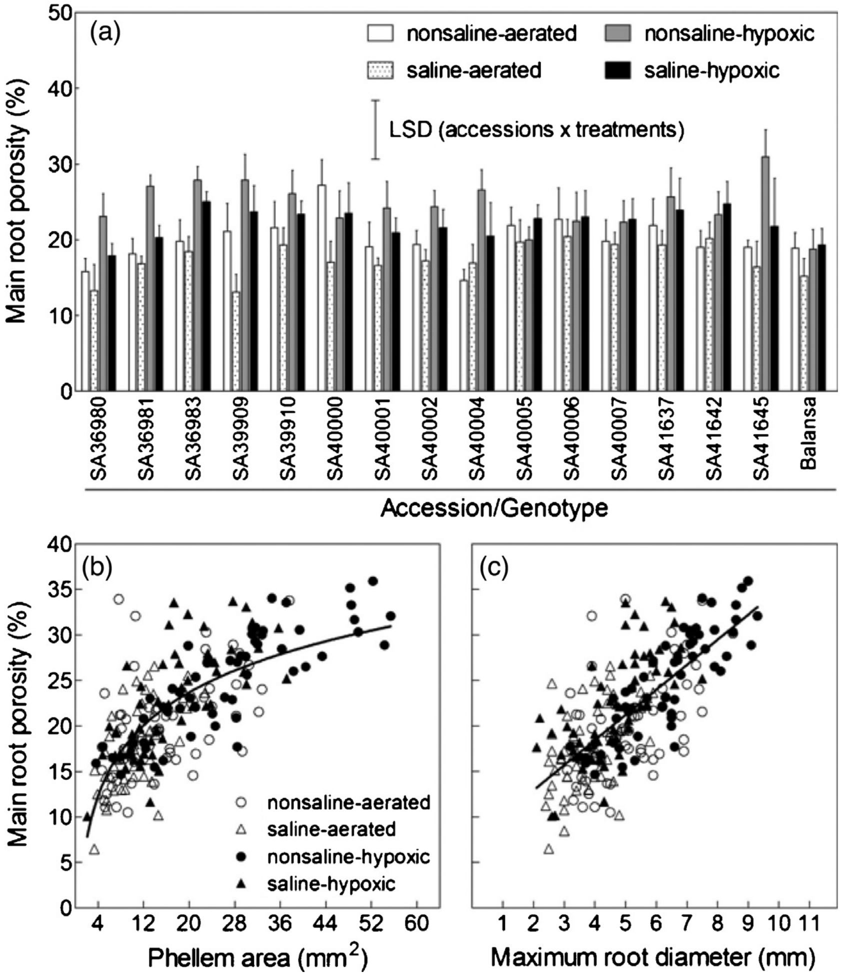

Fig. 3. Root aeration characteristics in *M. siculus*: (a) variation in main root porosity of 15 accessions, (b) relationship between main root porosity and phellem cross sectional area, and (c) relationship between main root porosity and maximum root diameter. *Melilotus siculus* was grown for 21 days with nonsaline–aerated, saline–aerated, nonsaline–hypoxic or saline–hypoxic treatments in nutrient solution. Data for *Trifolium michelianum* ('Balansa') are also shown in part (a) for comparison. Maximum root diameter and cross-sectional area of phellem were not measured for *T. michelianum* as it does not have phellem. In part (a), values are means of four replicate pots with four plants per pot. The error bar indicates the SEM. The LSD is the Fisher's protected least significant difference at $P = 0.05$ (for comparisons of means across accessions and treatments).

(v/v) (Table 1A). In comparison, for *T. michelianum* the porosity of the main root was ~19% under nonsaline–aerated conditions and there was no effect on this of the combination of salinity and hypoxia (Table 1B).

3.5. *Relationships between root porosity and shoot ion concentrations or shoot dry mass*

We conducted a range of correlations to test for relationships between potential for root aeration amongst *M. siculus* accessions shoot ion relations or shoot growth when under saline–hypoxic conditions. However, despite the ~7% (v/v) variation in porosity of the main root between the most porous and least porous accessions, there were no significant relationships between root porosity and average concentrations of Na^+, Cl^- or K^+ in the shoots, or root porosity and average shoot or root DM across the accessions (summarised in Table 2).

3.6. *Nature of the interactions between salinity and hypoxia*

Our data can be used to assess whether, as might be expected from previous literature (see Section 1), there were antagonistic interactions between salinity and hypoxia on shoot or root DM and ion concentrations in the two species (Table 3). Table 3 calculates for the range of measured parameters in both species, the relative effects of : salinity alone (column A), hypoxia alone (column B), the combination of salinity with hypoxia (column C), and the product of columns A and B (column D). The type of interaction can then be derived by comparing columns C and D.

For shoot and root DM, salinity alone had more adverse effects on *T. michelianum* than *M. siculus*, with relative effects of 0.2–0.4 and 0.6, respectively (column A). By contrast, hypoxia alone had little or no adverse effect on both species, with relative effects of 0.8–1.3, respectively (column B). Comparisons of the relative effects of salinity with hypoxia (column C) with the product of these two individual stresses (column D), showed that for both shoot and root DM of both species, column C ≈ column D. Thus, the case for antagonistic interactions between salinity and hypoxia cannot be made based on the average shoot or root DM in either species.

This kind of analysis can also be used to assess the possibility of additive or antagonistic interactions between salinity and hypoxia on ion concentrations in the shoots (Table 3). Antagonistic interactions between salinity and hypoxia on Na^+ and Cl^- in shoots are indicated if the relative concentration under saline–hypoxic conditions (Table 3—column C) is greater than the product of the relative concentrations of each stress individually (Table 3—column D). For *M. siculus*, there were additive interactions between salinity and hypoxia on Na^+ and Cl^-; salinity alone caused large increases in the respective concentrations relative to control (values in column A = 6.9 and 6.4), hypoxia had nearly no effect relative to control (values in column B = 1.0 and 1.1), and both stresses together had relative effects (values in column C = 7.5 and 7.4) that were only 9–11% higher than the product of the relative effects of the stresses separately (values in column D = 6.8). With K^+ there was a slight antagonistic interaction; salinity alone caused large decreases in the concentration relative to control (value in column A = 0.55), hypoxia had nearly no effect relative to control (value in column B = 1.04), and both stresses together had a relative ion concentration (value in column C = 0.40) that was 30% lower than the product of the relative effects of the stresses separately (value in column D = 0.57) (Table 3). By contrast to *M. siculus*, with *T. michelianum* the effects of salinity combined with hypoxia on shoot Cl^- and Na^+ were moderately antagonistic; with both stresses together the respective values in column C were 10.7 and 11.0, which were 70% and 36% higher than the product of the relative effects of the stresses separately (values in column D = 6.3 and 8.1, respectively). For K^+, the two stresses together had a slightly antagonistic interaction, with a column C value of 0.21, which was 20% lower than the product of the relative stresses separately (value in column D = 0.27; Table 3).

4. Discussion

This investigation has shown that under saline–hypoxic conditions, variation in shoot DM amongst 15 accessions of *M. siculus* was negatively correlated with concentrations of Na^+ and Cl^- and positively correlated with concentrations of K^+ in the shoots under these conditions (Fig. 2). Based on analyses of the relationships between ion concentrations, root porosity and shoot DM, we demonstrate for the first time that the variation in ion concentrations under saline–hypoxic conditions was related to variation in the innate ion regulatory capabilities of these accessions (Hypothesis 1), and was not due to differences in root aeration (Hypothesis 2). The discussion below focuses on the nature of the interactions between salinity and hypoxia in *M. siculus* and *T. michelianum*, and the role in these interactions in: (a) variation in innate ability to regulate ions (shoot Cl^-, Na^+ and K^+) in saline conditions, and (b) variation in root porosity for internal aeration.

4.1. *Were there antagonistic interactions between salinity and hypoxia?*

Physiological stresses can potentially interact on plant growth and various traits in antagonistic, additive or synergistic manners (see Section 1). Based on a recent review of the literature (Barrett-Lennard and Shabala, 2013), it was expected that antagonistic interactions between salinity and hypoxia would occur for *M. siculus* and *T. michelianum*, which would be apparent as greater decreases in shoot and root DM with the combined saline and hypoxic treatment than from the product of the responses to theses stresses individually. This would be likely caused by the combination of: (a) more elevated concentrations of Na^+ and Cl^- in shoots with the combined saline and hypoxic treatment than from the product of the stresses individually, and (b) more decreased concentrations of K^+ in shoots with the combined stress than from the product of the stresses individually.

Highly antagonistic interactions did not occur for shoot or root DM in either *M. siculus* or *T. michelianum* (Table 3). For shoot and root DM, the interactions between salinity and hypoxia were additive in both species after 21 days of treatment, i.e. multiplying the relative effects of each stress individually (column D in Table 3) gave approximately the same value as the relative effect of the combination of stresses (column C in Table 3). However, when considering the impact of the interaction on shoot ion concentrations, for *M. siculus* the effects were slightly antagonistic, with values in column C for Cl^- and Na^+ being only 9–11% higher than values in column D, and the value for K^+ in column C being 30% lower than the value in column D. For *T. michelianum*, the effects on Cl^- and Na^+ were more antagonistic with values in column C being 70% and 36% higher, respectively than the equivalent values in column D (calculated from data in Table 3). However, for K^+ the interaction was only slightly antagonistic as the value in column C was only 20% lower than the value in column D (calculated from data in Table 3). It is useful to compare these data with the example of the highly antagonistic interaction in wheat mentioned in Section 1 (Barrett-Lennard et al., 1999). In this case the effects of the interaction on shoot ion concentrations were even more antagonistic than for *M. siculus* or *T. michelianum*; for Cl^- and Na^+ the values in column C were 415% and 78% higher, respectively, than in column D, and for K^+ the value in column C was 34% lower than in column D

Table 3
Comparison of types of interactions between salinity and hypoxia on the dry mass (DM) of roots and shoots, and ion concentrations in shoots, of *M. siculus* and *T. michelianum* (data from this paper) and wheat (data from Experiment 3, Barrett-Lennard et al., 1999). For the *M. siculus* (values are means of 15 accessions) and *T. michelianum* ('Balansa', one genotype), plants were grown for 21 days with combinations of 0 or 200 mM NaCl under aerated or hypoxic (stagnant) conditions. For the wheat, plants were grown for 33 days with combinations of 0 or 60 mM NaCl under aerated or hypoxic (N_2 bubbled) conditions (Barrett-Lennard et al., 1999). Column A shows the effect of salinity alone relative to control (i.e. $\text{Value}_{\text{saline–aerated}}/\text{Value}_{\text{nonsaline–aerated}}$). Column B shows the effect of hypoxia alone relative to control (i.e. $\text{Value}_{\text{nonsaline–hypoxic}}/\text{Value}_{\text{nonsaline–aerated}}$). Column C shows the effect of the combination of salinity with hypoxia relative to control (i.e. $\text{Value}_{\text{saline–hypoxic}}/\text{Value}_{\text{nonsaline–aerated}}$). Column D shows the value of column A × value of column B.

Species	Trait	Ratio of stress value to control value . . .			A × B (D)	Nature of interaction[1]
		. . . for salinity alone (A)	. . . for hypoxia alone (B)	. . .for salinity with hypoxia (C)		
Plant dry mass						
M. siculus	Shoot DM	0.61	1.06	0.70	0.65	Additive
	Root DM	0.58	1.07	0.65	0.62	Additive
T. michelianum	Shoot DM	0.23	0.84	0.28	0.19	Additive
	Root DM	0.40	1.27	0.59	0.50	Additive
Ion relations						
M. siculus	$Cl^-{}_{shoot}$	6.86	0.98	7.50	6.75	Additive
	$Na^+{}_{shoot}$	6.40	1.06	7.41	6.78	Additive
	$K^+{}_{shoot}$	0.55	1.04	0.40	0.57	Slightly antagonistic
T. Michelianum	$Cl^-{}_{shoot}$	6.34	0.99	10.71	6.31	Moderately antagonistic
	$Na^+{}_{shoot}$	7.28	1.11	11.00	8.09	Moderately antagonistic
	$K^+{}_{shoot}$	0.29	0.91	0.21	0.27	Slightly antagonistic
Wheat[2]	$Cl^-{}_{shoot}$	3.55	0.52	9.48	1.84	Highly antagonistic
	$Na^+{}_{shoot}$	8.09	2.59	37.39	20.96	Highly antagonistic
	$K^+{}_{shoot}$	1.15	0.94	0.71	1.08	Highly antagonistic

[1] In each case, interactions are additive if value in column C ≈ value in column D. For shoot and root DM, antagonistic interactions occur if the value in column C ≪ the value in column D. For Na^+ and Cl^- concentrations, antagonistic interactions occur if the value in column C ≫ the value in column D. For K^+ concentrations, antagonistic interactions occur if the value in column C ≪ value in column D. More details are given in Section 4.

[2] Data of Barrett-Lennard et al. (1999).

(calculated from the data of Barrett-Lennard et al., 1999; reproduced for comparative purposes in Table 3).

4.2. Ion relations and variation in innate ability to regulate ions

The case for the variation in growth of *M. siculus* accessions under saline–hypoxic conditions being most affected by variation in the innate ability of the accessions to regulate shoot ions is strongly supported by the range of correlations tested (Table 2) and presumably occurs because of the high waterlogging tolerance of all accessions. Under saline–hypoxic conditions the variation in shoot DM was not only correlated with variation in ion concentrations in shoots under saline–hypoxic conditions, but was also correlated with variation in ion concentrations in shoots under saline–aerated conditions, and with variation in shoot DM under saline–aerated conditions. We therefore conclude that variation in growth amongst the *M. siculus* accessions under saline–hypoxic conditions occurs because of genetic variation in the ability of these accessions to regulate ions, with these differences being just as important under saline–aerated conditions as under saline–hypoxic conditions.

In general, salinity affects the growth of non-halophytes because of immediate adverse effects on plant water relations, and delayed effects due to increases in Na^+ and Cl^-, and decreases in K^+, in the tissues (Greenway and Munns, 1980; Munns, 1993; Shabala and Cuin, 2007; Munns and Tester, 2008). Within *M. siculus* there was impressive variation in shoot DM production amongst accessions under saline–aerated and saline–hypoxic conditions, with the largest accessions having about twice the shoot DM of the smallest accessions. In a review of the importance of K^+ homeostasis in plant adaptation to environmental stress, Anschütz et al. (2014) have suggested that it is the cytosolic ratio of K^+/Na^+ rather than the actual Na^+ concentration that is crucial for saline toxicity. It is therefore interesting that in the present study, variation in shoot DM was clearly associated with differences in ion relations (Fig. 2; Table 2), and that under both saline–aerated and saline–hypoxic conditions, high shoot DM was most strongly correlated with high K^+/Na^+ (Fig. 2), although cytoplasmic K^+/Na^+ would be expected to differ significantly from tissue K^+/Na^+ owing to differences in ion concentrations between vacuoles and cytoplasm (Munns and Tester, 2008). This tissue ion ratio did not appear to be the only factor affecting shoot DM as correlations of the combined data from the saline treatments (saline–aerated and saline hypoxic) shoot DM against shoot K^+/Na^+ resulted in the formation of two parallel lines of the same slope (the Supplementary materials; Fig. S5).

4.3. Impacts of variation in root porosity

One of the themes implicit in the literature on plant adaptation to combined salinity and waterlogging is that enhanced production of aerenchyma (measured here as an increase in root porosity) improves root aeration in a hypoxic medium, enabling plants to maintain ATP production in the roots, thereby maintaining ion regulation and growth. This case was first argued in studies of impaired nutrient uptake under conditions of waterlogging (e.g. Trought and Drew, 1980; Barrett-Lennard et al., 1988) and has been adopted more recently by researchers of the interaction between waterlogging and salinity (e.g. Teakle et al., 2007; Malik et al., 2009; Alamri et al., 2014). While the link between greater aerenchyma formation and better ion regulation seems plausible (reviewed for roots in non-saline hypoxia by Colmer and Greenway, 2011), proving the benefits of increased amounts of aerenchyma as an adaptation to the interaction of salinity and hypoxia requires comparisons of closely related accessions differing in root porosity across the range likely to impact on ATP production. The lack of strongly antagonistic interactions on the shoot growth of both species studied here suggests that the root porosities were not sufficiently low to strongly impair ATP production, altering the functioning of the H^+-ATPases responsible for membrane depolarisation (c.f. Buwalda et al., 1988; Teakle et al., 2013). Over the time frame of this study, the interactions between salinity and hypoxia on Na^+, Cl^- and K^+ in shoots were additive to slightly antagonistic in *M. siculus*.

How did our measurements of root porosity compare with other data from the literature? In a survey of 91 plant species, Justin and Armstrong (1987) found that plants collected from landscapes in which the soil was saturated for most of the year had root porosities

(95% confidence interval) of 10–16%, when grown under drained conditions, and 16–23% when grown under waterlogged conditions. In contrast, plants adapted to well-drained habitats had root porosities of only 1–10%, when grown under drained conditions, and of 4–15% when grown under waterlogged conditions (see calculations by Mullan and Barrett-Lennard, 2010). In the present study, we observed higher average porosities in the main roots of *M. siculus* (18–20% in aerated solutions and 22–25% in hypoxic solutions—Table 1A) than in *T. michelianum* (15–19% in aerated solutions and 19% in hypoxic solutions—Table 1B). In *M. siculus*, but not *T. michelianum*, the aerenchyma in the older parts of the roots occurs in a specialised spongy secondary tissue called phellem and the primary cortex remaining in younger root portions contains a schizogenous-type aerenchyma (Teakle et al., 2011; Verboven et al., 2012). Our measures of the porosity of the main root were likely to have been indicative of total root porosity in *T. michelianum*, but not *M. siculus* as phellem is more prevalent on the main root than lateral roots. However using data from Rogers et al. (2011) we estimate that *M. siculus* would have had an average porosity of the total root system of ~14% under aerated conditions and ~19% under hypoxic conditions.

Given all of this, the likely effects of root porosity can now be summarised as follows. For species like wheat, adapted to well-drained habitats (root porosities of 4–15% under waterlogged conditions—cf. Justin and Armstrong, 1987) we would expect to see highly antagonistic interactions between salinity and hypoxia on growth, particularly at the lower end of this porosity range. By contrast, with species like *M. siculus* and *T. michelianum* adapted to soil saturation for most of the year (root porosities of 16–23% under waterlogged conditions—cf. Justin and Armstrong, 1987) we would expect to see additive interactions between salinity and hypoxia on growth.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2014.08.006.

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