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Variable response of three *Trifolium repens* ecotypes to soil flooding by seawater

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Abstract

- *Background and Aims* Despite concerns about the impact of rising sea-levels and storm surge on coastal ecosystems, there is remarkably little information on the response of terrestrial coastal plant species to seawater inundation. Our aim was to elucidate responses of a glycophyte (*Trifolium repens*) to short duration soil flooding by seawater and recovery following leaching of salts.
- *Methods* Using plants cultivated from parent ecotypes collected from a natural soil salinity gradient; we examined the impact of short-duration seawater soil flooding (8 or 24 hrs) on short-term changes in leaf salt ion and organic solute concentrations, and longer-term impacts on plant growth (stolon elongation), and flowering.
- *Results* There was substantial Cl^- and Na^+ accumulation in leaves, especially for plants subjected to 24 h soil flooding with seawater, but no consistent variation linked to parent plant provenance. Proline and sucrose concentrations also increased in plants following seawater flooding of the soil. Plant growth and flowering were reduced by longer soil immersion times (seawater flooding followed by drainage and freshwater inputs), but plants originating from more saline soil responded less negatively than those from lower salinity soil.
- *Conclusions* The accumulation of proline and sucrose indicates a potential for solute accumulation as a response to the osmotic imbalance caused by salt ions, while variation in growth and flowering responses between ecotypes points to a natural adaptive capacity for tolerance of short-duration seawater soil flooding in *T. repens*. Consequently, we suggest that selection for tolerant ecotypes is

24 possible should the predicted increase in storm surge flooding events frequency
25 unfold.

26 **Key Words –** Flooding; Osmotic stress; Salinity; Saline soil waterlogging; Salt
27 ions; Sea-level rise; Stress metabolites; Storm surge; White Clover

28

Introduction

A combination of sea level rise and the increased likelihood of storm surge events associated with anthropogenic climate change is likely to result in more frequent and severe episodes of salt water inundation into low-lying coastal vegetation (Nicholls and Cazenave, 2010; Martin *et al*., 2011; Zappa *et al*., 2013). Coastal habitats are both economically and ecologically important. Not only do they provide protection against the sea for urban areas and agro-ecosystems in-land (Hanley *et al*., 2014), they offer refuge for many plant and animal species excluded by intensive agriculture (Rhymer *et al*., 2010; Fisher *et al*., 2011). Consequently the ecological response of these coastal habitats to seawater inundation may have important ramifications not only for conservation, but also shoreline management.

The potential impact of storm surge events may be particularly acute for grazing marsh, sand dunes and coastal pasture since unlike salt marshes, these ecosystems are not naturally susceptible to the periodic intrusion of sea water. Salt stress and inundation regimes are known to affect productivity and plant zonation in estuarine marsh/grassland transitions (Guo and Pennings, 2012; Janousek and Mayo, 2013), and also influence plant survival, growth, and reproduction in freshwater macrophytes (Van Zandt and Mopper, 2002; Van Zandt *et al*., 2003; Middleton, 2009; Pathikonda *et al*., 2010). However, our understanding of the impact of salt stress on coastal mesophytes is limited to the effects of sea spray on cliff and strandline vegetation (Malloch *et al*., 1985; de Vos *et al*., 2010; Rogers and Wiser, 2010). This contrasts markedly with a rich literature documenting salinity tolerance

in halophytes (Zhu, 2001; Flowers and Colmer, 2008; Wetson *et al*., 2012) and the impact of salinity on growth and yield of crop species (Munns and Tester, 2008; Flowers *et al*., 2010).

The tolerance of wetland plants to freshwater flooding has been studied extensively (see reviews by Bailey-Serres and Voesenek, 2008; Colmer and Voesenek, 2009), but knowledge of plant response to seawater flooding is limited (Colmer and Flowers, 2008; Bennett *et al*., 2009; Hanley *et al*., 2013) and for the most part focusses on how increased tidal flooding affects halophytes in salt marsh systems (e.g., Janousek and Mayo 2013; Valentim *et al*., 2013). In addition to impeded gas exchange and chemically-reduced compounds in flooded soils (Armstrong, 1979), flooding by seawater also imposes osmotic and ionic stresses; the osmotic stress limits the plant's ability to absorb water and the ionic stress can result in toxicity through the accumulation of Na^+ and Cl^- in tissues (Munns and Tester, 2008). In order to cope with the challenge of salinity the plant must prevent or alleviate damage caused by the high concentration of salt ions (Na^+ and Cl^-) and re-establish homeostatic function (e.g. for K^+). Salt tolerance is often achieved by the accumulation of stress metabolites (e.g. compatible solutes) and the regulation of tissue ions to exclude or compartmentalize the potentially damaging Na^+ and Cl^-, but even if successfully achieved, this can impose a cost on plant growth (Munns and Tester, 2008).

Any change in the frequency and severity of seawater flooding might therefore be expected to influence greatly the vegetation of low-lying coastal ecosystems. Nonetheless, our ability to predict the impact of anthropogenically-induced changes

74 in storm surge scenarios on coastal vegetation is limited by the paucity of
75 information on how component species respond to salt water inundation (Hoggart *et*
76 *al*., 2014; but see Redondo-Gómez *et al*., 2011; Hanley *et al*., 2013). Here we
77 investigate how one common and economically important coastal grassland species,
78 *Trifolium repens* L., responded to simulated short-term seawater inundation of the
79 soil. Although some coastal populations of this species have been shown to be
80 relatively tolerant of salinity, all previous work has exposed plants to a range of salt
81 concentrations imposed for several weeks (Ab-Skukor *et al*., 1988; Rogers *et al*.,
82 1997), rather than looking at the effects of a simulated, short-duration seawater soil
83 flooding event and subsequent recovery. Given the lack of information on plant
84 response to salinity stress following storm surge events, our primary goal was to
85 elucidate how exposure to a relatively short pulse of seawater soil flooding affected
86 plant survival, immediate onward growth, and flowering. In addition, as plant
87 performance and response are likely influenced by the accumulation of salt ions and
88 organic solutes (Munns and Tester, 2008; Flowers and Colmer, 2008), we
89 investigated post-immersion changes in the tissue concentrations of Na^+, Cl^- and K^+
90 and organic solutes (sugars, sugar alcohols, and proline).

91 We also examined whether response to seawater immersion varied for plants
92 cultivated from clonal fragments taken from a natural salinity gradient, since we
93 hypothesised that likely natural adaptation for salt tolerance (Munns and Tester,
94 2008) would influence the response of *T. repens* plants to short-duration soil
95 immersion in seawater. Environmental gradients across even small distances can
96 facilitate the evolution of local ecotypes; i.e., genetically distinct populations

adapted to local environmental conditions (Turesson, 1922). Population-specific variation in morphological or physiological responses to environmental factors such as climate are well-known (Fernández-Pascual *et al*., 2013; Quilot-Turion *et al*., 2013), but our understanding of ecotype-specific variation in response to salinity stress is largely confined to halophytes (Huiskes *et al*., 1985; Blits and Gallagher, 1991 – but see Ab-Skukor *et al*., 1988; Van Zandt *et al*., 2003). Ours is the first study to examine ecotypic variation in response to simulated seawater soil flooding in a terrestrial, coastal glycophyte.

Materials and Methods

Plant species, collection and cultivation

Naturally distributed throughout Europe, North Africa and Asia, *T. repens* has been widely introduced elsewhere as a pasture forage plant (Grime *et al*., 2007). It is also a very common component of coastal grasslands and dune systems likely to be affected by sea-level rise and storm-surge events. In June 2011, we collected parent plants from grassland pasture at South Efford Marsh near Aveton Gifford, Devon, England (50°18'14"N, 3°50'59"W). Plants were collected at locations approximately 25, 200, and 700 m away from the most southerly point of the site. At 25 m, seepage under the sea wall favoured the development of a semi-halophytic community (containing *Puccinellia maritima* and *Spergularia media*) as well as common pasture species like *T. repens* and *Agrostis stolonifera*. At 200 m the plant community was more typical of a terrestrial pasture (dominated by *A. stolonifera* and *Ranunculus repens*), and at 700 m, the sward was dominated by similar species,

but also including common pasture grasses such as *Holcus lanatus* and *Cyanosurus cristatus*. The variation in the plant community with distance from the sea wall suggested a salinity gradient (confirmed by electrical conductivity measurements of the soil - described below), with which to test the hypothesis that natural adaptation for salt tolerance would influence plant response to short-duration soil immersion in seawater.

In mid-June 2011 we sampled 12 individual plants per distance, collecting large, branched (circa 100 mm diameter) fragments with multiple rooting points. All parent plants were at least 5 m apart and taken from distinct patches to reduce the likelihood of collecting from the same individual (Ab-Shukor *et al*., 1988). The plant fragments were transplanted into 110 × 110 × 120 mm plastic pots containing John Innes No. 2 potting compost and cultivated in a sheltered outdoor area. Three soil cores (10 cm depth, 5 cm diameter) per distance were also collected at random intervals to quantify soil electrical conductivity. Three 20 g subsamples were taken from each core and mixed with 100 ml deionised water on a rotational mixer to provide a 1:5 soil:water extract (British Standards Institution, 1997). An electrical conductivity reading was then obtained for each subsample using a WTW Cond 330i handheld conductivity meter with a WTW TetraCon 325 probe (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). One-way ANOVA was used to determine variation in soil electrical conductivity from these samples.

Experimental treatments

In November 2011 we removed 10 stolons (about 20 mm long with 1 root node) from each of 12 parent 'stock' plants per distance group and individually transplanted these into 70 x 70 x 80 mm pots containing John Innes No. 2 potting compost. Plants were subsequently grown in a naturally lit, heated greenhouse in Plymouth, England (mean daily temperatures varying between 10.7 ^{o}C (± 0.2) min and 26.3 ^{o}C (± 0.6) max, with daily watering with tap water), until late April 2012.

On April 24th 2012, established ramets were selected such that each of 12 parent 'stock' plants was represented by up to 6 clones, each uniform in size and appearance. Clones were allocated to a different treatment group such that we aimed to subject two ramets from each parent 'stock' plant to the same soil immersion treatment with seawater collected from Plymouth Sound (electrical conductivity = 47.9 mS/cm at 23.8 ^{o}C) for 8, or 24 h, or were retained as untreated controls. This arrangement ensured that genetic variation across all soil immersion treatments was minimised within all distance classes. However, due to some plants failing to establish, replication fell below the target of 6 clones per parent for the 200-m and 700-m distance groups, affecting the number of replicates used in the onward growth and flowering experiment (see below). By immersing to pot-level (in large plastic tubs) we simulated short-term soil waterlogging. Although we recognise that seawater inundation following a storm-surge event would potentially also result in shoot submergence, our approach allowed us to focus on the effect of ionic imbalance in the root-zone rather than the combined effects that could also arise if shoots experienced oxygen deficiency by submergence. In addition, while seawater flooding following storm surge events might also be expected to persist longer than

24 h, records from along the UK coast suggest a 1-d long seawater flooding event is typical (Environment Agency 2014).

Immediately after immersion in seawater, pots were allowed to fully drain before being arranged randomly on a wire mesh-topped bench inside the greenhouse; the wire mesh allowed free drainage and prevented cross-contamination of any leachates between pots.

Tissue ion and metabolite analyses

One fully expanded, non-senescent leaf was removed from each of 12 individual clones per ecotype per immersion treatment 2 d after soil immersion. These samples were frozen in liquid nitrogen and freeze-dried. Samples were then stored in sealed containers with desiccant in a freezer at -80 °C until analysis. These plants were then discarded.

Dried samples were digested in 5 mL dilute (0.5 M) nitric acid while suspended on a shaker in dark conditions for 2-d at room temperature prior to determination by flame photometry of Na^+ and K^+ and by chloridometry of Cl^- (Munns et al., 2010). This procedure was applied to 4 replicate leaf samples per soil immersion treatment/ecotype group (minimum sample mass = 26.7 mg). A reference tissue taken through the procedures confirmed the reliability of these measurements.

The remaining leaf samples were bulked (by adding two samples together) such that 4 replicate samples were created for each soil immersion treatment/ecotype group (minimum sample mass = 54.3 mg). Tissue metabolites were extracted from these

using 5% (w/v) perchloric acid and neutralised extracts were analysed using HPLC (Fan *et al.* 1993). Neutralised extracts were filtered (0.22 µm) and stored at -80 ºC. The initial HPLC analysis of organic solutes (glycinebetaine, proline, prolinebetaine and trigonelline), soluble sugars (fructose, glucose, sucrose) and sugar alcohols (sorbitol, mannitol and pinitol) was adapted from Slimestad and Vågen (2006). The HPLC system (Waters, Milford, MA) consisted of a 600E pump, 717plus autosampler and a 996 photo-diode array detector (PDA). As detection of fructose, glucose and sucrose with the PDA at 195 nm is very insensitive, an Alltech (Deerfield, IL, USA) evaporative light scattering detector (ELSD) was also used to improve sensitivity by minimum 100 fold. Separation was achieved at 22 ± 1.0 ºC on a Prevail ES Carbohydrate column (250 x 4.6 mm i.d. with 5 µm packing; Alltech) using a gradient elution profile of acetonitrile (Eluent A) and water (Eluent B) at 1 ml min^{-1}. Samples in the autoinjector were held at 10 ºC, the ELSD drift tube held at 85 ºC and eluent nebulisation with high purity N_2 gas at a flow rate of 2.6 l min^{-1}.

Quantification was based on PDA peak area for organic solutes, and ELSD peak area for soluble sugars and sugar alcohols. Calibration curves were generated from peak area versus the mass of standard analyte injected, with linear relationship for the PDA and a power relationship for the ELSD output. A standard was analysed every 10 samples to check for any instrument/detector drift. Retention times of standards were used to identify analytes in the sample extracts with the PDA spectral data and peak purity used to confirm organic solutes. Typical sample

injections were 20 µl and runtime was 20 min per sample with Empower™ 2 software (Waters) used for data acquisition and processing.

Due to the fact that pinitol co-eluted with fructose, a second HPLC approach was needed to separate and quantify these two analytes and the method described by Naidu (1998) was followed. The Sugar-Pak column (300 × 6.5 mm i.d.) was held at 90 ± 0.5 ºC and separation achieved using a mobile phase of 2.5 mg l^{-1} Ca-EDTA at 0.6 ml min^{-1}. Detection and quantification of pinitol was undertaken with the PDA as this offered good sensitivity as well as peak spectral and purity comparisons with the standards.

Following application of the Cochran test for homogeneity of variance, and (log_N) data transformation where necessary (Underwood, 1997), the effect of immersion on tissue ion and solute concentrations was examined using a two-way ANOVA with 'Immersion Time' and 'Distance' as factors, with the tests for an 'Immersion Time' × 'Distance' interaction included to examine any evidence for distance-specific variation in response to immersion duration.

Plant survival, growth and flowering

Plants not used for chemical analysis were watered to capacity 48 h after salt water immersion (using tap water), and then every two days thereafter for a further 70 d (5^{th} July 2012). Greenhouse air temperatures during this phase of the experiment were: 11.3 ^{o}C (± 0.3) min and 29.9 ^{o}C (± 0.9) max. Due to some clones failing to establish before immersion, replication fell below the target of 12 plants for the 200-

m (0-hr = 9 plants, 8-hr = 9 plants, 24-hr = 8 plants) and 700-m (24-hr = 11 plants) distance groups.

The number of inflorescences on each plant were monitored weekly, so that the total number of inflorescences produced per plant ('Flowering Effort') and the percentage of plants that produced open inflorescences ('Percentage Flowering') could be quantified at the end of the experiment. Elongation of a representative, randomly selected shoot on each plant, measured from a node marked with loosely tied cotton thread, was quantified at 14 days and 42 days ('Stolon Growth'). Plant mortality was recorded daily.

Subsequently, the effects of 'Immersion Duration' and 'Distance' on 'Flowering Effort' were examined using a two-way ANOVA with 'Immersion Duration' and 'Distance' as factors, with the tests for an 'Immersion Duration' × 'Distance' interaction included to examine any evidence for distance-specific variation in response to immersion duration. The effects of 'Immersion Duration' and 'Distance' on 'Stolon Growth' at 14 d were analysed in the same way. Data transformation of the 42 d 'Stolon Growth' data could not avoid deviations from normality and homogeneity of variance; therefore Kruskal Wallis tests were applied. Pearson's chi-squared test was performed on 'Percentage Flowering' data to assess the effects of immersion within 'Distance' groups, where assumptions of the statistical test were met.

In order to quantify the leaching of salts from the potting compost, four pots were randomly chosen from each soil immersion treatment and water passing through collected and electrical conductivity measured as described above for 5 weeks.

Results

Electrical conductivity of soil samples from transects

One-way ANOVA indicated significant variation in soil electrical conductivity (of 1:5 soil:water extract, $EC_{1:5}$) between sample sites ($F_{(2,27)}$ = 39.61, P <0.01). Post-hoc SNK comparisons revealed that soil electrical conductivity declined with distance from the sea wall embankment, with a mean $EC_{1:5}$ of 6.96 (± 1.35 SE) mS/cm at 25 m, 1.29 (± 0.40 SE) mS/cm at 200 m and 0.19 (± 0.02 SE) mS/cm at 700 m. We therefore conclude that soil salt concentrations were higher at 25 m due to likely sea water seepage through the sea wall and/or deposition of salts from sea spray.

Accumulation in leaves of salt ions and stress metabolites

There was substantial accumulation of Cl^- and Na^+ in leaves following soil immersion in sea water (Table 1). Post-hoc SNK tests showed that leaf tissue Cl^- concentrations significantly increased following 24 h sea water immersion compared with both control and 8 h treatments, irrespective of original parental distance from the sea wall. There was also a significant increase in Cl^- concentrations in the 8 h treatments compared with control plants. A similar effect of 'Immersion Time' was evident for Na^+ although there were also additional 'Distance' and 'Immersion

Time' × 'Distance' effects. However, SNK tests indicated that these were a manifestation of relatively high leaf Na^+ levels in 200 m control plants, and generally relatively low leaf Na^+ levels in 25 m plants following soil immersion. There was no effect of 'Immersion Time' on K^+ concentrations; the significant 'Distance' effect denoted the inherently higher leaf K^+ concentrations in plants cultivated from the 200 m parent population.

We found detectable amounts of the amino acid proline, the sugars fructose, glucose and sucrose, and the sugar alcohol pinitol, in leaf extracts (Table 2). Of these, proline and sucrose increased in all distance groups following soil immersion, but no other treatment-specific trends were located for organic solutes.

Plant survival, growth, and flowering

Water collected from control plants remained at an average electrical conductivity (EC_w) of 2.37 ± 0.03 mS/cm. Leachates from pots immersed for 8 and 24 h recorded similar levels of EC_w 2 d post-immersion (12.53 ± 3.28 mS/cm and 11.05 ± 5.61 mS/cm respectively) and declined at a similar rate until 35 d. At this point, stabilisation of EC_w occurred in both treatments (Fig.1).

Only four plants died during the experiment, these were all in the 24 h sea water soil immersion treatment; three from the 700 m ecotype and one from the 200 m ecotype. All plants from the 25 m ecotype survived 24 h soil immersion. At 14 d after the short-durations of soil flooding, 'Stolon Growth' was greatly reduced by 'Immersion' for all distance groups ($F_{(2,91)} = 50.25$, $P < 0.01$), with post-hoc SNK tests indicating the greatest reduction after 24 h soil immersion treatment ($P < 0.01$)

290 (Fig. 2a). 'Distance' also had a significant effect on 'Stolon Growth' ($F_{(2,91)}$ = 3.40,
291 P <0.05); likely due to a generally greater stem growth of 200 m plants and much
292 reduced growth of 700 m plants following soil immersion. There was no interaction
293 between 'Immersion' and 'Distance' ($F_{(4,89)}$ = 1.78, P > 0.05). The loss of cotton
294 thread markers used to identify stolons reduced effective replicate number (see
295 Figure 2b) at 42 d, nevertheless the 'Immersion' effect remained apparent ($H_{(2)}$ =
296 35.22, P <0.01), but 'Distance' had no significant effect on 'Stolon Growth', as
297 average growth of 25 m plants increased to similar rates as 200 m ($H_{(2)}$ = 2.15, P >
298 0.05). However, average stolon growth of 700 m plants appeared to be much
299 reduced even at 42 d after the longer duration (24 h) of soil immersion (Fig. 2b).

300 While both 'Immersion' ($F_{(2,92)}$ = 14.18, P <0.01), and 'Distance' ($F_{(2,92)}$ = 6.12, P
301 <0.01) had a significant effect on 'Flowering Effort' (Fig. 3), there was no
302 interaction between 'Immersion' and 'Distance' ($F_{(4,90)}$ = 0.69, P >0.05). Post-hoc
303 SNK tests revealed that the mean number of inflorescences per plant ('Flowering
304 Effort') produced by the end of the experiment were reduced in both the 8 and 24 h
305 soil immersion treatments (P < 0.01). The significant 'Distance' effect could be
306 explained by greater number of inflorescences produced by the 200 m and 700 m
307 control plants compared with the 25 m control plants, suggesting that these
308 populations naturally produce more flowers. However, 'Flowering Effort' in the 200
309 m and 700 m plants was nonetheless greatly reduced by 24 h soil immersion. Similar
310 patterns were observed for 'Percentage Flowering' (Fig. 4); 700 m plants produced
311 more open flowers than plants in the groups collected at 200 m and 25 m. Pearson's
312 chi-squared test revealed that 'Percentage Flowering' in the 25-m plants was

313 reduced by soil immersion ($X^2_{(36,2)} = 16.94$, $P < 0.01$). Although the same test could
314 not be performed on 200 m and 700 m plants (over 50% of cells in the test matrix
315 had expected values of less than 5), there was a trend for reduced flowering in both
316 these distance groups following soil immersion.

317 ## Discussion

318 Our experiment to simulate the effects of transient saline soil waterlogging yielded
319 consistent ecophysiological and plant performance responses; two days after soil
320 immersion, all plants exhibited higher leaf concentrations of Na^+ and Cl^-,
321 concentrations of some organic solutes significantly changed (e.g. sucrose and
322 proline increased), and longer-term plant growth and flowering potential were
323 reduced. The fact that growth and flowering responses also varied according to the
324 location of the parent plants along a natural salinity gradient, suggests that our study
325 plants were displaying natural ecotypic variation to the short-duration salinity stress.

326 The accumulation of Na^+ and Cl^- in leaf tissues is commonly observed in salt-
327 stressed plants which in-turn leads to the accumulation of organic solutes to counter
328 the resulting osmotic imbalance (Wyn Jones and Gorham, 2002; Flowers and
329 Colmer, 2008). It was interesting to note, however, that despite having different
330 growth and flowering responses to seawater soil immersion, the accumulation of
331 Na^+ and Cl^- in leaf tissues did not vary for plants from different locations along the
332 natural salinity gradient. Unlike Rogers *et al*. (1997) who documented that tolerance
333 of *T. repens* to longer exposures to salinity was associated with 'exclusion' of Cl^-
334 from the shoot, the apparent salinity tolerance of plants we collected from the 25 m

population could not be linked to differences in leaf ion concentrations (the trend in the present study for lower leaf Na^+ and Cl^- in ecotypes collected from closer to the sea wall was not statistically significant - Table 1). Moreover, in addition to regulation of leaf ion concentrations, other traits such as differences in "tissue tolerance" can also influence overall plant salinity tolerance (Munns and Tester, 2008). Maintenance of tissue K^+ concentration, which can decrease in plants exposed to excess Na^+, to retain a favourable K^+/Na^+ ratio of the cytoplasm for enzyme functioning, is also of importance for salt tolerance (Maathuis and Amtmann, 1999), but for the short-term salinity exposures in the present experiment on *T. repens* there was no significant treatment effect on leaf K^+ (Table 1). In addition to regulation of leaf ion concentrations, organic solutes that are compatible with enzymes and therefore accumulated in the cytoplasm are also of importance for adaptation to salinity (Munns and Tester, 2008; Flowers and Colmer, 2008). Organic solutes in plants can include various sugars, sugar alcohols, betaines, and amino acids, many of which accumulate with increasing plant exposure to NaCl, but the types and concentrations vary in different species (Flowers and Colmer, 2008). *T. repens* leaves contained constitutively high concentrations of pinitol, whereas proline and sucrose concentrations rose dramatically in the salt-stressed plants (Table 2). The increased organic solute accumulation is likely a response to provide the osmotic balance needed in the cytoplasm for Na^+ and Cl^- accumulation in the vacuole.

The reduction in plant growth reported here mirrors that of plants in salinized agricultural systems (Bennett *et al*., 2009; Flowers *et al*., 2010). Simulated seawater

358 soil flooding not only reduced vegetative growth, measured here for *T. repens* as
359 stolon growth, but had the added effect of causing reduced flowering potential.
360 Exposure of the roots to seawater is expected to exert a negative effect on plant
361 growth and reproductive allocation via ionic and osmotic stresses; salt stress impacts
362 on cell division and photosynthesis as an osmotic imbalance leads to stomatal
363 closure and reduced CO_2 uptake and so less photosynthesis (Zhu, 2001). Therefore,
364 greater stress will be exerted on plants enduring longer root immersion as Na^+ and
365 Cl^- ions have more time to accumulate in leaf tissues (as was observed here for all
366 plants subject to 24 h soil immersion when compared to the 8 h treatment). This is
367 reflected by the greatest reduction in stolon growth and flowering potential of *T.*
368 *repens* occurring as a result of 24 h seawater soil immersion despite the fact that salt
369 levels in the growing media were roughly comparable for 8 and 24 h treatments after
370 removal from seawater and subsequent leaching due to watering.

371 Growth and flowering responses to seawater soil immersion also varied according to
372 the location of the parent plant along a natural salinity gradient. After 24 h of
373 seawater soil immersion, stolon growth of 700 m plants was severely limited, while
374 25 m and 200 m plants were less affected and seemed to return to similar rates of
375 growth by 42 d. Similarly, although 'Flowering Effort' and the percentage of plants
376 with flowering inflorescences were reduced for all distances, the effects were
377 particularly marked for 700 m plants. Ecotype-specific variation in flowering as a
378 consequence of long-term salinity exposure is well known for halophytes like *Aster*
379 *tripolium* (Gray *et al*., 1979) and *Sporobolus virginicus* (Blits and Gallagher, 1991),
380 but ours is this first study to demonstrate this effect for a glycophyte from a natural

381 salinity gradient when exposed to short-duration seawater soil flooding. Our results
382 indicate that the 25 m- and to some degree the 200 m-ecotype, were resilient to
383 transient seawater soil immersion presumably as a consequence of a local population
384 adaptation to the higher soil salinity near to the sea wall embankment. Interestingly,
385 a trade-off between salinity tolerance and reproductive allocation might be evident,
386 as flowering potential in the 25 m ecotype was lower than in the 700 m population.

387 Plant adaptation to salinity has been shown for species in many hypersaline semi-
388 arid/arid areas (Munns and Tester, 2008), but such local adaptation also occurs in
389 coastal plant populations exposed to salt spray and long-term accumulation of salt
390 ions in the soil (Lowry *et al*., 2009), including *T. repens* (Ab-Shukor *et al*., 1988;
391 Rogers *et al*., 1997). Our study is considerably different, however, in that we applied
392 a one-off, short-term, salinity pulse via soil flooding in an attempt to mirror seawater
393 inundation in a storm surge event. Consequently, our plants experienced an
394 immediate salinity shock followed by a gradual reduction of salt ions around the
395 roots as freshwater diluted and leached salt from the potting media. In flooded
396 ecosystems, the dissipation of salt ions over time may also allow plants to recover
397 from the initial impact of ionic and osmotic stress, an effect not only likely to vary
398 between plant species and ecotypes, but also with season and local weather
399 conditions. The effects of seasonality and species identity remain to be elucidated,
400 but we demonstrate here population-level variation in plant response to seawater soil
401 flooding. Although ecotype-specific response to salinity stress has been
402 demonstrated at the regional scale for *T. repens* (Ab-Shukor *et al*., 1988), our study
403 evidences the presence of different salinity-tolerant ecotypes over relatively small

404 distances within a single coastal site. If salinity-tolerant ecotypes are a widely-
405 distributed feature of coastal plant species, supra-littoral vegetation may be naturally
406 buffered to some extent against predicted sea-level rise and increased incidence of
407 storm-surge events over coming decades. The existence of flooding-tolerant
408 ecotypes may also be important in developing salt-tolerant cultivars for use in saline
409 soils (Ab-Shukor *et al*., 1988; Rogers *et al*., 1997; Bennett *et al*., 2009), and any
410 coastal pastures susceptible to future episodes of seawater flooding.

411 Given the increased likelihood of storm surge events over coming decades (Martin
412 *et al.*, 2011; Zappa *et al.*, 2013), the international conservation importance of low-
413 lying coastal vegetation and the role these areas play in coastal defence (Rhymer *et*
414 *al*., 2010; Fisher *et al*., 2011; Hanley *et al*., 2014), a more detailed understanding of
415 the structural and functional responses of coastal vegetation to periodic seawater
416 flooding is particularly pressing. We demonstrate here that one common plant
417 species component of many coastal grasslands and dune systems, *T. repens*,
418 responds poorly to simulated seawater soil flooding, but that the response may be
419 population-, i.e. ecotype-, specific and that the species consequently has an adaptive
420 capacity to withstand short periods of soil inundation by seawater. Whether the
421 likely increased selection pressure for plants resistant to seawater flooding impacts
422 on other plant traits (e.g. growth, N-fixing capacity, anti-herbivore defence,
423 reproductive potential) and thus the ecological role and economic value of this
424 species is worthy of future attention. In addition, the impact of short-duration
425 seawater flooding on multi-species mixtures and subsequent community assembly
426 and function could yield many useful insights into the likely responses of coastal

427 vegetation to rising sea-levels and the anticipated increased frequency and severity
428 of saline flooding events.

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435 Literature Cited

436 **Ab-Shukor NA, Kay QON, Stevens DP, Skibinski DOF. 1988.** Salt tolerance in
437 natural populations of *Trifolium repens* L.. *New Phytologist* **109**: 483-490.
438 **Armstrong W. 1979.** Aeration in higher plants. *Advances in Botanical Research* **7**:
439 225–332.
440 **Bailey-Serres J, Voesenek LACJ. 2008.** Flooding stress: acclimations and genetic
441 diversity. *Annual Reviews in Plant Biology* **59**: 313-339.
442 **Bennett SJ, Barrett-Lennard EG, Colmer TD. 2009.** Salinity and waterlogging as
443 constraints to saltland pasture production: a review. *Agriculture, Ecosystems*
444 *and Environment* **129**: 349-360.
445 **Blits KC, Gallagher JL. 1991.** Morphological and physiological responses to
446 increased salinity in marsh and dune ecotypes of *Sporobolus virginicus* (L)
447 Kunth. *Oecologia* 87: 330–335.

448 **British Standards Institution 1997.** *Part 3: Chemical methods*. British Standard
449 7755, British Standards Institution, London, UK.
450 **Colmer TD, Flowers TJ. 2008.** Flooding tolerance in halophytes. *New Phytologist*
451 **179**: 964-974.
452 **Colmer TD, Voesenek LACJ. 2009.** Flooding tolerance: suites of plant traits in
453 variable environments. *Functional Plant Biology* **36**: 665-681.
454 **de Vos AC, Broekman R, Groot MP, Rozema J. 2010.** Ecophysiological response
455 of *Crambe maritima* to airborne and soil-borne salinity. *Annals of Botany*
456 **105**: 925-938.
457 **Environment Agency UK 2014.** DataShare. Available from
458 http://www.geostore.com/environment-agency/. Accessed 4th April 2014.
459 **Fan TWM, Colmer TD, Lane AN, Higashi RM. 1993.** Determination of
460 metabolites by ^{1}H NMR and GC: analysis for organic osmolytes in crude
461 tissue extracts. *Annals of Biochemistry* **214**: 260–271.
462 **Fernández-Pascual E, Jiménez-Alfaro B, Caujapé-Castells J, Jaén-Molina R,**
463 **Díaz TE. 2013**. A local dormancy cline is related to the seed maturation
464 environment, population genetic composition and climate. *Annals of Botany*
465 **112**: 937–945.
466 **Fisher B, Bradbury RB, Andrews JE, *et al*. 2011**. Impacts of species-led
467 conservation on ecosystem services of wetlands: understanding co-benefits
468 and tradeoffs. *Biodiversity Conservation* **20**: 2461-2481.
469 **Flowers TJ, Colmer TD. 2008.** Salinity tolerance in halophytes. *New Phytologist*
470 **179**: 945-963.

471 **Flowers TJ, Gaur PM, Gowda CLL, Krishnamurthy L, Samineni S, Siddique**
472 **KHM, Turner NC, Vadez V, Varshney RK, Colmer TD. 2010.** Salt
473 sensitivity in chickpea. *Plant, Cell and Environment* **33**: 490–509.
474 **Gray AJ, Parsell RJ, Scott R. 1979.** The genetic structure of plant populations in
475 relation to the development of salt marshes. In: Jefferies RL, Davy AJ, eds.
476 *Ecological Processes in Coastal Environments.* Oxford: Blackwell, 43–64.
477 **Grime JP, Hodgson JG, Hunt R. 2007.** *Comparative Plant Ecology* 2nd edn.
478 Castlepoint Press, Dalbeattie, UK.
479 **Guo HY, Pennings SC. 2012.** Mechanisms mediating plant distributions across
480 estuarine landscapes in a low-latitude tidal estuary. *Ecology* **93**: 90-100.
481 **Hanley ME, Hoggart SPG, Simmonds DJ, *et al*. 2014.** Shifting sands? Coastal
482 protection by sand banks, beaches, and dunes. *Coastal Engineering* **87**: 136-
483 146.
484 **Hanley ME, Yip PYS, Hoggart SPG, Bilton DT, Rundle SD, Thompson RC.**
485 **2013.** Riding the storm: The response of *Plantago lanceolata* to simulated
486 tidal flooding. *Journal of Coastal Conservation* **17**: 799-803.
487 **Hoggart SPG, Hanley ME, Parker DJ, et al., 2014** The consequences of doing
488 nothing: The effects of seawater flooding on coastal zones. *Coastal*
489 *Engineering* **87**: 169-182.
490 **Huiskes AHL, van Soelen J, Markusse MM. 1985.** Field studies on the variability
491 of populations of *Aster tripolium* L. in relation to salt marsh zonation.
492 *Vegetatio* **61:** 163–169.

493 **Janousek CN, Mayo C. 2013.** Plant responses to increased inundation and salt
494 exposure: interactive effects on tidal marsh productivity. *Plant Ecology* **214**:
495 917-928.
496 **Lowry DB, Hall MC, Salt DE, Willis JH. 2009.** Genetic and physiological basis of
497 adaptive salt tolerance divergence between coastal and inland *Mimulus*
498 *guttatus*. *New Phytologist* **183**: 776-788.
499 **Malloch AJC, Bamidele JF, Scott AM. 1985.** The phytosociology of British sea-
500 cliff vegetation with special reference to the ecophysiology of some maritime
501 cliff plants. *Vegetatio* 62: 309-317.
502 **Martin J, Fackler PL, Nichols JD, *et al.* 2011.** Structured decision making as a
503 proactive approach to dealing with sea level rise in Florida. *Climatic Change*
504 **107**: 185-202.
505 **Maathuis FJM, Amtmann A. 1999.** K^+ nutrition and Na^+ toxicity: the basis of
506 cellular K^+/Na^+ ratios. *Annals of Botany* **84**: 123–133.
507 **Middleton EA. 2009**. Regeneration of coastal marsh vegetation impacted by
508 hurricanes Katrina and Rita. *Wetlands* **29**: 54-65.
509 **Munns R, Tester M. 2008.** Mechanisms of salt tolerance. *Annual Review of Plant*
510 *Biology* **59**: 651-681.
511 **Munns R, Wallace PA, Teakle NL, Colmer TD. 2010.** Measuring soluble ion
512 concentrations (Na^+, K^+, Cl^-) in salt-treated plants, in Sukar, R. (Ed.), *Plant*
513 *Stress Tolerance*. Methods in Molecular Biology 639 Springer, Berlin, pp
514 371- 382.

Naidu BP. 1998. Separation of sugars, polyols, proline analogues, and betaines in stressed plant extracts by high performance liquid chromatography and quantification by ultra violet detection. *Australian Journal Plant Physiology* **25**: 793–800.

Nicholls RJ, Cazenave A. 2010. Sea-level rise and its impact on coastal zones. *Science* **328**: 1517–1520.

Pathikonda S, Meerow A, He ZX, Mopper S. 2010. Salinity tolerance and genetic variability in freshwater and brackish *Iris hexagona* colonies. *American Journal of Botany* **97**: 1438-1443.

Quilot-Turion B, Leppälä J, Leinonen PH, *et al*. 2013. Genetic changes in flowering and morphology in response to adaptation to a high-latitude environment in *Arabidopsis lyrata. Annals of Botany* **111**: 957–968.

Redondo-Gómez S, Andrades-Moreno L, Parra R, Mateos-Naranjo E, Sánchez-Lafuente AM. 2011. Factors influencing seed germination of *Cyperus capitatus*, inhabiting the moving sand dunes in southern Europe. *Journal of Arid Environments* **75**: 309-312.

Rogers GM, Wiser SK. 2010. Environment, composition and conservation of coastal turfs of mainland New Zealand. *New Zealand Journal of Botany* **48**: 1-14.

Rogers ME, Noble CL, Halloran GM, Nicolas ME. 1997. Selecting for salt tolerance in white clover (*Trifolium repens*): chloride ion exclusion and its heritability. *New Phytologist* **135**: 645-654.

Rhymer CM, Robinson RA, Smart J, Whittingham MJ. 2010. Can ecosystem services be integrated with conservation? A case study of breeding waders on grassland. *Ibis* **152**: 698–712.

Slimestad R, Vågen IM. 2006. Thermal stability of glucose and other sugar aldoses in normal phase high performance liquid chromatography. *Journal of Chromatography A* **1118**: 281-284.

Turesson G. 1922. The genotypical response of the plant species to the habitat. *Hereditas* **3**: 211–350.

Underwood AJ. 1997. *Experiments in Ecology*. Cambridge University Press, Cambridge, UK.

Valentim JM, Vaz N, Silva H, Duarte B, Caçador I, Dias JM. 2013. Tagus estuary and Ria de Aveiro Salt marsh dynamics and the impact of sea level rise. *Estuarine, Coastal and Shelf Science* **130**: 138-151.

Van Zandt PA, Mopper S. 2002. Delayed and carry-over effects of salinity on flowering in *Iris hexagona* (Iridaceae). *American Journal of Botany* **89**: 1847–1851.

Van Zandt PA, Tobler MA, Mouton E, Hasenstein KH, Mopper S. 2003. Positive and negative consequences of salinity stress for the growth and reproduction of the clonal plant, *Iris hexagona*. *Journal of Ecology* **91**: 837–846.

Wetson AM, Zoerb C, John EA, Flowers TJ. 2012. High phenotypic plasticity of *Suaeda maritima* observed under hypoxic conditions in relation to its physiological basis. *Annals of Botany* **109**: 1027-1036.

Wyn Jones G, Gorham J. 2002. Intra- and inter-celluar compartments of ions, in Läuchli, A., Lüttge, U. (Eds.), *Salinity: environment-plant-molecules*, Kluwer, Dordrecht, the Netherlands. pp. 159-180.

Zappa G, Shaffrey LC, Hodges KI, Sansom PG, Stephenson DB. 2013. A multi-model assessment of future projections of North Atlantic and European extratropical cyclones in the CMIP5 climate models. *Journal of Climate* **26**: 5846–5862.

Zhu J-K. 2001. Plant salt tolerance. *Trends in Plant Science* **6**: 66-71.

Distance	Treatment	N	Chloride (µmol g^{-1} DM)		Potassium (µmol g^{-1} DM)		Sodium (µmol g^{-1} DM)	
			Mean	SE	Mean	SE	Mean	SE
25 m	Control	4	134^{A}	12	476	41	216^{A}	18
	8 h	4	966^{B}	47	383	82	663^{B}	46
	24 h	4	1261^{C}	233	502	81	1049^{C}	129
200 m	Control	4	138^{A}	16	797	30	273^{A}	29
	8 h	4	1025^{B}	77	712	112	929^{B}	34
	24 h	4	1598^{C}	67	693	76	1126^{C}	30
700 m	Control	4	110^{A}	17	466	66	202^{A}	28
	8 h	4	1047^{B}	36	457	66	814^{B}	39
	24 h	4	1805^{C}	59	518	20	1454^{C}	49
Results of two-way ANOVA	Factor		*F*	*P*	*F*	*P*	*F*	*P*
	Distance (DF = 2,27)		1.1	0.358	14.9	**0.000**	5.0	**0.014**
	Immersion time (DF = 2,27)		471.0	**0.000**	0.7	0.498	289.6	**0.000**
	Interaction (DF = 4,27)		2.3	0.084	0.5	0.753	3.2	**0.027**

Table 1: Ion concentrations of leaf tissues taken from *Trifolium repens* plants 2-d after root-zone immersion in seawater. Significant differences (post hoc S-N-K tests, $P < 0.05$) between treatment means are denoted by different letters following two-way ANOVA showing how immersion time (0, 8 or 24 h) and parent plant location along a natural salinity gradient (25 m most saline, 700 m least saline; described in 'Results' section) affected accumulation of Cl^-, Na^+ and K^+.

Distance	Treatment	N	Proline (µmol g^{-1} DM)		Fructose (µmol g^{-1} DM)		Glucose (µmol g^{-1} DM)		Sucrose (µmol g^{-1} DM)		Pinitol (µmol g^{-1} DM)	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
25 m	Control	4	ND	-	84	11	57	5	9^{A}	2	166	31
	8 h	4	14^{AB}	8	74	16	75	12	155^{B}	19	138	18
	24 h	4	28^{B}	10	85	42	74	16	148^{B}	32	125	16
200 m	Control	4	ND	-	42	5	36	3	14^{A}	2	160	28
	8 h	4	16^{B}	3	68	12	63	8	54^{B}	10	148	13
	24 h	4	45^{C}	5	30	9	45	6	61^{B}	9	163	8
700 m	Control	4	ND	-	77	33	55	15	21^{A}	7	137	11
	8 h	4	22^{B}	4	61	18	57	6	142^{B}	15	131	9
	24 h	4	55^{C}	8	45	22	58	8	107^{B}	18	118	16
Results of two-way ANOVA	**Factor**		***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
	Distance $_{(DF = 2,27)}$		3.3	0.054	1.9	0.174	3.4	**0.047**	13.8	**0.001**	1.8	0.183
	Immersion time $_{(DF = 2,27)}$		44.0	**0.000**	0.4	0.649	1.9	0.171	73.9	**0.000**	0.9	0.436
	Interaction $_{(DF = 4,27)}$		1.6	0.198	0.5	0.735	0.6	0.697	4.8	**0.005**	0.4	0.790

Table 2: Organic solute concentrations of leaf tissue taken from *Trifolium repens* plants 2-d after root-zone immersion in seawater. Significant differences (post hoc S-N-K tests, $P < 0.05$) between treatment means for each solute are denoted by different letters following two-way ANOVA showing how immersion time (0, 8 or 24 h) and parent plant location along a natural salinity gradient

(25 m most saline, 700 m least saline; described in 'Results' section) affected solute accumulation. ND denotes failure to detect any quantity above the detection limit, which were: proline (12 µmol g^{-1} DM), fructose (14 µmol g^{-1} DM), glucose (20 µmol g^{-1} DM), sucrose (6 µmol g^{-1} DM), and pinitol (36 µmol g^{-1} DM).

1 **Figure Legends**

2 **Figure 1:** Average electrical conductivity (mS cm^{-1}) of water passing through pots (i.e. of collected leachates following watering)
3 after sea water soil immersion for the durations of 0 (control), 8 and 24 h.

4 **Figure 2:** Mean stolon extension (mm ± SE) of *Trifolium repens* clones cultivated from parent plants from different locations along a
5 natural salinity gradient (25 m is most saline, 700 m least saline; described in 'Results' section), (a) 14 d and (b) 42 d after the root-
6 zones of plants were immersed in sea water.

7 **Figure 3:** The effects of immersion time on the mean (± SE) number of inflorescences ('Flowering Effort') produced by *Trifolium*
8 *repens* clones cultivated from parent plants from different locations along a natural salinity gradient (25 m is most saline, 700 m least
9 saline; described in 'Results' section) 70 d after root-zone immersion in sea water.

10 **Figure 4:** The effects of immersion time on the proportion of *Trifolium repens* plants that had flowered by 70 d after root-zone
11 immersion in sea water. Clonal fragments were cultivated from parents from different locations along a natural salinity gradient (25
12 m is most saline, 700 m least saline; described in 'Results' section).

13

Figure 1

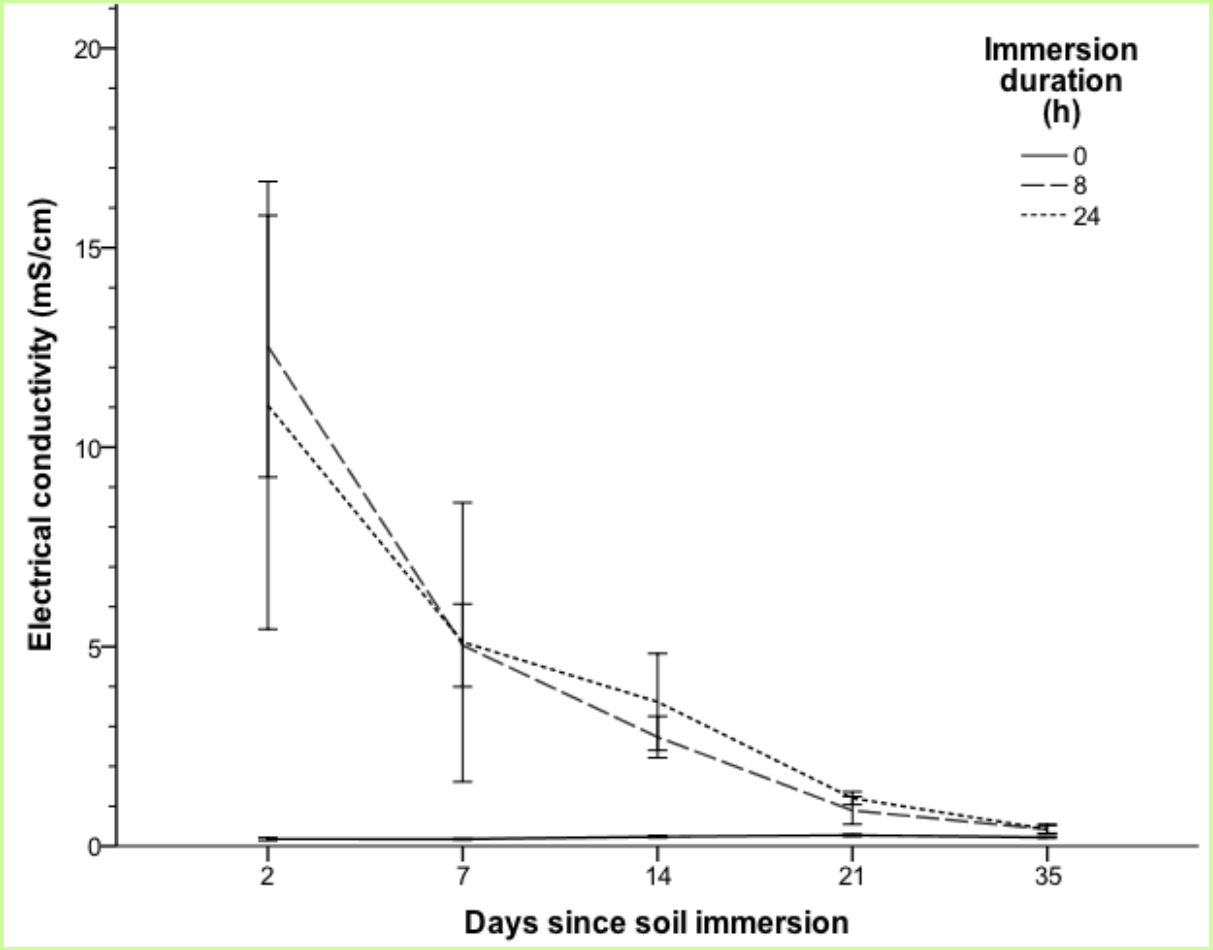
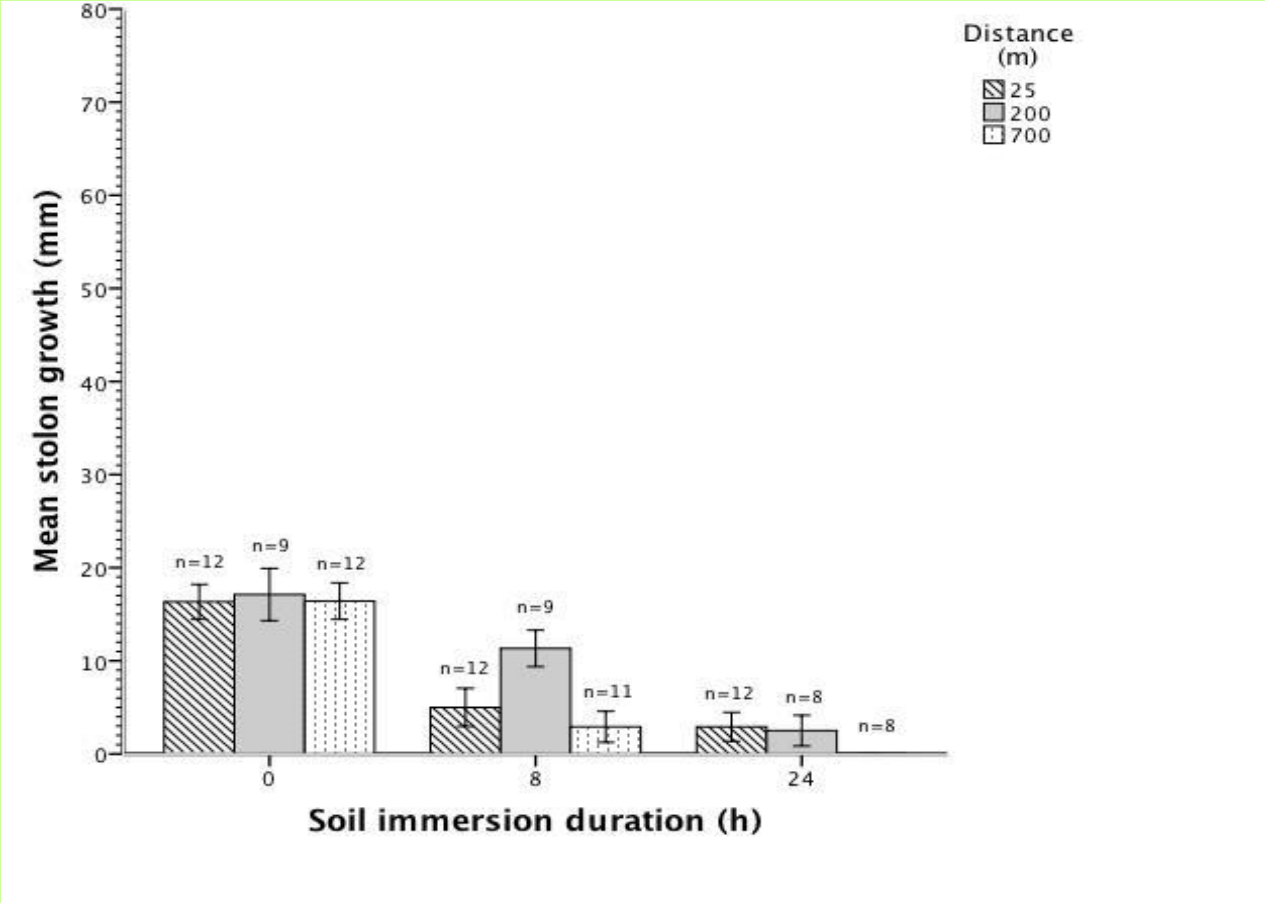
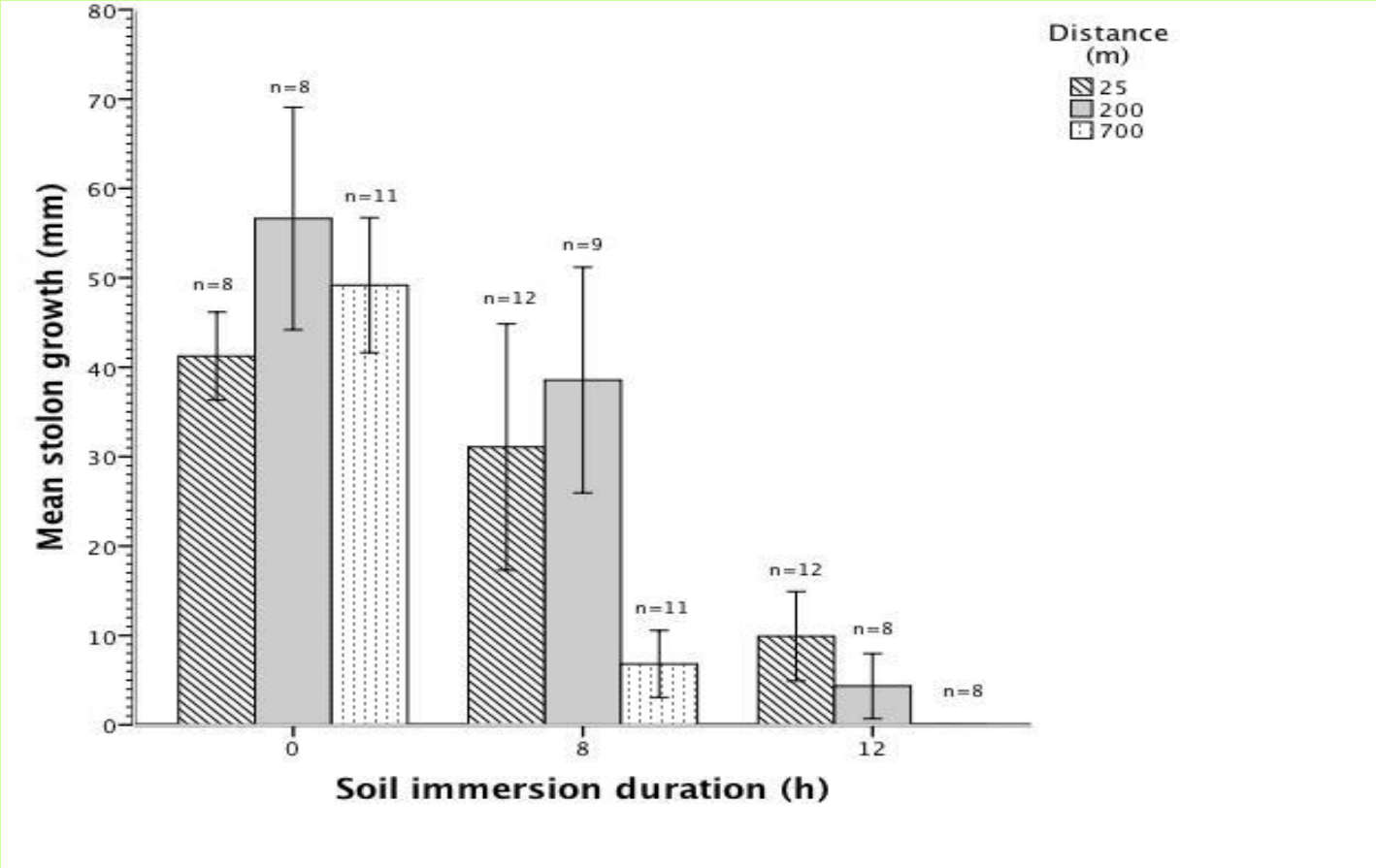

Immersion duration (h)
0
8
24
Electrical conductivity (mS/cm)
20
15
10
5
0
2
7
14
21
35
Days since soil immersion

Figure 2a


Distance
(m)
25
200
700
Mean stolon growth (mm)
80
70
60
50
40
30
20
10
0
n=12
n=9
n=12
n=12
n=9
n=11
n=12
n=8
n=8
0
8
24
Soil immersion duration (h)

1 Figure 2b

2



3
4

Figure 3

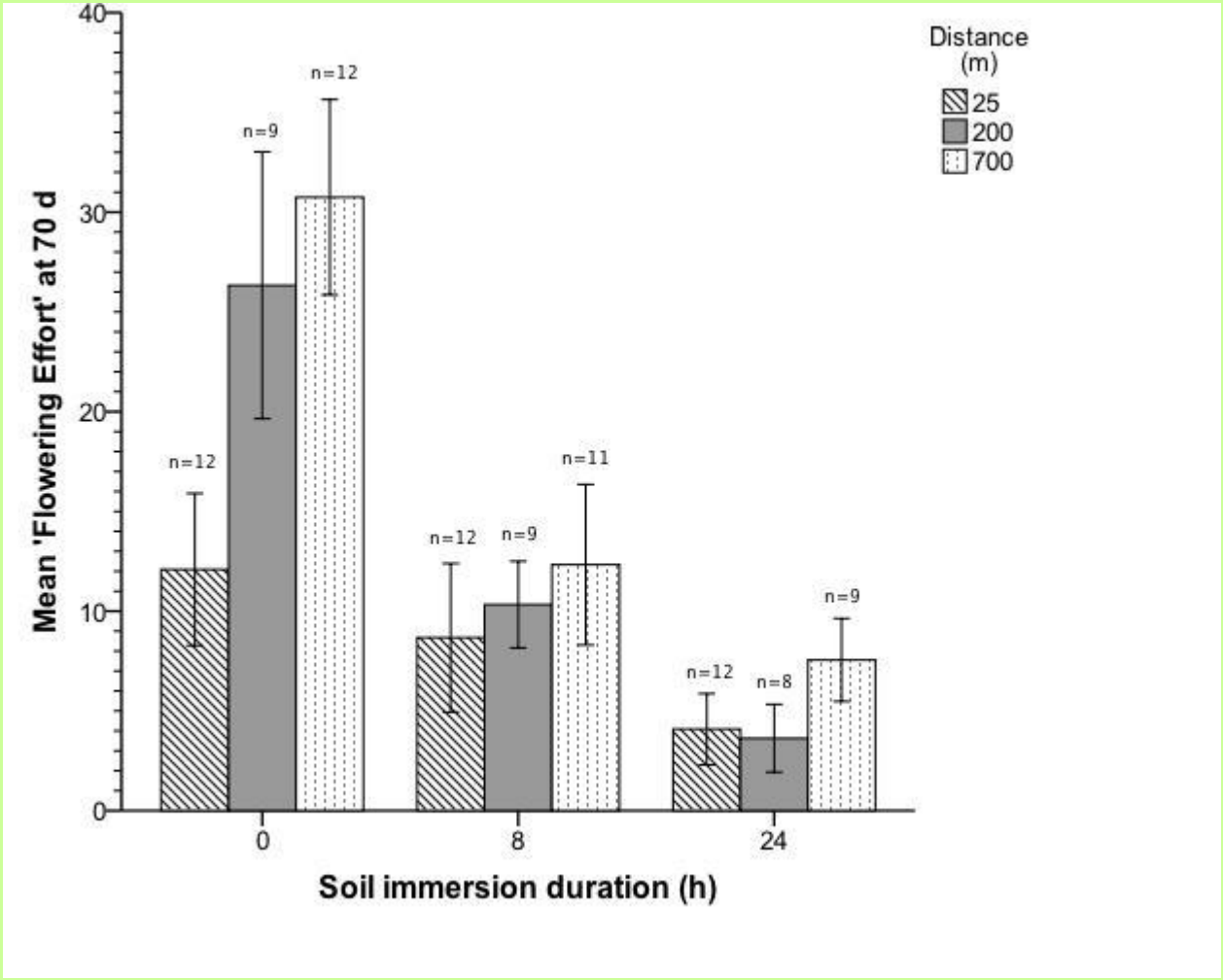
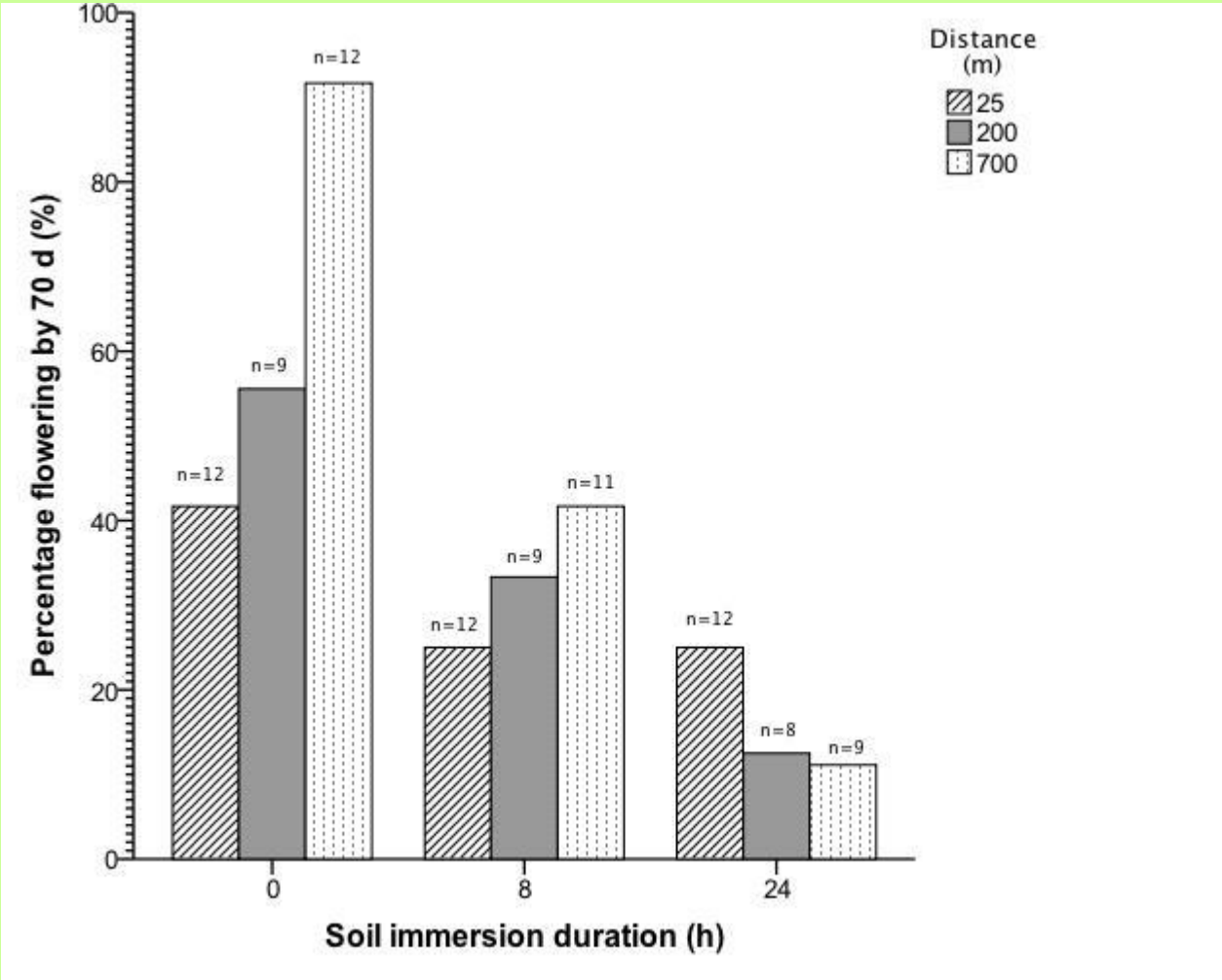

Distance (m)
25
200
700
Mean 'Flowering Effort' at 70 d
40
30
20
10
0
n=12
n=9
n=12
n=12
n=12
n=9
n=11
n=12
n=8
n=9
0
8
24
Soil immersion duration (h)

Figure 4


Distance (m)
25
200
700
Percentage flowering by 70 d (%)
100
80
60
40
20
0
n=12
n=9
n=12
n=12
n=9
n=11
n=12
n=8
n=9
0
8
24
Soil immersion duration (h)