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Responses of Transpiration to Salinity and Vapor Pressure Deficit (VPD) in Five Chinese Songnen Grass Species

Master thesis submitted by

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Abstract

Salt stress and high atmospheric vapor pressure deficit (VPD) commonly occur in the semi-arid Songnen Grassland. Stomata function controls the water pathway in the soil-plant-air continuum, which is affected by environmental variables. In the first experiment, five native species: C_3 and perennials (*Leymus chinensis*, *Medicago sativa*). C_3 and annuals (*Melilotus officinalis*), C_4 and annuals (*Chloris virgata*, *Setaria viridis*) were treated with mixed salts (NaCl : Na_2SO_4=1:1; Na concentration of 60, 120, 180, 240 mM) for 2 weeks to investigate the effects of salt stress on biomass production, mass partitioning, leaf morphological moderation and patterns of ion accumulation. In the second experiment, the instantaneous transpiration response to progressively increasing VPD (0.5-3.5 kPa) was measured in an environmentally controlled chamber. Recorded whole-canopy water loss was used to calculate transpiration rate and canopy conductance. The objectives of this study were to clarify the difference of morphological/physiological salinity tolerance in five grass species, and to identify the response of transpiration rate to short-term atmospheric VPD changes. Perennial grasses showed stronger salt tolerance than annuals in terms of reduction in leaf area and total dry biomass, with more preferential biomass partitioning to roots. SLA was decreased by salinity and differed between species. Na^+ and Cl^- increased progressively with increasing external salt concentration, while K^+ was relatively stable and was slightly reduced by high salinity. Salinity decreased K/Na ratio in different tissues as compared with non-saline controls. C_3 species have relatively higher transpiration rate than C_4 species under moderate salinity (below 120 mM Na^+), probably due to the larger stomata aperture of C_3 species for carbon dioxide diffusion. At higher salt stress (180 and 240 mM), response of transpiration to increasing VPD was not significantly different between functional groups of photosynthetic pathways, rather differed between salt-including and salt-excluding species. C_3 species showed limitations on transpiration rate with a VPD threshold. Salinity changed the regression slope and the emergence of the breakpoint, which differed between species. Transpiration rate of C_4 species increased linearly to VPD at salinity≤120 mM Na^+, a threshold in canopy conductance

emerged to restrict the increment of transpiration at a lower rate at higher salinity. This study provided initial investigation of the transpiration response of grass species to salinity and short-term VPD changes. A comprehensive knowledge about stomatal functioning under co-occurring salt and atmospheric humidity stresses may provide theoretical instructions for the conservation of natural vegetation and the improvement of forage production.

Keywords: VPD, salinity stress, transpiration rate, canopy conductance, Songnen Grassland, C_3 and C_4 grasses, perennials and annuals

1. Introduction

More than 800 million hectares of land throughout the world are salt affected, accounting for more than 6% of the world's land (Munns and Tester 2008). The Songnen plain is one of the three regions in the world characterized by saline-sodic soil, covering an area of approximately 170,000 km^2 in the central part of northeastern China. Increasing demand for agricultural land has resulted in degradation and salinizaion of the grassland since the 1960s (Wang and Ripley 1997). Approximately 70% of the natural grasslands are affected by salinity and alkalinity, with an annual increase of 1.5-2% of the total area (Shang et al. 2003). Substantial reduction in canopy cover combined with soil compaction due to overgrazing increased evapotranspiration and runoff while decreased percolation. The saline ground-water rises and evaporates, resulting in salt accumulation in the top soil (Wang and Ripley 1997).

Understanding the effects of environmental variables on stomatal conductance (g_s) is a central focus of plant physiological research since g_s is directly linked to water use and carbon gain. Apart from soil salinity, plants are often subjected to periods of soil and atmospheric water deficits during their life cycle (Chaves et al. 2009). Salinity is a common feature of arid and semiarid lands, and NaCl is the most soluble and widespread salt (Wang et al. 2009; Munns and Tester 2008). High salt concentrations in the substrate can cause adverse effects on plant growth and development at the molecular, biochemical, and physiological levels (Parida and Das 2005). Salinity affects plant growth mainly by reducing plant water potential, disturbing ion homeostasis and causing toxicity (Munns 2002). Saline conditions markedly increased root hydraulic resistance (Azaizeh and Steudle 1991), the osmotic component of salinity restrained the ability of roots to extract water from the soil and transport it to the shoot, thus decreasing leaf water potential, stomatal conductance and leaf relative water content in salt-treated plants (Farquhar 1978). The speed and amount of water moving from the root to shoot in turn determine the concentration of solutes arriving at the shoot (Markhart and Smit 1990). Stomatal conductance (g_s) is well correlated with leaf-specific hydraulic conductance (Addington et al. 2004). Drought or the osmotic

effect of salinity can cause variation in the response of g_s to atmospheric vapor pressure deficit (VPD) by changing hydraulic conductance.

Increasing VPD results in increased atmospheric evaporative demand, and consequently higher crop transpiration (Sinclair et al. 2007). A large body of research showed that higher leaf-to-air VPD decreased stomatal conductance (Bunce 2006; López-Berenguer et al. 2006). The resulting stomatal closure restricts the transpiration rate to a plateau and sometimes a decrease at high VPD (Oren et al. 2001). Therefore, stomatal closure avoids a corresponding decrease in plant water potential, and thus prevents excessive dehydration and physiological damage. However, it also limits the CO_2 supply to the leaf and thus reduces carbon assimilation rates. The magnitude of the reduction (the slope of gs vs VPD) indicates the sensitivity of the response (Oren et al. 1999). The sensitivity of stomatal response is related to maximum gs; species that have high gs at low VPD are more likely to close stomata at increasing VPD (Ohsumi et al. 2008). Alteration in stomatal conductance is often depicted as a nonlinear decline whereas C_4 and C_3 species showed substantial differences in their response and sensitivity to VPD. While the C_4 species lacked sensitivity to VPD, C_3 species showed limitations in transpiration rate at high VPD (Wherley and Sinclair 2009). While more recent study showed that C_4 and C_3 species share common relationships of hydraulic capacity and stomatal sensitivity to increasing VPD (Ocheltree et al. 2013).

The relative importance of stomatal and boundary layer conductance in controlling canopy transpiration has been investigated at leaf level and whole plant level. Whether the single leaf responses precisely reflect the entire organism is poorly understood. Wullschleger et al. (1998) pointed out that relying on porometric data alone could lead to misinterpretation of the significance of stomatal movements to whole-plant transpiration. Therefore, canopy transpiration may be more indicative of leaf water status under natural conditions (Franks and Farquhar 1999). The Northern Chinese Songnen grasslands are located in semi-arid regions where VPD is high and salinity commonly occurs. The dominant species there are a mix of C_3 and C_4 species, as well perennial and annual species. The effect of salinity on transpiration rate of natural grasses subjected to increasing VPD has not been investigated to date. This study

examined the salt resistance strategies of five common species and their responses of transpiration rate to atmospheric VPD by exposing the whole plant to changes of VPD in an environmentally-controlled chamber.

2. Literature Review

2.1 Songnen plain and its saline and alkaline problems

Songnen plain covers an area of about 170,000 km^2 in the central part of northeastern China (43° 30′ to 48° 40′ N and 121° 30′ to 127° 00′ E), and is a big basin surrounded by mountains from three sides: Changbai mountain in the east, Xiaoxing'an Mountain Ranges in the north and Daxing'an Mountain Ranges in the west (Wang et al. 2009). The climate in this region is classified as the transition of sub-humid and semi-arid, thus it is the transitional zone of agriculture and grazing. The grasslands dominated by *Leymus chinensis* is one of the best suited in northern China for grazing and forage (Wang et al., 1997). However, increasing demand for agriculture land and the intensive grazing of livestock have resulted in substantial reduction of canopy cover in much of the grassland (Wang et al. 2009). The direct results of surface cover clearing and compaction are increased evaporation and diminished infiltration of water into the soil (Guo 1989). More recent data shows that the salt-affected land has reached 3.2×10^6 ha, and the newly salinized and alkalized land increased about 20.0×10^3 every year (Wang et al. 2009). The causes of soil salinization and alkalization in the Songnen Plain are mainly attributed to natural and anthropogenic factors. Natural factors include topographic positions, parent materials, semi-arid/sub-humid climate and freeze-thaw action. Human-related factors are population pressure, overgrazing and improper agricultural policies (Wang et al. 2009).

Map of the Songnen Plain in Northeast China (cited from Wang et al. 2009)

2.2 Major environmental factors limiting plant growth in Songnen Grasslands

2.2.1 Salinity and alkalinity

Saline soil (mainly containing NaCl and Na_2SO_4) and alkaline soil (mainly containing $NaHCO_3$ and Na_2CO_3) are two typical types of salt-alkaline soils in Songnen Plain (Shi and Wang 2005). High salt concentrations of NaCl and Na_2SO_4 and alkaline stress caused by carbonates are economically important threats for the the survival and growth of crops in these types of soil (Li et al. 2010). The saline-alkali soils in Songnen Plain are mainly caused by alkali rocks which are parent materials of soil. The weathering rocks with lots of dissoluble saline (NaCl and Na_2SO_4) and alkali compounds (Na_2CO_3 and $NaHCO_3$) move downward with the runoffs into the plain. Since the Songnen Plain is surrounded by mountains and the land form in the plain is very flat, the drainage is blocked and salts are accumulated in the lower landscape positions (Gao et al. 1996). In the Songnen Plain, groundwater flows slowly and the ion's content is very high, salinity easily occurs in soil surface with water evaporation and vegetation transpiration (Wang et al 2009). Salinity shows significant seasonal variations, which are leached to the deeper soil horizons in rainfall periods and ascend to superficial horizons during drought periods. The average annual evaporation

(1200-1800mm) is 2-3.5 times more than the annual precipitation, so the upward capillary movement of soil water is stronger than infiltration and gravity movement; the soluble saline compounds are accumulated in the soil surface.

2.2.2 Seasonal atmospheric vapor pressure deficit (VPD)

The climate in Songnen Plain is semi-arid and sub-humid, characterized with a cold, dry, windy spring; a warm, wet summer with uneven precipitation and frequent droughts; early autumn frosts and a long and cold winter with relatively little snowfall (Yao et al. 2006, Wang et al. 2009). The little precipitation during winter is because the majority of the region is dominated by the Mongolian anticyclone (high pressure) system and the southeast ocean monsoon is blocked out by Changbai Mountain. In the summer monsoon, the anticyclone breaks down; warm and moist oceanic air is drawn into the region. Therefore, more than 80% of the rains occur mainly during the summer monsoon (between June and September), causing a moisture deficit during plant seedling and harvest periods (Wang et al. 2009).

2.3 Information on the species studied

Setaria viridis (common name: green foxtail) is member of the tribe *Paniceae*. *S. viridis* has a number of desirable traits, such as small size (10-15 cm), short life cycle (6-9 weeks decided by photoperiod), and abundant seed production (about 13,000 seeds per plant). *S. viridis* serves ideally as model organisms to study the underlying mechanisms of abiotic stress tolerance (Li and Brutnell 2011). *S. viridis* originated in Eurasia (around latitude 15°N) and has spread widely to temperate, tropical, and subtropical regions. The simple growth requirements and large seed yield perhaps contribute to its successful history of invading and adapting to new environments.

Chloris virgata is a natural annual halophyte species. The high protein content makes this grass a high quality forage plant. *C. virgata* is very alkali tolerant and can grow on heavily alkalized soil with pH over 10 and normally colonize bare alkaline patches as a pioneer species, contributing to the restoration of the retrogressed grassland

ecosystem (Zheng and Li 1999).

Melilotus officinalis, also called as yellow sweet clover or yellow melilot, is a dicotyledonous summer annual or biennial herb and belongs to the *Fabaceae* family. The *Melilotus* genus, which originates from Eurasia, is closely related to the *Medicago* and *Trigonella* genera and includes approximately 25 species of annuals and biennials/perennials (Allen and Allen 1981). *Melilotus* species tend to be moderately winter-hardy, drought resistant and can be valued as pasture forage (Rogers et al. 2008).

Alfalfa (*Medicao sativa* L.) is one of the most important forage crops and has high protein and highly digestible fiber contents. It is perennial forage, with crude protein content reaching approximately 16-22%. *M. sativa* has been reported to be relatively salt tolerant (Ehsanpour and Fatahian 2003) and has been cultivated in moderate salt-alkaline soils as an economic crop worldwide (Li et al. 2010). Alfalfa has become increasingly important as the number of livestock increases in north China.

Leymus chinensis (Trin.) Tzvel., a rhizomatous perennial species of *Poaceae*, is widely distributed at the eastern end of the Eurasian steppe zone. It is a dominant species of the grasslands of Chinese Songnen Plain and the eastern part of the Inner Mongolian plateau. *L. chinensis* is of high palatability and ideal for grazing and forage (Zhu 1993). It is relatively drought-sensitive compared to other species, such as perennial bunchgrasses in the same region (Bai et al. 2004).

2.4 Effects of salinity on growth of grasses

2.4.1 Concept of salinity

Soils are classified as saline when the EC≥4 dS/m, which is equivalent to approximately 40 mM NaCl and generates an osmotic pressure of approximately 0.2 MPa (Munns and Tester 2008). In general, salt concentrations higher than 45 mM NaCl can decrease yields of many crops (Shannon et al. 1994). Previous reports have suggested that salt stress in the natural Songnen Plain can be defined as the stress of neutral salts (NaCl and Na_2SO_4) and alkali stress is the stress of alkaline salts ($NaHCO_3$

and Na_2CO_3) (Shi and Wang, 2005). Simple salt stress (only NaCl) has been used in a number of salt stress biology studies (Li et al. 2010). However, the existence of alkali stress has been shown to be more severe than single salt stress, therefore, the alkali stress should be considered and separated from salt stress (Brand et al. 2000; Shi and Wang 2005).

2.4.2 Two phases of salt stress

The reduction of plant growth to salinity can occur in two distinct phases: the osmotic effect of the salt in the soil and the toxic effect of the salt within the plant (Munns and Tester 2008). The osmotic effect starts immediately after the salt concentration in the rooting medium increases to a threshold level, as a result the rate of shoot growth decreases significantly. Ion-specific phase is a slower response and starts when salt accumulates to toxic concentrations in the old leaves. The impact of ionic stress on growth much is much later, thus have less effect than the osmotic stress at low to moderate salinity levels (Munns and Tester 2008; Chaves et al. 2009).

2.4.3 Physiological adaptations to salt stress

The effects of salinity on plants at the morphological, physiological, biochemical, and molecular levels have been comprehensively synthesized (see reviews of Gorham et al. 1985; Parida and Das 2005; Munns and Tester 2008). The limited growth of salt-affected plants is attributable to (a) the osmotic effect resulting from increased osmotic potential in the rhizosphere causing disturbances in the water balance, reduction in turgor and stomatal aperture; (b) ion toxicity through excessive uptake of chloride and sodium affecting plant leaf growth and water status; (c) nutrient imbalance associated with the competition of chloride and sodium ions with potassium, nitrate, or phosphate (Gorham et al. 1985).

Increasing salt concentration in the root medium causes the rate of ion uptake to increase, which lowers the root water potential and stimulates water absorption in order to maintain cell turgor and the turgidity of the plant tissues (Sanchez-Diaz et al. 1982).

Plants either avoid excessive salt uptake or compartmentalize the ions in vacuoles to maintain metabolic functions through osmotic adjustment (Zhu 2003). A salt-inducible enzyme Na^+/H^+ antiporter is important in the process of removing sodium from the cytoplasm or partition of sodium into the vacuoles (Apse et al. 1999). Plants also maintain low concentrations of Na^+ and high concentrations of K^+ in the cytosol by modulating the expression and activity of K^+ and Na^+ transporters, as well as H^+ pumps (Zhu 2003). Under high salinity elevated cytosolic Ca^{2+} facilitates stress signal transduction and leads to higher K^+/Na^+ selectivity, therefore meliorating the toxic effects of salinity (Liu and Zhu 1997; Knight et al. 1997). Other mechanisms of salt regulation involve salt secretion. Salt glands secrete salt from leaves as a means to decrease internal ion concentration. Many halophytes regulate the salt content of leaves through salt exclusion by roots (Chaves et al. 2009).

It has been intensively reported that glycophytic and halophytic plant species exhibit considerable interspecific varation in ion regulation (Malcolm et al. 2003). Glycophytes restrict ion movement to the shoot by controlling ion flux into root xylem, while halophytes require higher external salinity concentration for optimal growth and tend to take up Na. Halophytes survive and grow in saline environments due to either exclusion of sodium and chloride through glands and bladders, or osmotic adjustment through intracellular compartmentation that partitions toxic ions into vacuoles (Munns and Tester 2008). The comparison of tolerance between different species is complicated by differences in growth rate, growth habit and life cycle. The degree of tolerance changes with development stage, relative humidity, temperature and ionic balance (Gorham et al., 1985).

2.5 Effects of vapor pressure deficit (VPD) on stomata functioning and transpiration

2.5.1 Mechanisms of stomatal responses to VPD

VPD is the difference between saturation vapor pressure at the leaf temperature and

the actual water vapor pressure at the outside air temperature, which is the driving force for water movement from the inside leaf to the outside air. The epidermis of leaves is covered by a waxy outer layer, the cuticle, which is an effective barrier to both water and CO_2 diffusion. Stomata are the openings at the leaf surface that enable the control of water efflux and CO_2 infux between the inside leaf and the ambient air (Buckley 2005). While stomatal closure effectively conserves water, the decreased stomatal conductance strongly limits net photosynthesis, and in turn cumulative carbohydrates and biomass productivity (Ohsumi et al. 2008). Two contrasting mechanisms of stomatal response to VPD have been proposed. The feedforward hypothesis states that stomatal conductance decreases directly as VPD increases because stomata are somehow able to sense an increasing VPD (Farquhar 1978). This is an apparent beneficial adaptation by which plants can minimize transpiration water loss.

The other hypothesis is a feedback response based on the fact that a decrease in stomatal conductance is caused by a direct increase in transpiration as VPD increases (Mott and Parkhurst 1991; Monteith 1995). A high transpiration may induce stomatal closure by lowering bulk leaf water potential or by increasing the water potential gradient between guard cells and other epidermal cells (Meinzer et al. 1997). Because increasing VPD would increase transpiration, it seems logical to assume that the resulting stomatal closure is caused by a reduction in water potential somewhere in plants (Bunce 2006).

2.5.2 Plant hormone (ABA) in stress adaptation and stomatal regulation

Changes in environmental factors such as a decrease in soil water potential and an increase in atmospheric vapor pressure deficit (VPD) induce drops in whole-plant water potential and a decrease in stomatal conductance (Peak and Mott 2011). Abscisic acid is the major mediator involved in this response (Christmannn et al. 2007). ABA functions in the mechanism of salt tolerance, such as root-to-shoot biomass allocation, cellular signaling and the regulation of stomatal conductance (Davies et al. 2005), as well as modifications of root hydraulic conductivity (Ruggiero et al., 2004; Sinclair et al. 2008).

ABA originating from the mesophyll of the leaves or arriving from the roots in the xylem stream could modify stomatal behavior. The study of Tardieu and Davies (1992) further pointed out that the extent of the stomatal response to any ABA signal will depend on the water status of the epidermis. ABA was suggested to control stomatal conductance by means of change in the rate of delivery of ABA to the guard cells in the transpiration stream in response to VPD (Farquhar 1978). A reduction in water potential amplified the effect of a given concentration of ABA, whereas the water potentials alone did not directly impact stomatal aperture. ABA decreases stomatal conductance and down-regulates leaf hydraulic conductance. The effects of ABA on stomatal closure are dual: biochemical effect on guard cells; and indirect hydraulic effect through a decrease in water permeability within leaf vascular tissues (Pantin et al. 2013).

3. Objectives and Hypotheses

The effects of salt stress on canopy transpiration and conductance have not been examined in grass species of Songnen Grasslands. This study was carried out to (1) compare salt tolerance of five grass species in terms of morphological and physiological traits; (2) investigate the changes in canopy transpiration and conductance of five grass species in response to salt stress and high leaf-to-air VPD, which occur frequently in semi-arid Songnen Grasslands.

The five species in this study fall into two functional groups based on either photosynthesis metabolism (C_3 and C_4 grasses) or life form (perennials and annuals). The tolerance mechanisms developed by these plants to soil salinity and concurrent atmospheric water vapor deficit are investigated. The hypotheses are (1) Salt salinity affects the occurrence of a VPD breakpoint beyond which transpiration rate increases at lower rate, or changes the slope of transpiration rate vs. VPD regression. (2) The effect of salinity on the transpirational responses across increasing VPDs may depend on the salt-resistance strategy of individual species, or varies between functional groups.

4. Materials and methods

4.1 Plant materials

This study included five grass species dominantly found in the Chinese Songnen grassland ecosystem. The selected grass species can be grouped on the basis of either photosynthesis metabolism (C_3 and C_4 grasses) or life form (perennials and annuals) (Table 1). These species are genetically distinct and displayed differences in salt resistance (descriptions see literature review).

Table 1 Species (their photosynthesis metabolism, life form and corresponding family) of grasses from Northeastern Chinese Songnen Grasslands.

Species	Photosynthesis metabolism	Life form	Family
Leymus chinensis	C_3	Perennial herb	Poaceae
Medicago sativa	C_3	Perennial herb	Fabaceae
Melilotus officinalis	C_3	Annual and biennial herb	Fabaceae
Chloris virgata	C_4	Annual herb	Poaceaee
Setaria viridis	C_4	Annual herb	Poaceae

4.2 Salt stress experiment

Saline soil (mainly containing NaCl and Na_2SO_4) and alkaline soil (mainly containing $NaHCO_3$ and Na_2CO_3) are two typical types of salt-alkaline soils in China. Previous reports have suggested that salt stress can be defined as the stress of neutral salts (NaCl and Na_2SO_4) and the stress of alkali stress induced by alkali salts ($NaHCO_3$ and Na_2CO_3), and the deleterious effects of alkali stress were more severe than those of salt stress (Yang et al, 2008a; Li et al., 2010). This study only focused on the effect of neutral salts (1:1 molar ratio of NaCl and Na_2SO_4, pH at about 7.0) The experiment was conducted between April and August in a greenhouse at the University of Hohenheim (See table 2). A maximum photosynthetic photon flux density (PPFD) of approximately 500 umol m^{-2} s^{-1} was supplied for 12 h per day with lamp type: PL SON-K-400 (DHLicht GmbH, Wülfrath, Germany). Plant seeds were directly sown in nursery pots filled with washed sand and watered with nutrient solution, consisting of 0.7 mM K_2SO_4; 0.1 mM KCl; 0.1 mM KH_2PO_4; 0.5 mM $MgSO_4*7H_2O$; 2.0 mM $Ca(NO_3)_2*4H_2O$; 0.5 uM $MnSO_4*H_2O$; 0.1 uM $ZnSO_4*$ $7H_2O$; 0.2 uM $CuSO_{4*}$ $5H_2O$; 0.01 uM $(NH_4)_6Mo_7O_{24}*$ $4H_2O$; 10 uM H_3BO_3; 50 uM FeNaEDTA (Nutrient solution suggested by Ph.D. Linda Gorim, see the supplemented table). Considering the nutrient formula is used for hydroponic culture, the concentration shown above was used for young seedlings, but the concentration was doubled for fully developed plants because of their higher nutrient demand. When seedlings were in 4-leaf stage, about 2-4 weeks' old

depending on species, four young plants were transplanted into plastic pots (11.5cm height, 14.5cm length and 9.5cm width) filled with washed sand. According to the experience of nutrient application from other colleagues, nutrient solution was applied every day that had high evapo-transpiration demand, but were applied every other day during rainy and cloudy days. Because the washed sand contained almost no nutrition and the volume of daily irrigation was 80 ml, the plants would not be over-fertilized. The main point was to maintain the plants well-watered while avoid water-logging. When these plants were approximately 4 weeks old, they were subjected to salinity treatments.

The experiments were arranged in a complete block design. 75 pots were grouped into three blocks, in which five species and five salinity treatments were assigned at random. Thus there were three replicates for every species at each salinity level. Sowing and measurement dates were shown in Table 2.

Table 2 Dates of sowing, salt treatment, leaf area measurement and biomass harvest

	Leymus chinensis	***Medicago sativa***	***Melilotus officinalis***	***Chloris virgata***	***Setaria viridis***
Sowing	24 March	24 March	24 March	6 May	30 March
Transfer	6 May	2 May	2 May	2 July	2 May
Salt treatment	20 June	1 June	11 June	1 August	29 May
Destructive harvest	10 July	15 June	26 June	16 August	13 June

Natural salt stresses are mostly mixed salt stresses. Two neutral salts (NaCl and Na_2SO_4) were selected based on the salt components in the extant salt-alkaline soil of northeast China (Ge and Li 1990). NaCl and Na_2SO_4 were mixed in a 1:1 molar ratio to create sodium concentration of 0, 60, 120, 180, 240 mM (**Table 3**). Salts were added to the nutrient solution and the pH of the irrigation solution was neutralized to 7.0. Electrical conductivity of irrigation water (EC) was measured with an EC-meter (Cole-Parmer Instr., Chicago, Illinois). EC values varied from 2.5 to 22.1 dS m^{-1} and were linearly correlated to the total amount of salt applied (**Figure 1**). Control plants were irrigated with nutrient solution; meanwhile salt-treated plants were watered with

salty solution of corresponding salinity level. The salt treatment lasted for 14 days. To avoid salt shock of directly applying severe salt stress, salts were added in an increment of 60 mM per day to a final Na concentration of 240 mM. The daily water loss varied between plants and salt treatments. Each pot was watered with 80ml treatment solution everyday around five o'clock. During hot and sunny days, an extra of 20 ml distilled water was added to ensure sufficient moisture in rooting medium due to strong evapo-transpiration.

Table 3 Five salinity levels derived from two neutral salts (NaCl, Na_2SO_4) mixed at 1:1 molar ratio, five salinity levels were 0, 60, 120, 180, 240 mM Na^+ respectively).

Added Na^+ concentration (mM)		**0**	**60**	**120**	**180**	**240**
Salt component (g/L)	NaCl	-	1.17	2.34	3.51	4.68
	Na_2SO_4	-	2.84	5.68	8.52	11.38
EC (dS/m)		2.50	7.96	12.88	17.76	22.09
Total salt application (g)	NaCl	-	1.15	2.21	3.19	4.10
	Na_2SO_4	-	2.78	5.37	7.75	9.96

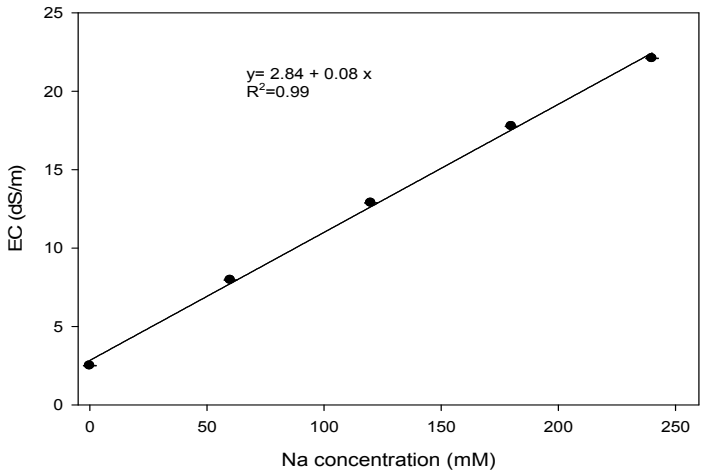


Figure 1: EC values of the irrigation water as a function of the Na^+ concentration (0, 60, 120, 180, 240 mM).

4.3 VPD chamber measurements

In the second experiment, transpiration responses of plants were measured in a closed chamber (80×80×100cm). The chamber was equipped with two computer box fans, one was blowing air into the chamber continuously, and the other was used to mix the air inside the chamber. LED light installed on the top of the chamber provided photosynthetic photon flux (PPF) of 600-1200 μmol m^{-2} s^{-1}. Continuous flow of air through the VPD chamber excluded the possibility of carbon dioxide deficit in the chamber. Temperature and relative humidity were recorded every minute using Tinytag data loggers (Type TGP-4500, Gemini data loggers, Chichester, UK) to calculate the actual atmospheric VPD in the chamber during measurements. Five individual balances with a resolution of 0.01 g (Model KERN KB 2400-2N d=0.01g) were evenly placed in the chamber and were connected to a data logger for continuous monitoring of pot weight by the computer using Grasslog Software. In the evening prior to measurements the plants were brought to the laboratory and watered until dripping. During chamber measurement, the pot surface was covered by dry gravel stones (around 150g) to eliminate soil evaporation into the chamber. VPD was initially maintained at approximately 0.5 kPa, and increased stepwise to approximately 3.5 kPa. Once the atmosphere in the chamber was equilibrated, the entire unit of plants and pot were weighed on a balance. Steady-state transpiration rate was determined based on the mass loss. Various humidity levels in the chamber were established by adjusting the flow rate of humid air and dry air. Measurements were initiated from the lowest VPD to the highest VPD settings to avoid any influence of exposure to high VPD on subsequent VPD tests. Transpiration rates were measured over 30 minutes for each VPD level, with an interval of 5 minutes for adjusting VPD until the starting of the higher VPD level. The measurement for each replicate consisted of five VPD levels, and around 3 hours in total. 10 relatively constant values (nine values following the smallest coefficient variation) were selected out of the measurement period of 30 minutes to calculate the

mean transpiration rate at each VPD level.

4.4 Data determination and calculation

4.4.1 Transpiration rate

Initially, the whole plant transpiration rates (TR) were converted to transpiration rates per unit leaf area (mmol H_2O m^{-2} s^{-1}) based on the change in pot weight of three minutes' interval, see equation (1). Since the plant canopy area cannot be accurately determined because leaves are rolling and curling in response to salt stress, especially when sodium concentration exceeds 120 mM, transpiration rate was also calculated as water loss per unit leaf dry weight, a more readily measurable parameter. See equation (2).

$$\mathrm{TR} = \frac{(\mathrm{W0} - \mathrm{W1})/180}{\mathrm{Time} \times \mathrm{Leaf\ Area}} \qquad (1)$$

$$\mathrm{TR} = \frac{(\mathrm{W0} - \mathrm{W1})/180}{\mathrm{Time} \times \mathrm{Leaf\ Dry\ Weight}} \qquad (2)$$

W0= the former weight

W1= weight after 180 seconds

Time= interval of 180 seconds

The recorded data for temperature and relative humidity were used to calculate the mean atmospheric VPD for each measurement. The data were analyzed by plotting TR against VPD. There was a large amount of scatter among data points; therefore, the data for each genotype were grouped into cohorts of 10 consecutive values of VPD. The mean of both VPD and TR for these cohorts were used in further analysis. Plotting methods refer to Wherley and Sinclair (2009).

4.4.2 Determination of leaf area and dry biomass

After measuring the transpiration, the plants were clipped at the stem base and separated into leaves and stems. The total leaf area was measured using a LI-3100 area meter. Roots together with sand medium were immersed in water to remove sand. Roots

were further washed with deionized water twice and sealed separately in labeled paper bags. All plant tissues were dried in an oven at 80□ for 48h to constant weight. The calculation of root-to-shoot ratio and specific leaf area (SLA) were based on these values.

4.4.3 Determination of plant ion concentrations

Oven dried tissues were finely ground in a ball mill (PAT COSHH Ltd, Kent, UK). 10 ml distilled water was added to weighted plant material and autoclaved for 1 h at 100 □ (Wolf SANOclav, Bad Überkingen Hausen, Germany). This extract was filtered (LLG filter paper folded, ø 150 mm, Lab Logistics group GmbH, Meckenheim, Germany) into volumetric flasks and filled up to 100 ml by adding distilled water. Leaf sodium and potassium content were measured using a flame photometer (Jenway, Bibby Scientific Limited, Essex, UK). The flame photometer was calibrated with flame photometry standards of 0, 12.5, 25, 50 and 100 ppm Na and K (Jenway, Essex, UK). Concentrations of chloride were indicated by peak hights compared with those of known standards.

4.4.4 Specific Leaf area

According to Lambers et al. (2008), Specific leaf area (SLA) was defined as the amount of leaf area per unit of leaf dry weight. SLA indicates leaf thickness.

$$\text{SLA} = \frac{\text{Leaf Area}}{\text{Leaf Dry Weight}}$$

4.4.5 Normalizing transpiration response to VPD

In this study, VPD in the chamber was controlled by hand through adjusting the relative humidity while keeping temperature constant, therefore the exact VPD could not be always kept at exactly 0.5, 1.0, 1.5, 2.5, 3.5 kPa. This makes it difficult to compare the transpiration responses of plants to VPD perturbations among species and among salt treatments within one species; therefore, transpiration rates (TR) at exact 0.5,

1.0, 1.5, 2.5, 3.5 kPa were extrapolated. Firstly, transpiration rate was calculated based on the recorded transpirational weight loss; then all the TR values were plotted against their respective VPD value to get a regression equation. Then TR values at exact 0.5, 1.0, 1.5, 2.5, 3.5 kPa were normalized according to the respective regression equation. Canopy conductance (Gs) was derived from the transpiration rate divided by respective VPD.

$$\mathrm{Gs} = \frac{\mathrm{TR}}{\mathrm{VPD}}$$

5. Data analyses

Data were analyzed by plotting transpiration rate (TR) data against VPD for each species under various salt concentrations. Initially, a two-segment linear regression using Piecewise Nonlinear Regression (SigmaPlot 10.0) was applied to the data:

If VPD < X_0, TR = a_1 (VPD) + b_1

If VPD = X_0, TR = a_2 (VPD) + b_2

Where X_0 is the breakpoint between the two line segments, a_1 and a_2 the slopes of the first and second line segments, respectively.

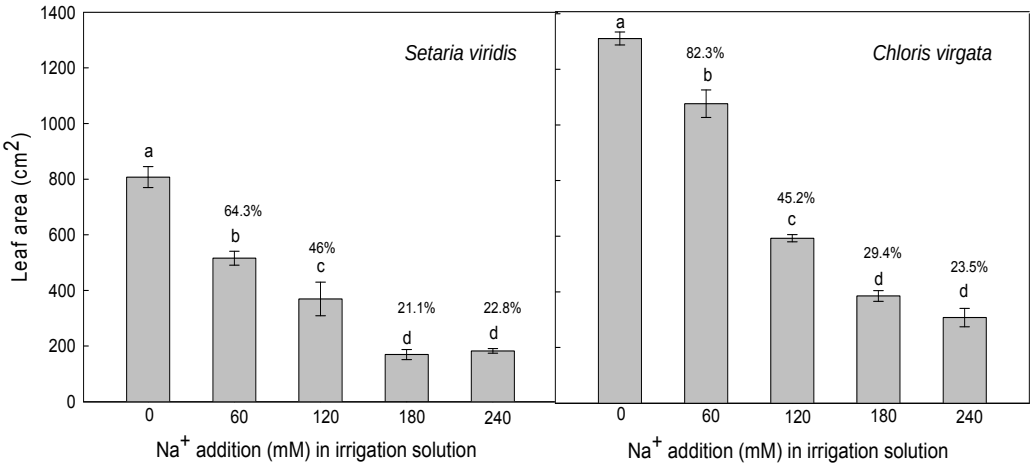
In the regression, the second line segment is constrained to intersect with the first line segment at X_0. Initially, the data were submitted to a two segment linear regression analysis. The slopes of the two linear regressions (a_1 and a_2) were statistically compared to determine if they were significantly different ($P<0.05$). The two-segment model was assumed if the slopes differed; otherwise a simple linear regression was fitted to all the data.

The experiment was a randomized complete block design with 3 replicates. Data were analyzed statistically using the SPSS 7.5 software package (Chicago, IL, USA). A two-way analysis of variance (ANOVA) was used to test the effects of salinity and species on the morphological and physiological variables. Differences between means were determined using Tukey's multiple range tests at the 0.05 confidence level. The relationships between TR and VPD were determined using Piecewise regression procedures in SigmaPlot software.

6. Results

6.1 Leaf area

Leaf area was strongly reduced across the salinity gradient for all species (Figure 2). Generally C_4 grasses (*Setaria*, *Chloris*) had larger leaf areas than C_3 grasses (*Melilotus, Medicago*, *Leymus*). Applying 240 mM Na^+ for two weeks reduced approximately 80% of the leaf area compared with the non-saline treatment for both C_4 species. The leaf area of *Chloris* was much larger than *Setaria* across all salinity treatments. A reduction of about 60% in leaf area was observed for *Melilotus*, and a reduction of 50% for *Leymus* and *Medicago*. The results indicate that the leaf development of C_4 species was more susceptible to salt stress than C_3 species. Comparing the leaf area from the perspective of life form, annual grasses (*Setaria*, *Chloris*, *Melilotus*) had larger leaf areas than perennial grasses (*Leymus*, *Medicago*), suggesting that C_4 and annual species grow faster. The smaller leaf area was attributed to the reduction in both leaf number and leaf size (data not shown).



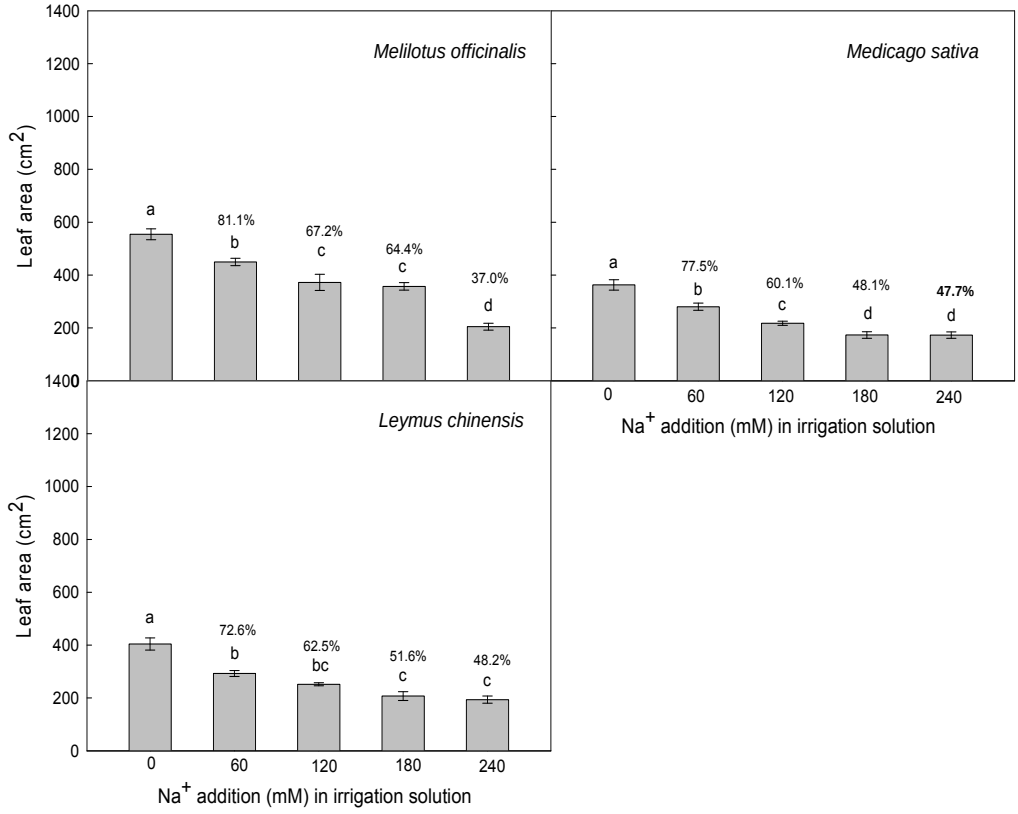


Figure 2: Leaf area (cm^2) of five species, subjected to different salinity treatments. No significant differences for values followed by the same letter, $p<0.05$.

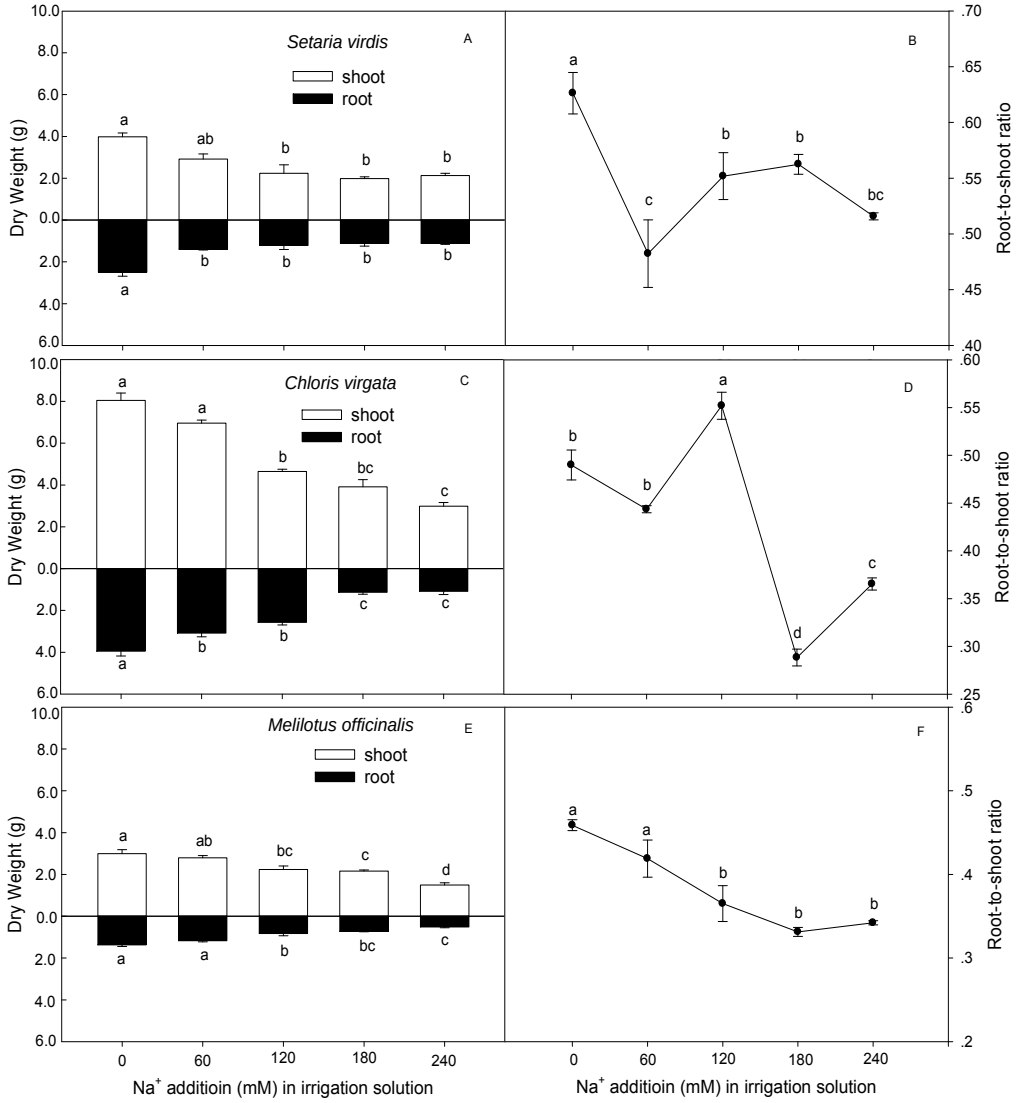
6.2 Plant biomass and root/shoot ratio

All species displayed a constant reduction in total biomass after two weeks of exposure to the different salt treatments (Figure 3). The reduction in total plant biomass indicated differences in salt tolerance between species. In annual species (*Setaria, Chloris, Melilotus*), 240 mM Na^+ induced a biomass reduction of more than 50% relative to the non-saline control. Total biomass of perennial grasses was less decreased, only 25% and 39% in *Medicago* and *Leymu*s respectively. Although annual species had greater total biomass than perennials, they were losing more biomass due to salinity. It's noteworthy that *Melilotus* can also grow as biennials, this explains that the biomass production of *Melilotus* was between the amount of perennials and annuals (Figure 3: E).

Analyzing shoot biomass and root biomass separately indicated differences in

biomass allocation among the species. Although all species displayed significant reductions in aboveground biomass when exposed to increasing salinity, belowground biomass of perennial grasses remained quite stable across all salt levels. The results may reveal that perennial grass species were more salinity-tolerant than annual species. C_4 species were more susceptible than C_3 species in terms of reduction in total biomass. Among the species studied, *Medicago* showed the strongest capacity to tolerate severe salt stress (Figure 3: G).

Changes in root-to-shoot ratios showed clear contrasts among species of different life forms. Increasing salinity resulted in an increasing trend of root-to-shoot ratio in perennial species, but decreased the ratio in annual species. Variations in root-to-shoot ratio were coinciding with the reduction in plant total biomass. Perennial species that had less biomass reduction displayed increasing root-to-shoot ratio, while annual species that had greater loss of biomass also decreased the root component relative to shoots.


Setaria virdis
A
shoot
root
Dry Weight (g)
B
Root-to-shoot ratio
Chloris virgata
C
D
Melilotus officinalis
E
F
0
60
120
180
240
Na+ additioin (mM) in irrigation solution

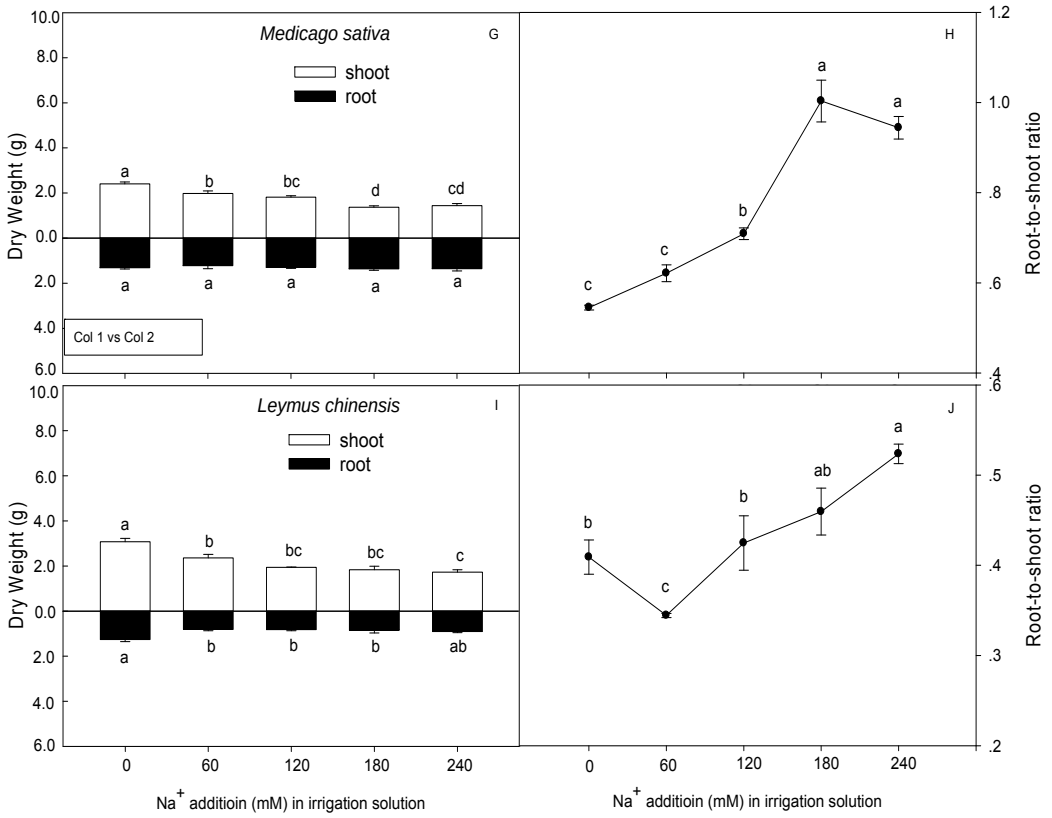


Figure 3 Shoot and root dry weight (g) and respective root/shoot ratio of five species subjected to different saline treatments. No significant differences for values followed by the same letter, $p<0.05$.

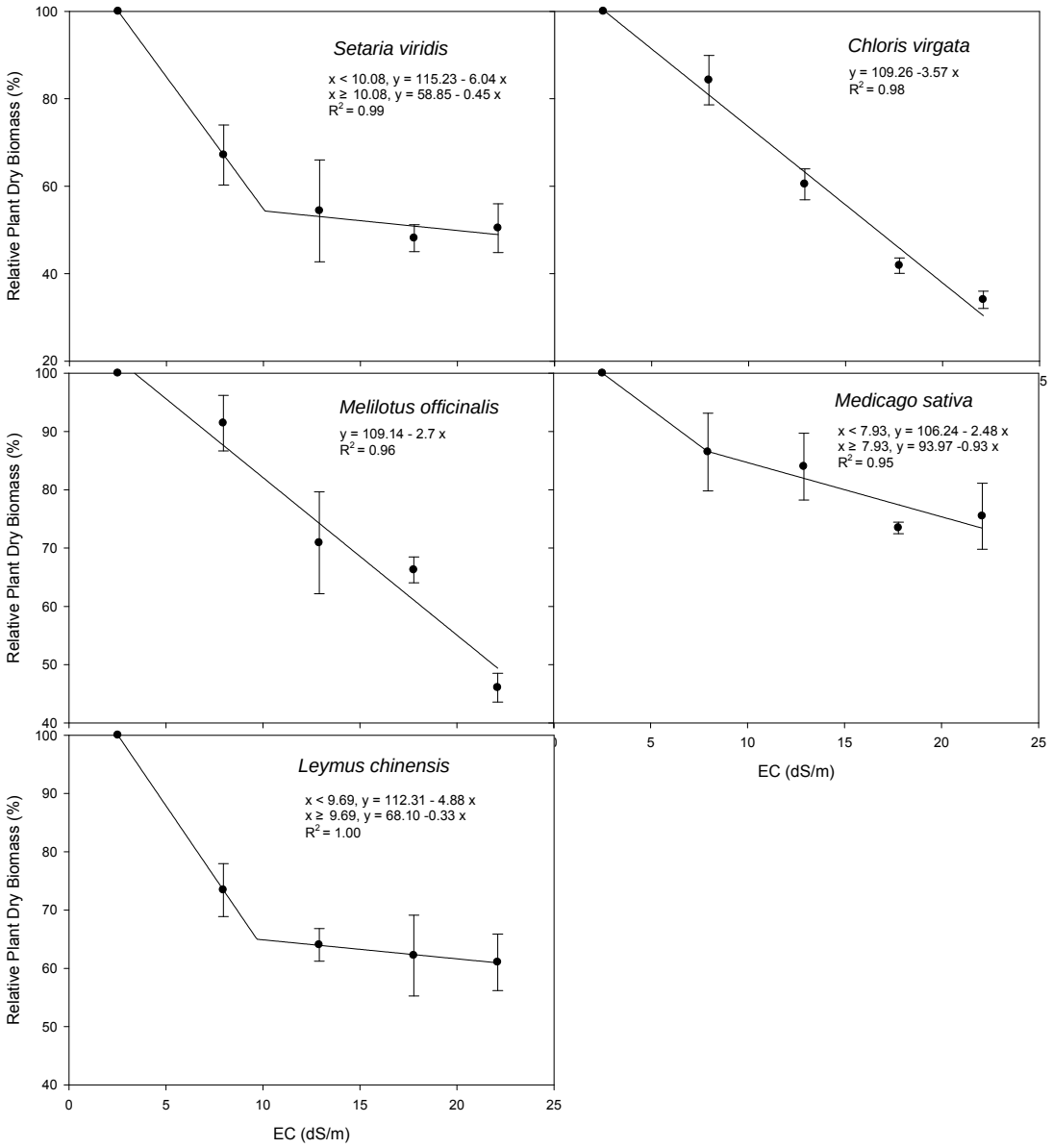


Figure 4 Response of plant total biomass expressed in percentage of controls to increasing EC value of irrigation solution.

Plant salt tolerance is generally evaluated by plotting the relative yield against root zone salinity, expressed as the electrical conductivity (EC) of either soil extract or irrigation water (methods refer to Maas and Hoffman 1997; Maggio et al. 2007). The relationship between the biomass under salinity and the electrical conductivity (EC) of irrigation solution was analyzed (Figure 4). The relative plant biomass was calculated as a percentage of the maximum dry mass obtained at non-saline conditions (EC=2.5 dS/m). Increasing EC resulted in a progressive reduction of the relative plant biomass.

The step-wise regression analysis revealed that the relative yield versus EC response was single linear for *Chloris* and *Melilotus*, with a slope of 3.6 and 2.7 respectively, while a bilinear reduction for *Setaria*, *Medicago* and *Leymus*. The two intersecting linear regions for both *Setaria* and *Leymus* changed at approximately 10 dS/m, with a sharp decrease in plant biomass (6% and 5% per dS/m respectively) until around 10 dS/m, afterwards only a slight decrease was observed (at 0.5% and 0.3% per dS/m respectively). In contrast, *Medicago* reduced biomass at 2.5% per dS/m before 8 dS/m and at 0.9 per dS/m beyond the threshold.

6.3 Specific leaf area (SLA)

Table 4 showed the changes of specific leaf area (SLA) in response to increasing salinity. SLA progressively decreased with an increase in salinity, suggesting that plants developed thicker leaves, although leaf area per plant decreased. But the threshold at which the decrease in SLA was statistically different between species. Generally, low salinity levels (0-60 mmol Na^+)) did not significantly reduce SLA in any species compared to their respective non-saline controls. SLA of perennial species (*Leymus, Medicago*) was relatively lower than those of annuals (*Melilotus, Chloris* and *Setaria*). These results indicate that perennial plants have lower leaf expansion rate and thicker leaves as compared with annual grasses.

Table 4 Specific leaf area (cm^2 g^{-1}) (means ± S.E.) for the five species in non-saline control and saline treatments (60, 120, 180, 240 mM Na^+, NaCl: Na_2SO_4=1:1)

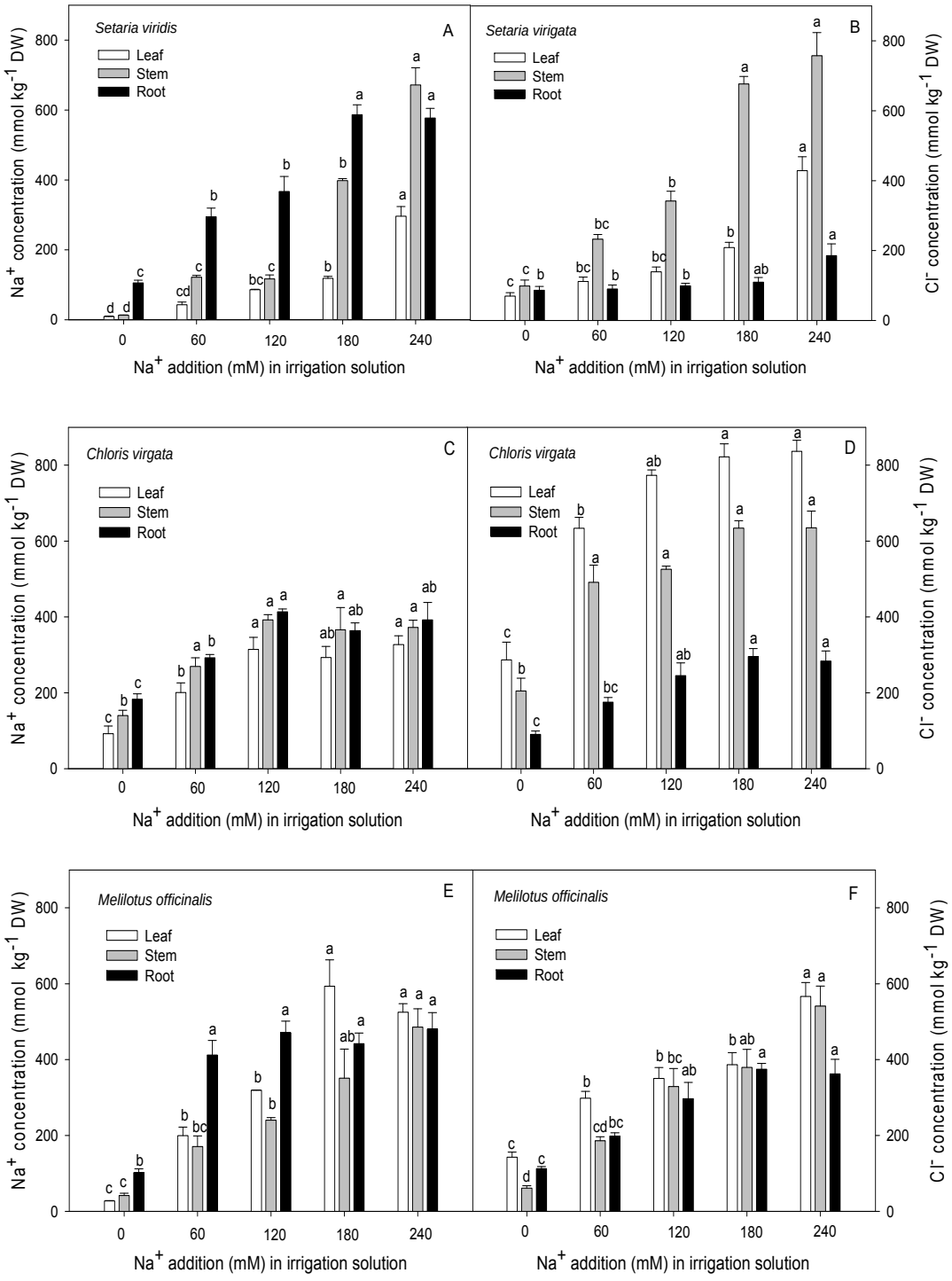
			Na^+ addition (mM) in irrigation solution		
Species	**0**	**60**	**120**	**180**	**240**
Leymus chinensis	261±0.5 a	229±6.1 ab	258±13.4 ab	222±6.9 b	230±6.3 ab
Medicago sativa	304±10.6 a	265±2.7 a	234±5.3 b	228±28.6 b	202±3.1 b
Melilotus officinalis	345±19.1 a	303±3.0 ab	286±6.5 ab	282±4.8 b	231±8.7 c
Chloris virgata	339±19.5 a	356±18.8 a	325±13.4 ab	261±9.6 bc	250±5.8 c
Setaria viridis	403±1.8 a	354±8.6 b	349±18.9 b	182±4.6 c	196±7.4 c

Means ± S.E. values followed by the same letter are not different at P = 0.05

6.4 Ion accumulation

At the end of the experimental period, salinity had led to a significant increase in Na^+ and Cl^- ions in all tissues (leaves, stems and roots) of treated plants. Ion allocation varied within the plant species, the salinity levels and the plant organs (Figure 5). For ***Setaria***, sodium concentration was highest in roots and lowest in leaves. Na in different plant tissues increased across the salinity gradient, which was most evident in shoots (leaves and stems) at the highest salt level (240 mM Na^+). Cl^- tended to accumulate in the stems, whereas the difference of chloride accumulation between leaves and roots increased with increasing salt stress. In comparison, sodium concentration in leaves, stems and roots of ***Chloris*** was not significantly different, the concentration increased from the non-saline control to 120 mM Na^+ (EC value = 12.9 dS/m), and remained more or less constant across the higher salinity levels. Enormously high amounts of chloride accumulated in leaves. The Cl^- concentration in stems was almost twice as high as that in roots. Salinity induced higher concentration of Na^+ in the leaves and stems of ***Melilotus***. The Na^+ concentration in roots almost doubled under salinity treatment but remained similar across the salt levels. Significantly high concentration of chloride in the shoot was only observed at the highest external salt application. ***Medicago*** and ***Melilotus*** are leguminous plants, the sodium concentration in the roots of both species showed similar pattern, which increased markedly under salinity and reached a plateau value across all salt levels. In contrast, the sodium concentration in the leaves of two species increased progressively with increasing salinity until 180 mM Na^+, sharing the highest concentration with treatment at 240 mM Na^+. The Na^+ concentration in the shoots of ***Medicago*** was significantly higher than that of ***Melilotus*** at 240 mM Na^+, indicating different capacity or strategy for salt accumulation. The concentration of Cl^- in different tissues of two species was observed to increase beginning with 60 mM Na^+ application and increased step wisely afterwards. Again, stronger accumulation of Cl^- in the shoots of ***Medicago*** was observed compared with ***Melilotus*** in the salinity range of 120 mM to 240 mM. It is noteworthy that ***Leymus*** showed different patterns of ion allocation among plant tissues. Sodium was mostly accumulated in roots and its

concentration was approximately twice as high as that in leaves or stems. Chloride concentration in roots was also significantly higher than that in leaves and stems at external sodium application of 60 and 120 mM, but the concentration in both leaves and stems was more than doubled as salinity increased up to 180 and 240 mM.



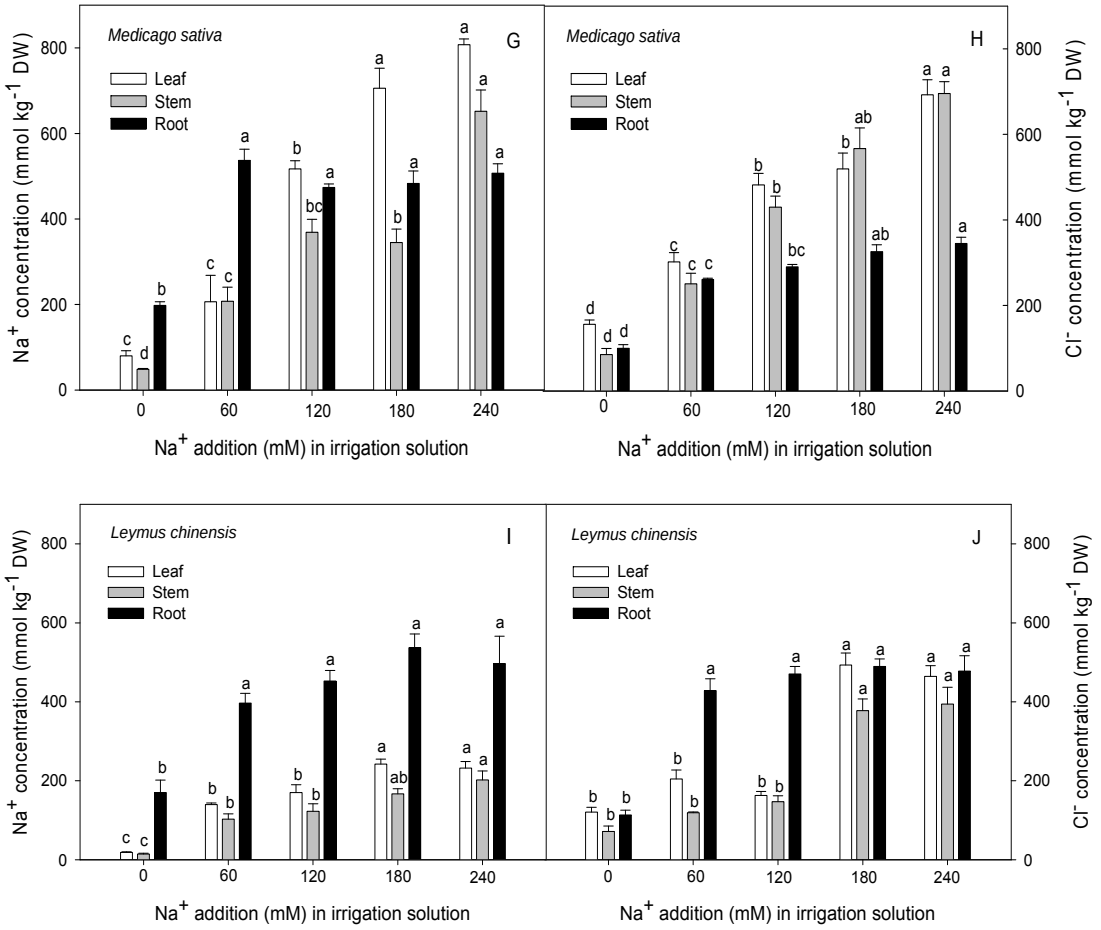
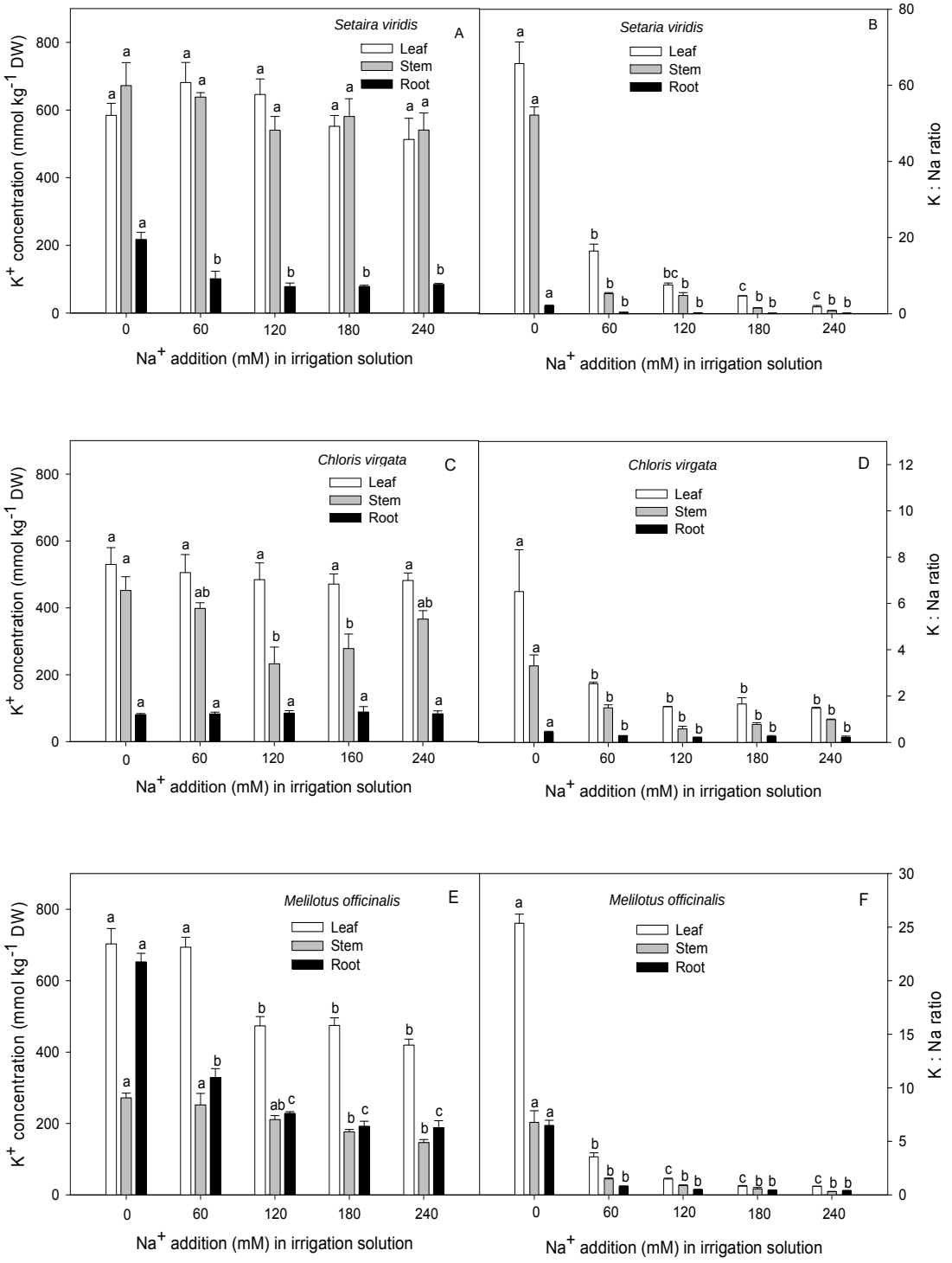


Figure 5: Na^+ and Cl^- concentrations (mmol kg^{-1} DW) in different tissues (leaves, stems and roots) of five species

In contrary to the concentration of Na and Cl, K concentration was less influenced by external salinity (Figure 6). K concentration of roots was lower than that of leaves or stems at all salt treatments, except for ***Medicago***. The potassium-to-sodium ratio in each plant tissue declined markedly when exposed to salt stress, and then decreased slightly but progressively across the salinity levels. K concentration in the roots of ***Setaria*** decreased under salinity compared to non-saline control; there was no significant difference in K concentration of the roots among different salinity levels. Shoot K concentration remained stable at different salt levels. K concentration in the leaves and roots of ***Chloris*** was hardly changed across all salt levels, while stem K was lower at salt treatments 120 and 180 mM in comparison with the non-saline treatment.

Application of 60 mmol sodium had no effect on the K content in the leaves and stems of ***Melilotus***, but strongly decreased the K concentration in roots. Salinity higher than 120 mM Na^+ did not further decrease K concentration in all tissues of *Melilotus*. For both ***Medicago*** and ***Leymus***, K concentration in different plant tissues showed no significant difference at each salt level compared to the non-saline treatments.



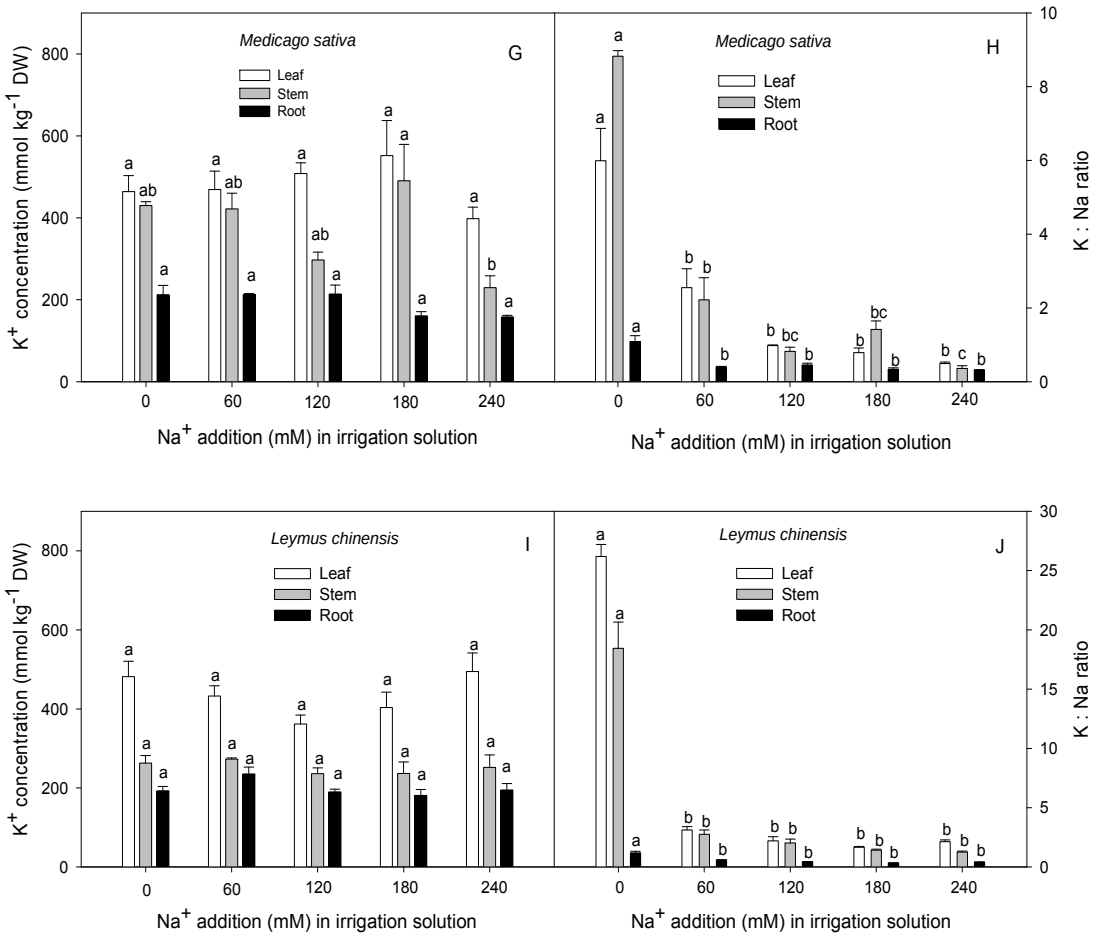


Figure 6 K^+ concentrations (mmol kg^{-1} DW) and respective K/Na ratio in different tissues (leaves, stems and roots) of five species

The increasing levels of salinity markedly reduced K/Na ratio in different plant tissues (leaves, stems and roots), accompanied by strong reduction in plant total biomass. We may wonder whether the ion molar ratios (K/Na ratio, Cl/K ratio or Cl/Na ratio) in leaves were correlated to total dry weight under salinity. The relationships between plant total dry weight under salinity and the molar ion ratio were examined according to the method referred by Asch et al. (2000). The y-axis indicates plant total dry weight under salinity as a percentage of the non-saline controls. The x-axis indicates values of ion ratios shown on a logarithmic scale (Figure 7 to Figure 9). Although the log-linear regression for plant total biomass and molar ion ratio was not statistically significant, the graphs showed great differences in biomass production and ion ratio among grass species. The difference in biomass induced by salinity indicates different

levels of salt resistance.

Generally, plant total biomass decreased as the leaf K/Na ratio decreased (Figure 7). This relationship was observed in *Medicago* and *Setaria*, less evident in other species. Especially for *Chloris*, the loss in biomass could not be explained by reduced K availability. *Medicago* had lower range of K/Na ratio compared with *Setaria*, whereas the total plant biomass was less affected by salinity.

The Cl/K ratio also indicated biomass reduction to some extent (Figure 8). Plant biomass decreased with the increased accumulation of chloride relative to potassium. *Medicago* was able to keep a high concentration of chloride relative potassium in tissues, whereas lost less total dry weight under salinity.

There seemed no clear relationship between Cl/Na ratio and reduction in plant total biomass (Figure 9). Especially for annual species (*Setaria*, *Chloris* and *Melilotus*), biomass reduction was independent of chloride-to-sodium ratio, probably because Cl and Na ions increased synchronously, their ratio might be similar at each salinity level, thus provided very poor indication of biomass reduction.

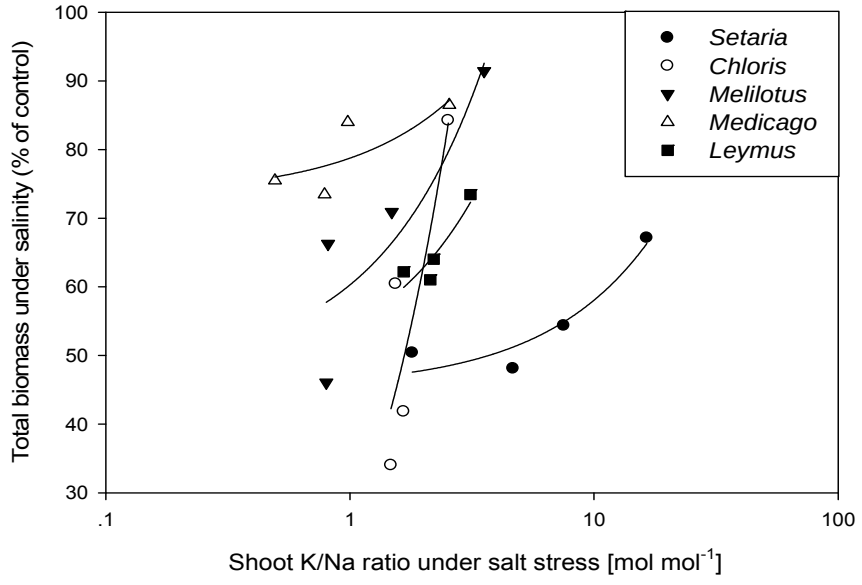


Figure 7: Relation between the mean leaf K/Na under salinity and total dry biomass under salinity relative to non-saline controls for the five species studied. Each data point represents a mean of three replicates.

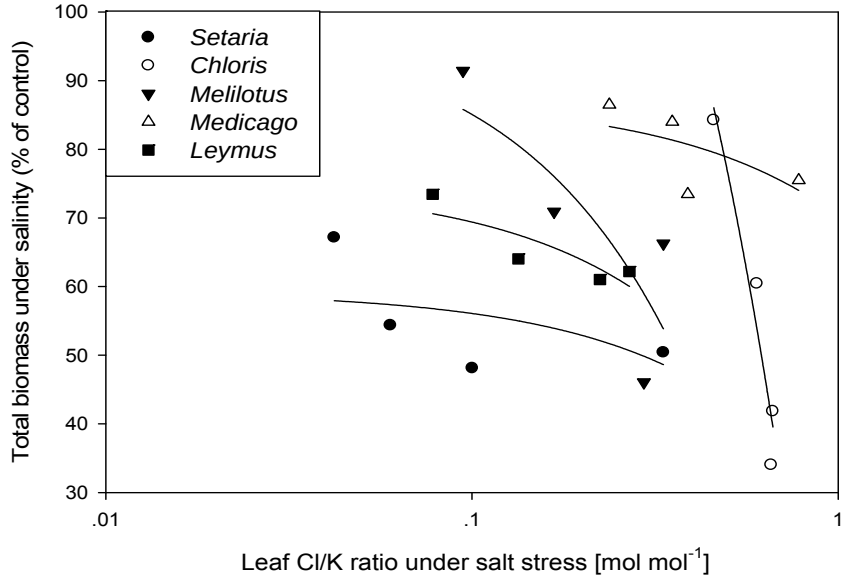


Figure 8: Relation between the mean leaf Cl/K under salinity and total dry biomass under salinity relative to non-saline controls for the five species studied. Each data point represents a mean of three replicates.

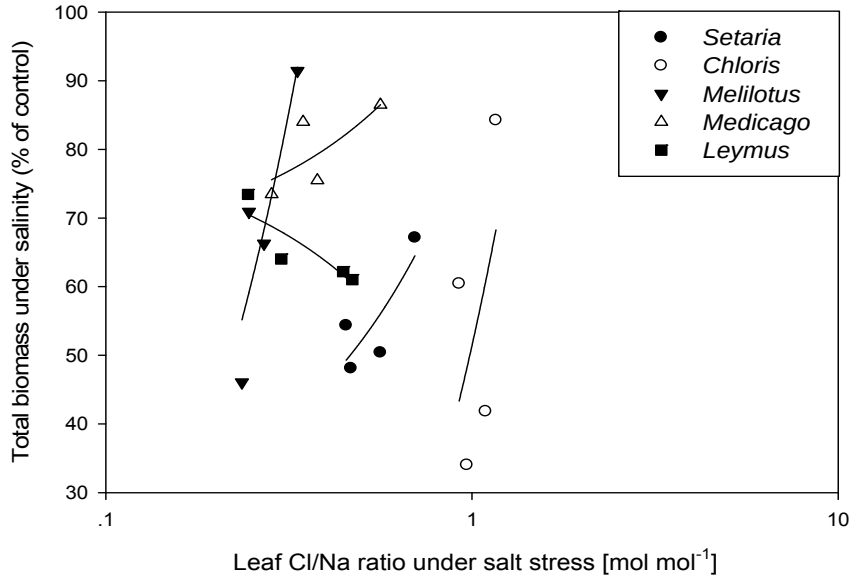


Figure 9: Relation between the mean leaf Cl/Na under salinity and total dry biomass under salinity relative to non-saline controls for the five species studied. Each data point represents a mean of three replicates.

6.5 Comparing salt includers and salt excluders

It's noteworthy that *Chloris* tried to restrict both sodium and chloride uptake into the shoots across increasing salinity levels (Figure 10 and Figure 11). The increment for sodium in leaves was less than five-fold and there was almost no increase observed at salinity higher than 120 mM Na^+. These results indicate that *Chloris* is a salt excluder. On the contrary, *Setaria* did not limit the uptake of salt ions, especially when the external salinity was added up to 180 mM. The sodium concentration increased by 50 and 30 times in the stems and leaves respectively at 240 mM, with less increment of chloride concentration (9 and 6 times in the leaves and stems respectively). As indicated by the incredibly high sodium load in the stem at 180 and 240 mM, *Setaria* mainly deposited Na in the stem tissue as an effort to protect photosynthetically active leaf tissues from excessive sodium concentrations. The capacity of other species (*Melilotus*, *Medicago* and *Leymus*) was much lower compared with *Setaria*.

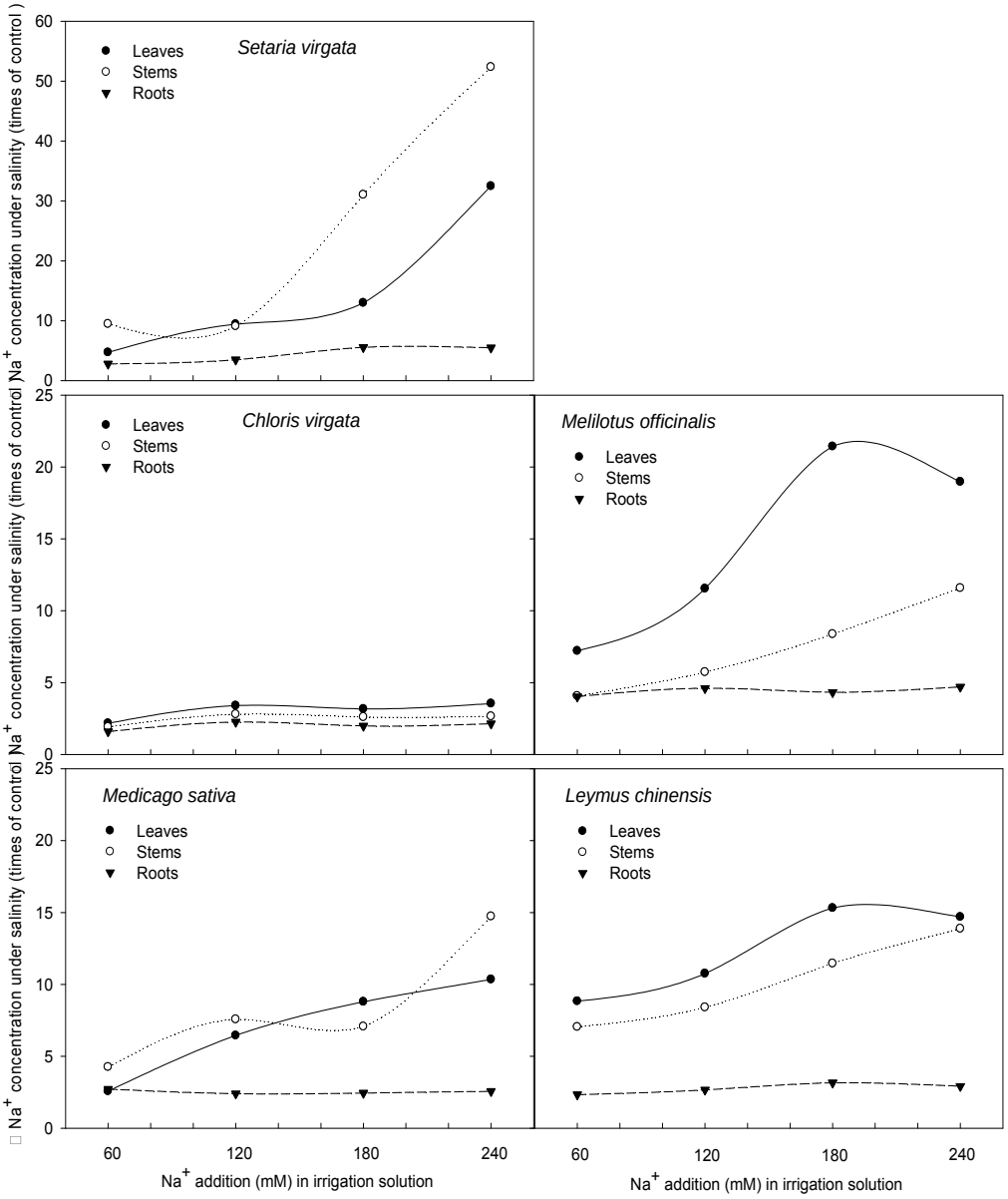


Figure 10: Na concentration at each salinity level, expressed as fold of non-saline controls. Comparison of Na accumulation within different plant tissues (leaves, stems and roots) of five species

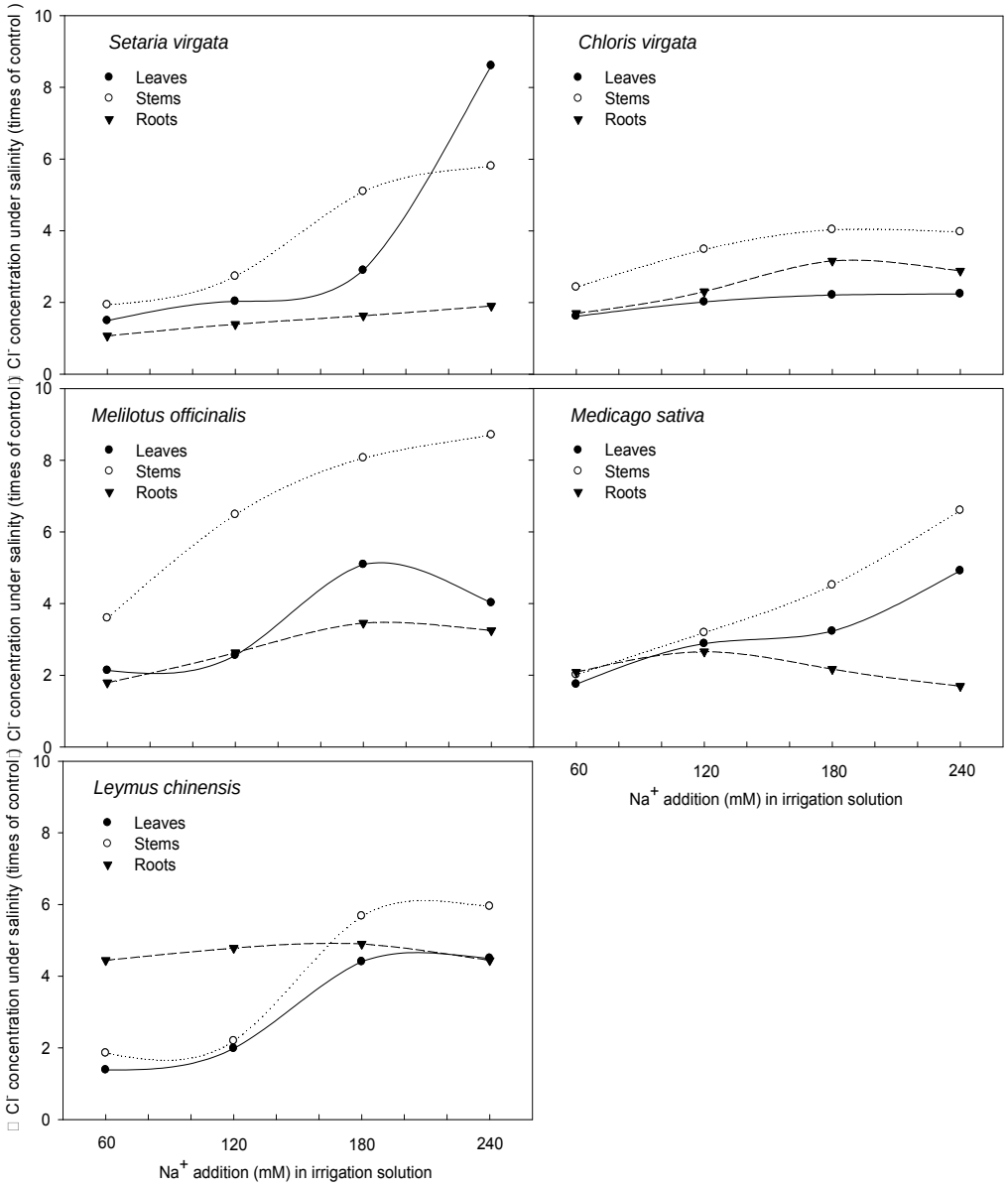


Figure 11: Cl concentration at each salinity level, expressed as fold of non-saline controls. Comparison of Cl accumulation within different plant tissues (leaves, stems and roots) of five species

6.6 Transpiration rate normalized by leaf area and its response of to increasing VPD

In the case of extremely high humidity (over 85% relative humidity), the calculated values of transpiration rate were negative at VPD 0.5 kPa, which occurred commonly for *Setaria* and *Chloris*. This might be because the two C_4 grasses species had larger leaf areas, on which the water vapor was more likely to condensate. Thus, the values for the transpiration rates at 0.5 VPD of *Setaria* and *Chloris* were discarded. The step-wise regression between transpiration rate and VPD was shown in **Figure 12 and Figure 13.** The regression at each salt treatment was compared with the non-saline treatment, indicated as control. Transpiration rate of *Setaria* under sodium treatment of 0, 60, 120 mM increased linearly over the range of tested VPD. These regression lines were roughly parallel, with slopes of 0.33, 0.35, 0.31 mmol H_2O m^{-2} s^{-1} kPa^{-1} respectively, although the transpiration rate across VPD levels at 120 mM Na^+ was slightly lower compared with the non-saline control. In contrast, the transpiration rate across all VPD levels increased when subjected to salinity addition of 180 and 240 mM. A two-segment linear regression provided the best fit at 180 and 240 mM Na+ treatment (R^2=0.72-0.79), with a breakpoint of 1.29, 1.24kPa respectively. The slope beyond the break-point in 240 mM-treated plants was more than doubled as compared to the 180 mM-treated plants.

For *Chloris*, a single linear regression provided the best fit for salinity treatments in the range of 0 to 180 mM. There was only slight difference of transpiration rates between non-saline and 60 mM-treated plants. However, transpiration rate was constrained at sodium addition of 120 and 180 mM, with less constraining effect at the latter salt concentration. At the highest salinity level 240 mM, transpiration rate was lower than the non-saline control prior to VPD 1.5 kPa, and increased to higher levels afterwards, accompanied with a limitation on transpiration rate at 2.6 kPa.

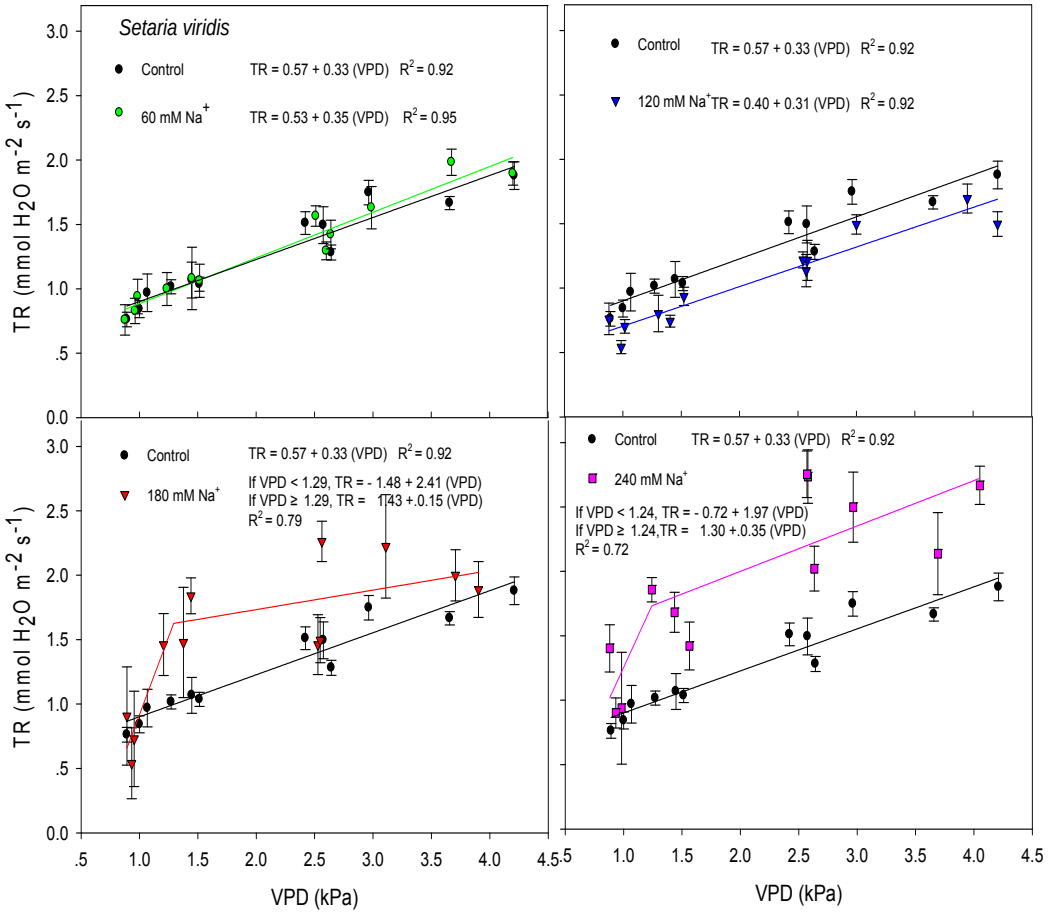


Figure 12: Whole-plant transpiration response (mmol H_2O m^{-2} s^{-1}) of Setaria viridis to increasing VPD in control (non-saline treatment) and in salt-treated plants (60, 120, 180, 240 mM Na^+ respectively).

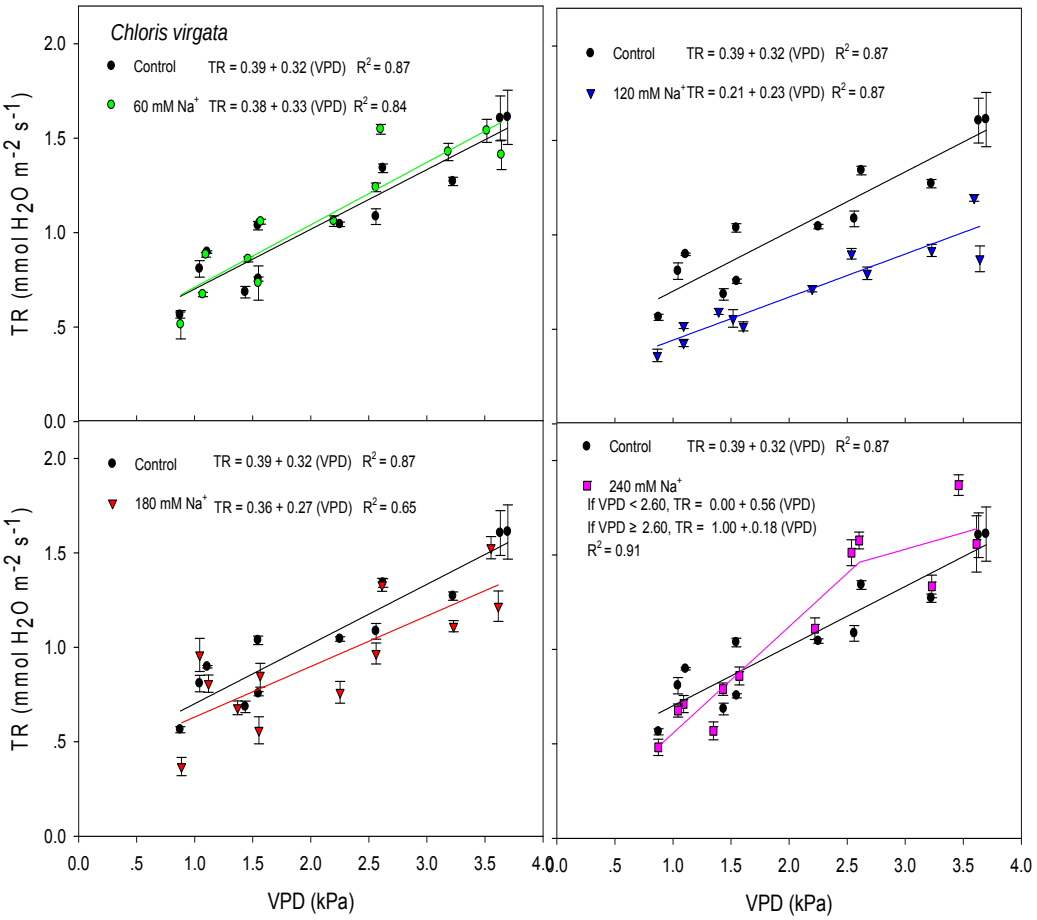


Figure 13: Whole-plant transpiration response (mmol H_2O m^{-2} s^{-1}) of C. virgata to increasing VPD in control (non-saline treatment) and in salt-treated plants (60, 120, 180, 240 mM Na^+ respectively).

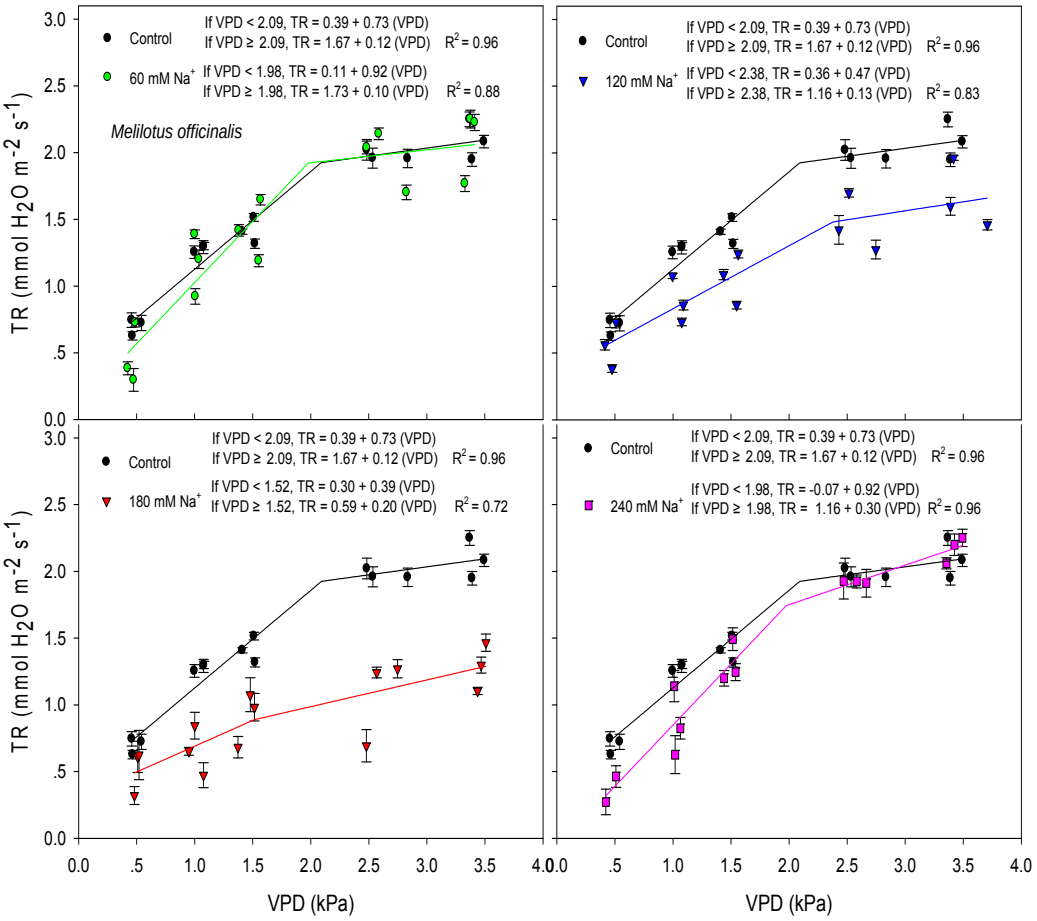


Figure 14: Whole-plant transpiration response (mmol H_2O m^{-2} s^{-1}) of *Melilotus officinalis* to increasing VPD in control (non-saline treatment) and in salt-treated plants (60, 120, 180, 240 mM Na^+ respectively).

The magnitude of transpiration rate between non-saline control and 60 mM treatment was quite similar, with a breakpoint at 2.09 and 1.98 kPa respectively (**Figure 14**). Increasing the salt concentration further suppressed the transpiration rate, and the effect was most evident at 180 mM Na^+. Consistent with the result observed in *Chloris* at salinity treatment of 240 mM, the transpiration rate was only constrained prior to the breakpoint at approximately VPD 2.0 kPa, then it reached the similar level as non-saline conditions.

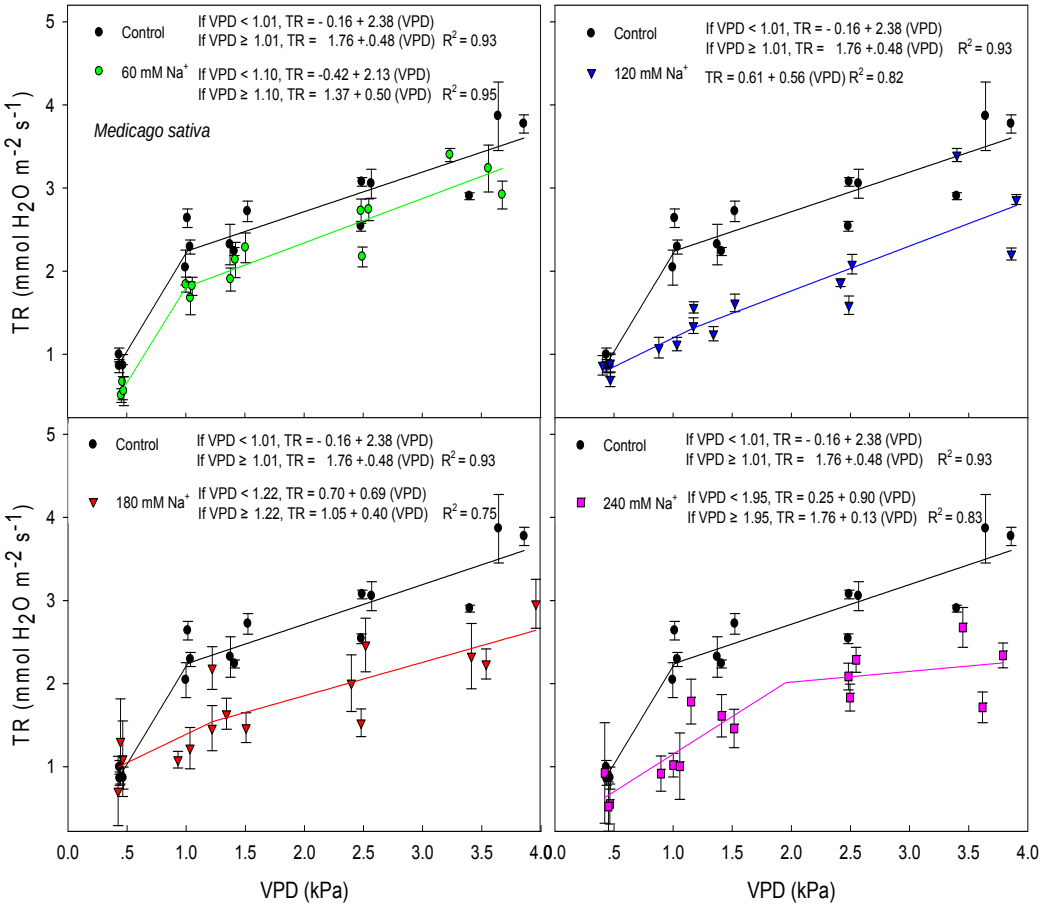


Figure 15: Whole-plant transpiration response (mmol H_2O m^{-2} s^{-1}) of Medicago sativa to increasing VPD in control (non-saline treatment) and in salt-treated plants (60, 120, 180, 240 mM Na^+ respectively)

In *Medicago*, transpiration rate of salt-treated plants was generally lower than under non-saline conditions and decreased with increasing salinity (**Figure 15**). Higher salt concentration treatments shifted the breakpoint to higher VPD (from 1 kPa at non-salinity to approximately 2 kPa at 240 mM-treatment), although no significant breakpoint was observed at salt level 120 mM. The constraining effect of salinity on transpiration rate was most manifest at 240 mM sodium stress, with extremely low transpiration (0.13mmol H_2O m^{-2} s^{-1} kPa^{-1}) at VPD higher than 2 kPa.

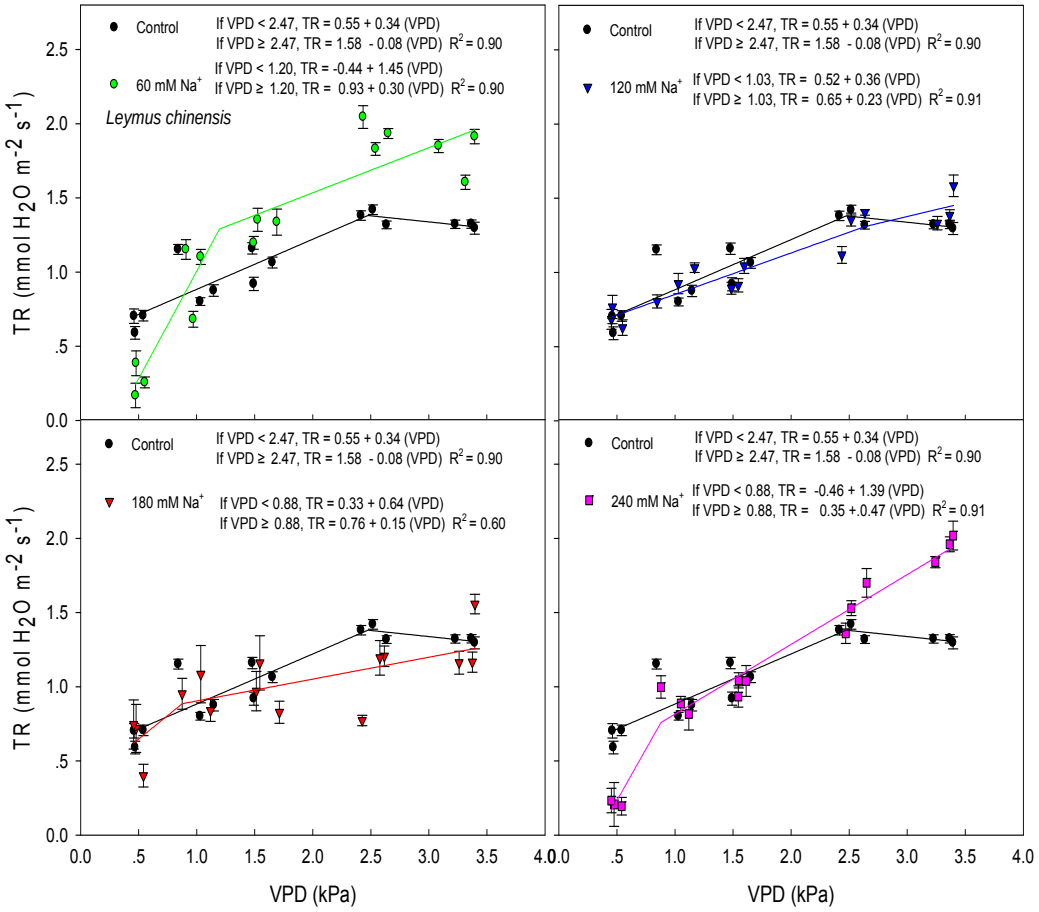


Figure 16: Whole-plant transpiration response (mmol H_2O m^{-2} s^{-1}) of Leymus chinensis to increasing VPD in control (non-saline treatment) and in salt-treated plants (60, 120, 180, 240 mM Na^+ respectively)

Transpiration rate in non-saline plants increased linearly with VPD up to 2.47kPa, above which an obvious limitation on transpiration rate was observed (**Figure 16**). Salinity imposed more complicated effect on the transpiration rate across VPD levels. The analyses of other four species showed that 60 mM Na^+ had little effect (in *Setaria, Chloris and Melilotus*) or slightly suppressed (in *Medicago*) transpiration rate, however, an unexpectedly higher transpiration rate in response to increasing VPD (over 1 kPa) was observed. Higher salinity (120 and 180 mM Na^+) suppressed transpiration rate compared with the non-salinity. The restrained transpiration rate at 240 was only observed prior the VPD breakpoint (0.88 kPa).

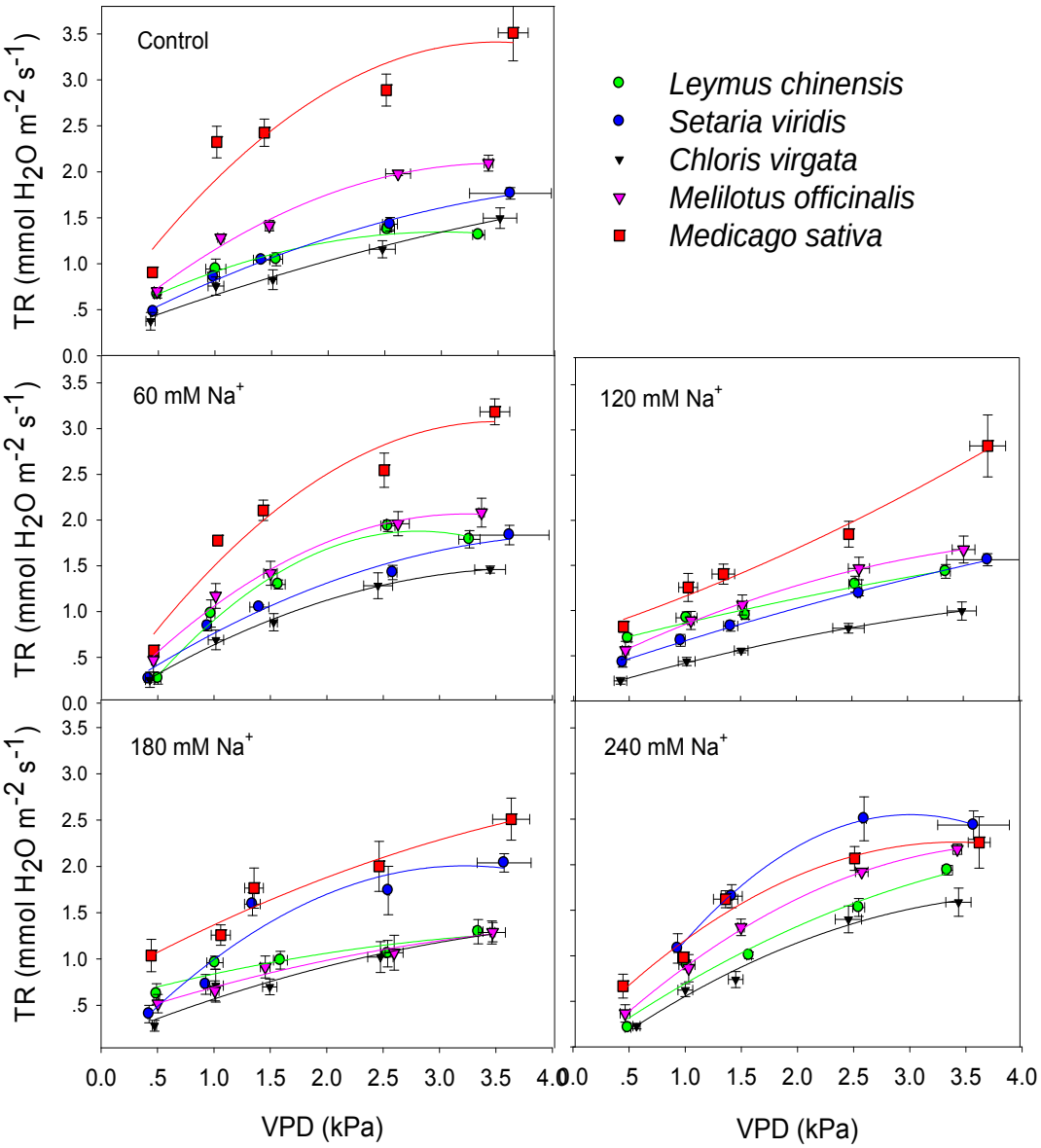


Figure 17: A comparison of whole-plant transpiration response (mmol H_2O m^{-2} s^{-1}) of five grass species to increasing VPD in control (non-saline treatment) and in salt-treated plants (60, 120, 180, 240 mM Na^+ respectively).

The responses of transpiration rate to increasing VPD differed between species and salt treatments. Generally, C_3 grass species (*Medicago, Melilotus, Leyums*) had higher transpiration rates than C_4 species (*Setaria and Chloris*) under non-salinity and moderate salt conditions (≤120 mM Na^+). However, the difference of transpiration responses to increasing VPD between functional groups was no longer observed under higher salinity levels (180 and 240 mM Na^+). The transpiration rate of *Setaria* was

increased to incredibly high level, even higher than that of C_3 grasses at salinity 240 mM. In contrast, another C_4 species (*Chloris*) maintained lowest transpiration rate across increasing VPDs at each salinity level. This may relate to the difference of resistance strategy against salt between the two C_4 grass species. The results also indicated that leguminous species were more prone to lose water with increasing VPD, while *Chloris* was most conservative in controlling canopy transpiration water loss. The relationship between accumulation of saline ions and transpiration responses to VPD changes will be discussed.

6.7 Transpiration rate normalized by leaf dry weight and its relationship to increasing VPD

It's important to note that leaves were curling and rolling under salinity, most commonly observed at salinity higher than 180 mM Na^+. Thus it was difficult to measure the exact transpiring surface. The rolled leaves were directly scanned to determine the leaf area because the wrapped leaf areas were not exposed to atmospheric VPD, from which transpiration could be minimized or avoided. However, if the measured canopy area was smaller than the actual transpiring leaf surface, the calculated transpiration rate (water loss per unit leaf area and unit time) under high salinity might be overestimated due to the smaller denominator. To testify this speculation, the transpiration rate was also calculated based on a more reliably measured parameter, the leaf dry weight (LW). **Figure 18-22** showed the comparison of transpiration response (mmol H_2O mg^{-1} LW s^{-1}) to increasing VPD among different salinity treatments in each grass species respectively. *Setaria viridis* and *Melilotus officinalis* showed a clear trend that transpiration rate was repressed with a stepwise salinity increase to 180 mM Na^+, however, was increased again at higher salinity 240 mM Na^+ when VPD increased. *Chloris virgata* and in *Leymus chinensis* also showed similar trait of transpiration response at salinity 240 mM Na^+, but put forward a new question: why transpiration at 60 mM Na^+ was higher than control? In contrast, the transpiration rate of *Medicago sativa* was consecutively decreased by increasing salinity

compared with control.

Whereas, calculating transpiration rate either as water loss per unit leaf area or water loss per unit leaf dry weight pointed to the same conclusion: moderate salinity (below 120 mM Na^+) decreased transpiration rate, however severe external salinity addition (240 mM Na^+) shifted up the magnitude of transpiration rate, the extent of increment differed between species.

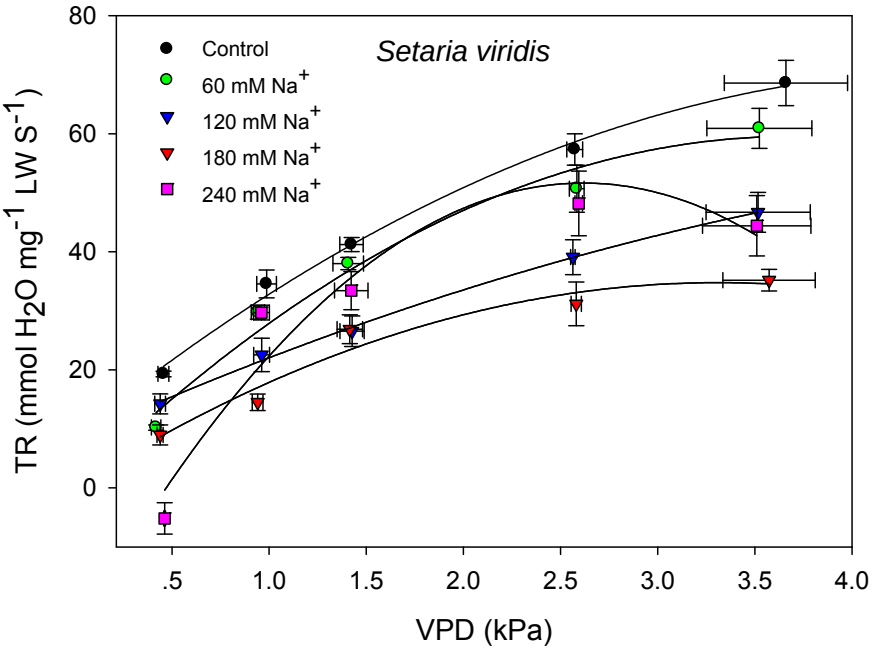


Figure 18: Transpiration rate (TR) calculated as water loss per unit leaf dry weight (mmol H_2O mg^{-1} LW s^{-1}). Comparison of TR response to increasing VPD among different salinity treatments in *Setaria viridis*.

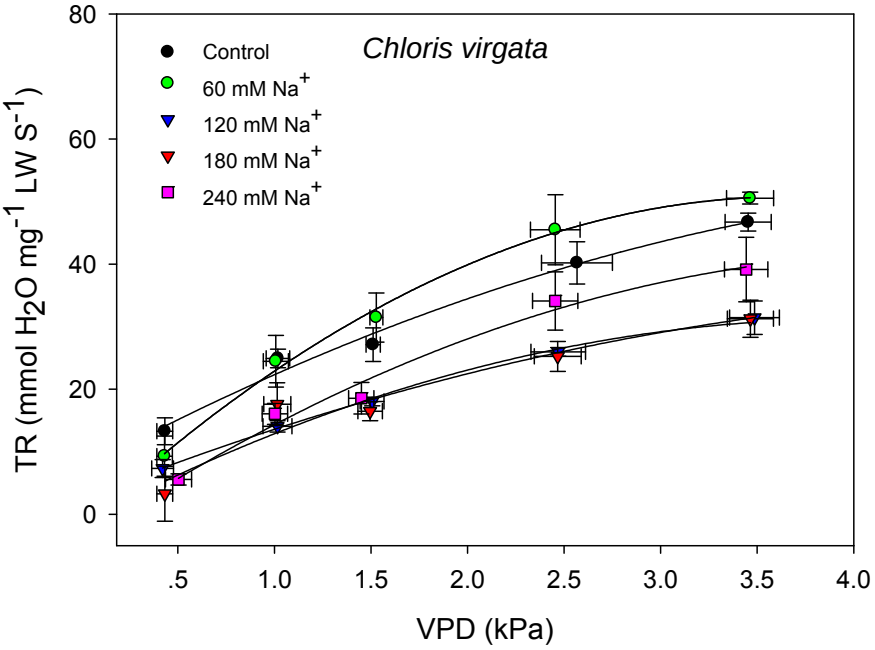


Figure 19: Transpiration rate (TR) calculated as water loss per unit leaf dry weight (mmol H_2O mg^{-1} LW s^{-1}). Comparison of TR response to increasing VPD among different salinity treatments in *Chloris virgata*.

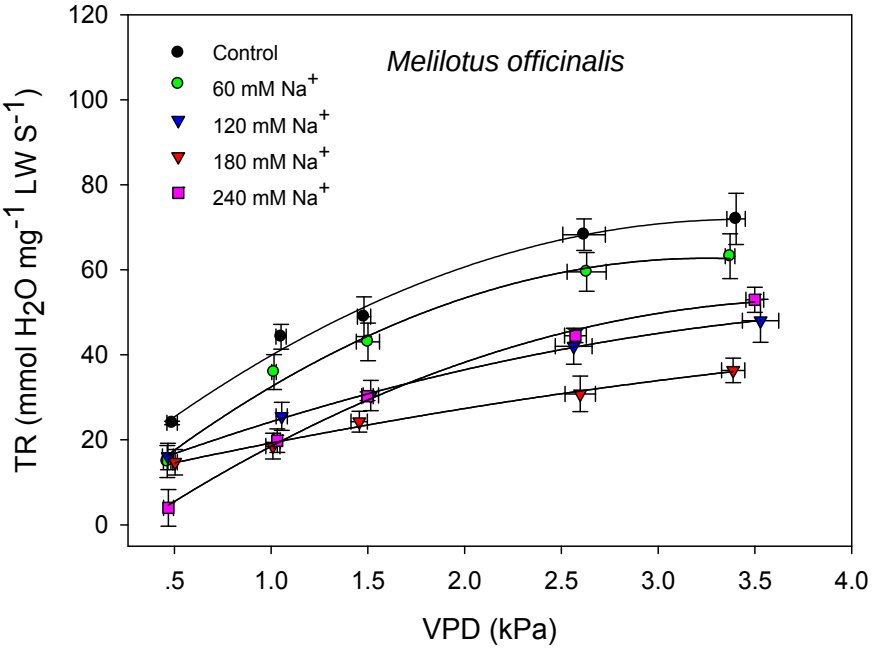


Figure 20: Transpiration rate (TR) calculated as water loss per unit leaf dry weight (mmol H_2O mg^{-1} LW s^{-1}). Comparison of TR response to increasing VPD among different salinity treatments in *Melilotus officinalis*.

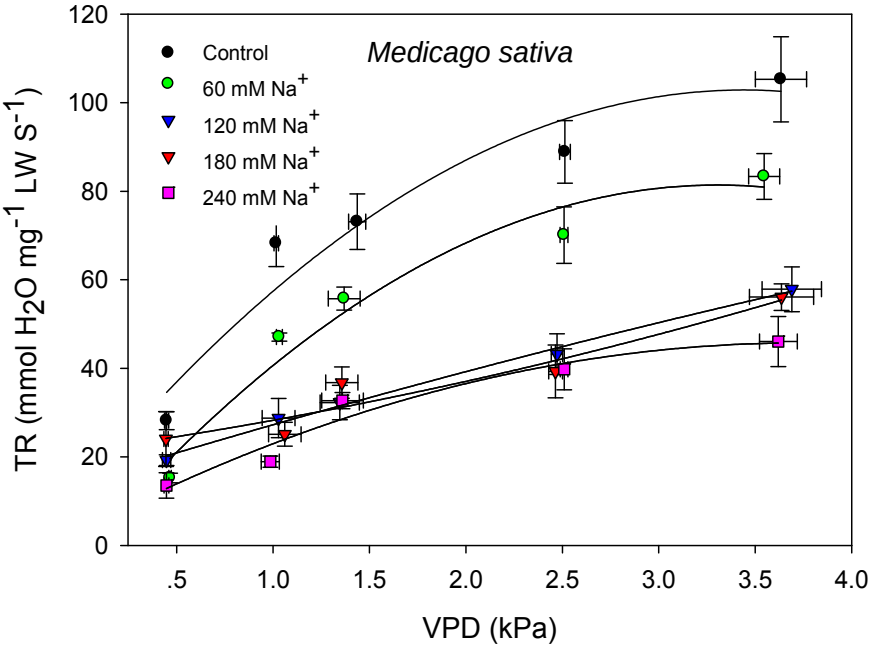


Figure 21: Transpiration rate (TR) calculated as water loss per unit leaf dry weight (mmol H_2O mg^{-1} LW s^{-1}). Comparison of TR response to increasing VPD among different salinity treatments in *Medicago sativa*.

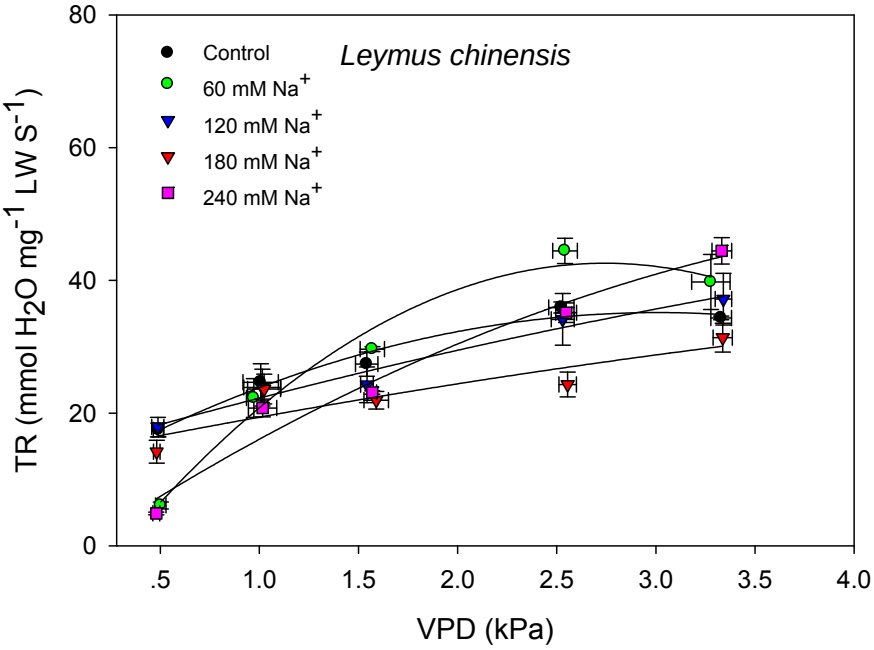


Figure 22: Transpiration rate (TR) calculated as water loss per unit leaf dry weight (mmol H_2O mg^{-1} LW s^{-1}). Comparison of TR response to increasing VPD among different salinity treatments in *Leymus chinensis.*

6.8 Normalized canopy conductance in response to VPD

Figure 23 shows the changes of canopy conductance (Gs) at different VPD in the five grass species studied. All species exhibited an overall trend of decreasing Gs with increasing VPD. This pattern was also observed across all salinity levels. The changes in canopy conductance fitted well with the responses of transpiration rates to VPD. Under moderate salinity (below 120 mM Na^+), Gs of *Mediago* was markedly higher among all the species, while *Chloris* had the lowest Gs, this was consistent with the rank of transpiration rate across VPDs among species. It was evident that species which had the highest Gs at a low VPD also possessed the highest Gs at higher VPD. Higher Gs at a low VPD tended to decrease more sharply as VPD increased, and the lowest Gs decreased only slightly at high VPDs. The differences in canopy conductance became smaller as VPD increased. Gs of species changed their ranks when exposed to severe external salinity (180 and 240 mM Na^+). *Setaria* exhibited significantly larger Gs at lower VPD but reduced Gs to a value lower than that of *Medicago* when VPD exceeded 2.5 kPa.

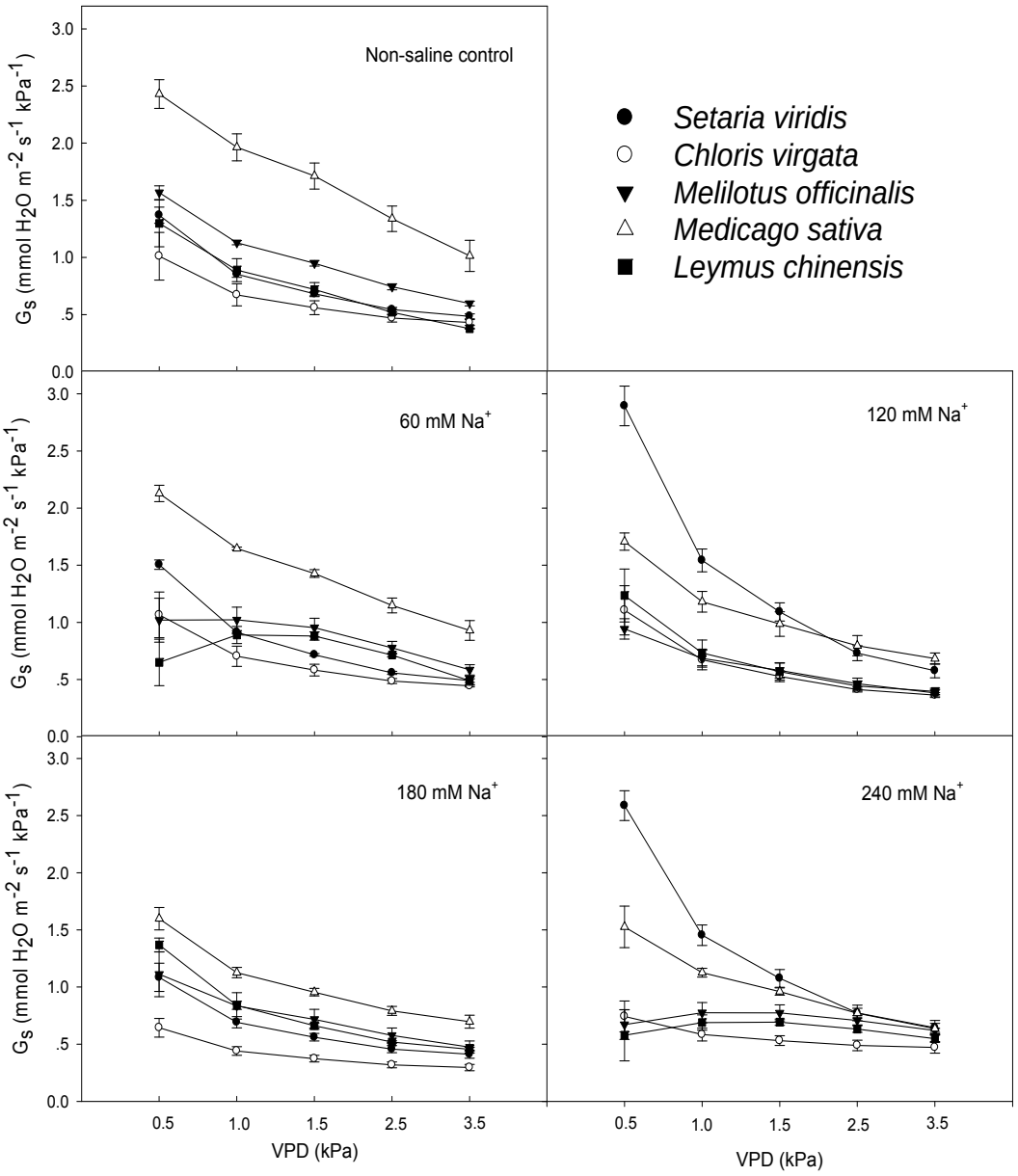


Figure 23: Extrapolated canopy conductance (Gs, mmol H2O m-2 s-1) at exact 0.5, 1.0, 1.5, 2.5 and 3.5 kPa. Changes of Gs in response to increasing VPD at each salinity levels, each point and bar represents the average of three replicates and S.E.

7. Discussion

7.1 Biomass reduction and partitioning in response to salinity

Dry weights (leaves, stems, roots) were strongly reduced under salinity compared to non-saline controls. Salinity had adverse effects not only on the biomass, but also on other morphological parameters such as plant height, number of leaves, root length and root-shoot ratio (Munns and Tester 2008). The deleterious effects of salt stress are commonly thought to result from a combination of osmotic and ionic stresses (Chen et al. 2008). Relative plant dry biomass (ratio of biomass exposed to salinity relative to control) has been reported to be an accurate indicator of relative salinity tolerance (Genc et al. 2007). Other studies also indicated that shoot biomass is a trait which showed differences more than other traits and has been used as an index for salt tolerance (Rahnama et al. 2010). The grass species in our study demonstrated different responses of root-to-shoot ratio to salinity, depending on life forms. Although salinity-tolerant perennial species had markedly less total biomass than the less salt-tolerant annual species, perennial C_3 grass species (*Leymus*, *Medicago*) responded to increasing salinity with an increasing root-to-shoot ratio. This was caused by a continuous decline in the shoot biomass but a relatively stable root dry weight. However, the annual grasses studied, including two C_4 species (*Chloris*, *Setaria*) and one C_3 species (*Melilotus*), showed a general decreasing trend in the root-to-shoot ratio under high salinity, especially at 240 mM Na^+. The difference of sensitivity between root growth and shoot growth indicates different strategies of annual and perennial grass species in response to salinity. Annual grasses do not have to store a pool of carbohydrates in roots for survival and re-growth in the following year; therefore they prioritize the growth of aboveground tissues.

The physiological significance of root-to-shoot ratio modification under salinity remains a topic for discussion, since the balance between water-obtaining and water-losing tissues determines the whole-plant water relations (Alshammary et al. 2004). An increase in root-to-shoot ratio is a tolerance mechanism against salinity

because the increased root-to-shoot ratio expands water-absorbing organs while reduces potential water loss (da Silva et al. 200). However, a reduced root-to-shoot ratio may improve salinity tolerance by restricting the flux of toxic ions to the shoot and consequently by delaying the onset of the tolerance threshold (Dalton et al. 2000). Besides, an adaptation to high salinity involves the premature abscission of the oldest leaves, which may partially explain why the share of shoots decreased. It should be noted that only slight visual symptoms of toxicity were observed in this study, such as senescence of primary leaves and extensive leaf rolling at 240 mM Na^+.

7.2 Leaf area and SLA affected by salinity

Leaf area is the most sensitive and readily observable growth parameter in response to salinity stress (da Silva et al. 2008). A reduction in leaf area development relative to root growth reduces the water use, thus allowing plants to conserve soil moisture and prevent an increase in the soil salt concentration (Munns and Tester 2008). Specific leaf area (SLA) is the ratio between leaf blade area and its dry weight, indicating leaf thickness. SLA has often been observed to decrease under salt conditions (Giuffrida et al. 2001; Rahnama et al. 2010). Thicker leaves have a higher density of chlorophyll and proteins per unit leaf area and therefore greater photosynthetic capacity per unit leaf area compared to thinner leaves. Leaf expansion depends on the ability to avoid excessive concentrations of ions in the transpiring tissues. Salinity affects leaf expansion earlier than photosynthesis. Different sensitivity of photosynthesis and leaf area expansion to soil salinity may explain the decrease in SLA (Lambers et al. 2008). SLA in perennials was relatively lower than in annuals. This may be explained by their different growth rates. Perennial grasses develop leaf area more slowly than annuals, thus have smaller leaf area, the accumulated photosynthetic products increase the leaf thickness.

7.3 Ion accumulation affected by salinity

Low Na^+ and high K^+ in the cytoplasm are essential for the maintenance of many

enzymatic processes (James et al. 2006). Potassium plays a key role in several physiological processes, such as osmotic regulation, protein synthesis and enzyme activation (Zhu 2003; Shabala and Cuin 2008)). One of the most damaging consequences of salt stress is an influx of Na^+ and a decrease of K^+ in plant tissues. This study showed that the concentration of Na^+ was remarkably higher in roots than in shoots. A high Na^+ concentration in roots, with the concurrent restricted Na^+ accumulation in leaves was speculated to be primarily associated with improved salt tolerance (Praxedes et al. 2009). This might also attribute to the contact with the rooting medium where sodium directly entered the root cells.

The absorbed Na^+ is either compartmentalized into the vacuole or excreted into the apoplastic space which is then transported upward following the transpirational water flow (Munns and Tester 2008). Stomatal closure is believed to function in mitigating ion fluxes to the shoots (Munns and Termaat 1986; Hasegawa et al. 2000). The consequently reduced canopy transpiration effectively protects leaves from excessively high Na^+ concentrations (Li et al. 2010). In the present study, we found that the Na and Cl^- concentration increased and the K^+ concentration slightly decreased, indicating competitive inhibition between the absorption of Na^+ and K^+ and the substitution of K^+ by Na^+ may lead to nutritional imbalance. The results are in agreement with the patterns of potassium and sodium also found in alfalfa by Li et al. (2010). The substantial increase in Na^+ and Cl^- concentrations in leaves might inhibit the leaf expansion, and subsequently decrease total leaf area and plant height. In contrast to Na^+ and Cl^-, K^+ generally remained constant at low-to-moderate (less than 120 mM Na^+) salinity but decreased at higher salt levels (over 180 mM Na^+), showing great differences among plant species. For all the species studied, K/Na ratio of different plant tissues was markedly reduced by salinity as compared to the non-saline controls, even under a weak concentration of 60 mM. Increasing salinity in the rhizosphere caused perturbation of ionic steady state for the K/Na ratio, because excessive sodium reduces the acquisition and intercellular influx of potassium (Hasegawa et al. 2000).

The relation between potassium and sodium in leaves was used to indicate seedling survival (Yeo and Flowers 1993; Genc et al. 2007) and grain yield (Asch et al. 2000)

under salinity. However, the effects of potassium and sodium concentrations either in the leaves or in the shoots on plant performance under salinity are controversially argued. Asch et al. (2000) reported that there was a significant logarithmic correlation between leaf K/Na at different salinity and relative grain yield to controls in rice. Following the same analytical method, the relation between total biomass under salinity relative to non-salinity and ion ratios (K/Na, Cl/K and Cl/Na ratios respectively) was examined. Contrary to their results, no significant linear correlation for any species was found. In comparison with K/Na ratio, the Cl/K ratio provides better indication of salt resistance and biomass reduction in this current study.

7.4 Comparing transpiration response of C_3 and C_4 species to VPD

Numerous studies investigated the influence of environmental parameters on stomatal response to VPD, such as the effects of humidity pre-treatments on the stomatal responses of C_3 and C_4 species (Kawamitsu et al. 1993); the sensitivity of stomata to altered CO_2 (Dai et al. 1992; Maherali et al. 2003), the effects of light (Bunce 2006) and changing temperatures (Sinclair et al. 2007). But no study comparing differences in transpiration response between C_3 and C_4 species exposed to different salinity conditions has been reported to date. This study generally showed that C_3 species had relatively higher transpiration rate than C_4 species under low to moderate salinity (sodium concentration below 120 mM). At higher salt stress (180 and 240 mM), response of transpiration to increasing VPD was not significantly different between functional groups of photosynthetic pathways, rather differed between salt-including and salt-excluding species. Water use efficiency (WUE) (ratio of photosynthesis rate to transpiration rate) is closely linked with different photosynthetic types. C_4 species have a photosynthetic advantage over C_3 plants because they are able to maintain the same net photosynthesis at smaller stomatal aperture with a CO_2 concentration pump based on phosphoenolpyruvate carboxylase (Kawamitsu et al. 1993). C_4 species typically have lower stomatal conductance and higher water use efficiency than C_3 species, coordinating with reduced hydraulic conductance per unit leaf area compared with C_3

species. This explains why C_4 species had lower transpiration rate across VPDs compared with C_3 species. Another explanation involves leaf surface resistance. Perhaps the outer epidermal surfaces of C_4 species are covered by a thicker layer of cuticle than C_3 species, but supporting information on leaf anatomy needs to be gathered. However, the latest study showed that C_3 and C_4 species shared common relationships between hydraulic conductance and responses to increasing VPD (Ocheltee et al. 2013), casting doubt on the findings that the axial transport of water through the xylem was lower in C_4 compared with C_3 species (Kocacinar et al. 2003 and 2008). Our results sided with the latter statement based on the measurements of exposing the whole plant to changes in VPD on short time scales.

Transpiration typically increases with increasing VPD and the response varies among species and genotypes (Fletcher et al. 2007; Sinclair et al. 2007). We have shown that C_3 species exhibited a breakpoint in the relationship of transpiration rate to VPD, while the transpiration rate for C_4 species increased linearly with increasing VPD under no or only moderate salinity. The results agree with previous findings in C_3 and C_4 turfgrasses (Wherley and Sinclair 2009). They also predicted that C_4 species would demonstrate a transpiration limitation if VPD was increased beyond 3.0 kPa, which was the upper limit in their study. However, contrary results were found in the current study. No breakpoint in C_4 species was detected even when VPD was increased up to 3.5-4.0 kPa.

Salt stress had a significant effect on the transpiration response to increasing VPD. Salinity altered the slope of the single linear regression in C_4 species (below 120 mM Na^+), and changed it into two-segment linear regression (at 180 or 240 mM Na^+). For C_3 species, the occurrence of VPD breakpoint that limited the increment of transpiration rate was affected by salinity, indicative of salt-induced changes in hydraulic conductance within the plants. Changes in leaf hydraulic conductance have been modeled to result in feedforward regulation in the response of transpiration to high levels of VPD (Buckley 2005). Root hydraulic conductance also affects the point at which plants reach their maximum rate of transpiration or begin to reduce transpiration in response to VPD (Sadok and Sinclair 2010). High hydraulic conductance of the root

system could minimize the water potential gradient from soil to leaf at high VPD, allowing plants to maintain higher rates of stomatal conductance and preventing a decline in transpiration in response to high VPD. However, root hydraulic conductance has not been tested at various salinity treatments in this study.

7.5 Magnitude of transpiration rate affected by salinity

The present study showed complex and species-specific behavior of canopy transpiration in response to increasing salinity. Transpiration of the two C_4 grasses *(Setaria, Chloris*) was slightly decreased at moderate salinity (60 and 120 mM Na^+) compared with plants under non-saline conditions, but increased at higher salinity (180 and 240 mM Na^+). However, *Melilotus* and *Leymus* restricted transpiration rate cross increasing VPDs as external salinity reached 180 mM Na^+, but lost stomatal control to high evaporative demand at salt stress of 240 mM. *Medicago* was more salt-tolerant than other species in terms of biomass reduction, its transpiration rate across the VPD levels was down-regulated by increasing salinity. It is known that stomatal conductance and transpiration decrease with increasing salt intensity in many plants, ranging from trees (Rajaona et al. 2012), thorny evergreen bush (Torrecillas et al. 2003), and grass species (Li et al. 2010). The transpiration response of salt-stressed plants is complicated by several interacting factors, such as growing environment, plant development stage, salt concentration and exposure time (Munns 2002). The water content, transpiration and stomatal conductanc decreased with increasing salt stress in *Medicago sativa* (Li et al., 2010) and in *Chloris virgata* (Yang et al. 2008a). But these studies did not involve the manipulation of atmospheric VPD, so how salinity affects stomatal functioning in the response to higher evaporative demand remains unclear. The reduced water content is suggested as a quick and economical approach to osmotic adjustment in response to osmotic stress induced by salinity (Lissner et al. 1999). Meanwhile, plants minimize transpiration water loss by reducing leaf expansion rates and stomata aperture (Brugnoli and Lauteri 1991; Munns 2002). The effects of salinity on the magnitude of transpiration differed greatly among the species studied, which may suggest differences

between salt-tolerant species and overall salt-sensitive species, or suggest different strategies for salt resistance.

The VPD chamber experiment in the current study only exposed plants to different VPD for about 3 hours with five increasing VPD gradients. It's hardly convincing to say that the salt concentration in any of the plant tissues is induced by the transpirational water loss at each VPD levels. However, the transpiration rate across increasing VPDs may indicate differences of resistance strategy against salt among the species. *Setaria* and *Chloris* both belong to C_4 and annual species, however, *Setaria* is a salt includer and *Chloris* is a salt excluder in terms of the degree of ion accumulation in the shoots. *Chloris* had the lowest rate of ionic increment in shoots compared with other four species, which corresponded well with the lowest transpiration rate across increasing VPDs at each salinity level. Canopy transpiration acts as the driving force for the transportation of salt ions from roots to shoots. Thus the relationship between transpirational water loss and the import of sodium into leaf tissue is highly relevant for salinity resistance strategies in plants (Munns and Tester 2008). *Chloris* limited the import of harmful ions by means of keeping low transpiration rate as compared with other species. In comparison, *setaria* is a salt includer, concentrations of sodium and chloride were enormously increased when applying salt stress up to 180 mM Na^+. The significant higher accumulation of salt ions coincided with the increasing transpiration rate under salt stress of 180 and 240 mM. Combined with the analysis of relative plant dry biomass, *Medicago,* the most salinity tolerant (least biomass reduction under salinity), demonstrated relatively high degree of transpiration rate. Its high transpiration rate relative to medium accumulation of salt ions also indicates *Medicago* as an effective salt excluder.

The effect of salinity on transpiration responses was analyzed individually by species (Figure 12 to Figure 16). *Medicago* showed progressively decreased transpiration rate with increasing salinity. Whereas in other species, transpiration rates across VPDs were not continuously decreased by increasing salinity. For two C_4 species, transpiration rates were restrained by moderate salinity up to 120 mM Na^+, however, increased again by high salinity (180 or 240 mM). This was most significant in *Setaria*,

whose transpiration rates at high salinity were even higher than that of non-saline controls. Higher potential of transpirational water loss to increasing VPD at 240 mM Na^+ was also commonly observed in *Melilotus* and *Leymus*. The transpiration response affected by salinity was instaneous, which might not reflect the long-term behavior. Most studies reported that transpiration rate tended to decline with increasing salinity because the lowered water potentials in the root triggered a signal (such as abscisic acid) from root to shoot to reduce the stomatal aperture (Robinson et al. 1997; Christmann et al. 2007). I presume that the different responses of transpiration rate to salinity may be related to the osmotic stress and toxicity characteristics of salt stress. Growth response to salinity over time has two phases, with first phase of growth reduction being due to osmotic effect of salt and the second due to toxic effects of slat that has accumulated over time in the leaves (Munns 1993 ; Munns and Tester 2008; James et al. 2008). Perhaps plants have accumulated enormous salt ions in the guard cells, impairing the normal function of stomata. Consequently stomata can close only partially or lose certain degrees of sensitivity to increasing leaf-to-air VPD, resulting in higher potential of transpirational water loss. However, future studies will be necessary to examine the function of stomata and directly measure the whole-plant hydraulic conductance, which may provide explanatory evidence for the increased transpiration potential at high salinity.

8. Conclusions and Outlook

(1) Annual grass species have greater total biomass and leaf area than perennial species at non-saline conditions, but had larger reduction rate in both parameters under salinity.

(2) Species of different functional groups shows significant difference in biomass partitioning in response to salt stress. High salinity resulted in greater root-to-shoot ratio in perennial species, but decreased the ratio in annual species.

(3) The transpiration response of C_3 species occurs in two phases with a VPD breakpoint: a rapid response to the increase in leaf-to-air VPD, and a slower response due to the reduction of canopy conductance. Salinity changes the regression slope and the emergence of the breakpoint.

(4) The TR-to-VPD relationship for C_4 species is a linear regression at salinity≤120 mM Na^+. At higher salinity, a breakpoint in canopy conductance emerges to restrict the increment of transpiration at a lower rate.

(5) C_3 species have relatively higher transpiration rate than C_4 species under low to moderate salinity (below 120 mM Na^+), probably due to the larger stomata aperture of C_3 species for carbon dioxide diffusion.

(6) At higher salt stress (180 and 240 mM), response of transpiration to increasing VPD was not significantly different between functional groups of photosynthetic pathways, rather differed between salt-including and salt-excluding species. However, the increased magnitude of transpiration (per unit leaf area) observed at salinity≥180 mM Na^+ is not precisely understood.

This study is a first attempt to investigate the transpiration response of grass species to salinity and short-term VPD changes. Some hints and improvements for future studies are summarized as follows: (1) Larger pots are more suitable for sand cultivation because sand has low water holding capacity. Increasing the volume of sand can compensate for the high rate of water run-off; therefore maintain sufficient moisture in the rooting medium. (2) The electrical conductivity (EC) of soil extracts should be determined to provide better indicatives of stress conditions. (3) The rolling and curling

symptom of salt-stressed leaves should be carefully considered when determining total canopy area. Measuring the actual area of transpiring surface is a tricky task. The curling leaves under high salinity reduce the transpiring surface exposed to high VPD, so measuring leaves after recovering turgor may overestimate the actual transpiring area. Besides, information on stomata density and distribution will be important for normalizing the transpiring surface. (4) Direct measurement of the whole-plant hydraulic conductance (Scholander bomb) and stomata conductance (gas exchange equipment) may provide more convincing explanation. (5) More replicates should be included. Three replicates might present some noise in the average that distracts us from the true response.

This study suggests that the growth decline under salt stress may involve stomatal sensitivity to VPD. Yet to what extent the stomatal conductivity relates to productivity is not known. For application to the agricultural context further experiments need to be carried out under various long-term VPD and salinity conditions to investigate the relevance between transpirational changes and yield. Understanding the morphological and physiological aspects of salinity adaptations will facilitate the conservation of natural grasslands and the improvement of forage production. Moreover, gaining a comprehensive knowledge of the importance and variation of adaptations to salinity and vapor pressure deficit between and within species is valuable for identifying stress-resistance traits and serving practical use from the perspective of crop breeding.

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Supplemented information

Formula of nutrient solution used in this study

Label	Chem. substance	Conc. stock	Molecular weight	Einwaage pro 1 L stock	Final conc.	Dil. factor	mL of stock for 1 L final solution
A	K_2SO_4 KCl	0.35 M 0.05 M	174.26 g 74.55 g	60.99 g 3.73 g	0.7 mM 0.1 mM	500	2
B	KH_2PO_4	0.1 M	136.09 g	13.61 g	0.1 mM	1000	1
C	$MgSO_4*7H_2O$	0.5 M	246.48 g	123.24 g	0.5 mM	1000	1
D	$Ca(NO_3)_2*4\ H_2O$	1 M	236.15 g	236.15 g	2.0 mM	500	2
E	$MnSO_4*\ H_2O$ $ZnSO_4*\ 7\ H_2O$ $CuSO_4*\ 5\ H_2O$ $(NH_4)_6Mo_7O_{24}*\ 4\ H_2O$	0.5 mM 0.1 mM 0.2 mM 0.01 mM	169.02 g 287.54 g 249.68 g 1235.86 g	84.51 mg 28.75 mg 49.94 mg 12.36 mg	0.5 uM 0.1 uM 0.2 uM 0.01 uM	1000	1
F	H_3BO_3	10 mM	61.83 g	618.3 mg	10 uM	1000	1
G	FeNaEDTA	50 mM	367.05 g	18.4 g	50 uM	1000	1