Q2

Motherly care: How *Leymus chinensis* ramets support their offspring exposed to saline-alkali and clipping stresses

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Abstract

***Background*:** Clonal integration can improve the performance of the rhizome plants, it remains unclear whether plants clonal integration strategy is different under contrasting clipping and saline-alkali homogeneous and heterogeneous environments. *Leymus chinensis* is a clonal grass native to the Songnen grassland where heavy grazing and patchy saline-alkali stress are serious environmental and ecological problems. We hypothesized that *L. chinensis* overcomes these stresses through clonal integration, in particular the transfer of nitrogen and carbohydrates.

***Methods*:** A pot experiment was carried out with ^{15}N isotope soil labeling method to study clonal integration strategy in the connected mother and daughter ramets of *L. chinensis*. The connected ramet pairs were grown in homogeneous (both connected ramets were treated) and heterogeneous (only daughter ramets were treated) environments with four treatments: control, clipping (60% aboveground biomass removal), saline-alkali (3.45 g of NaCl, $NaHCO_3$, and Na_2CO_3 per pot), and clipping × saline-alkali.

***Results*:** A significant amount (22.5%) of ^{15}N was transferred from mother to daughter ramets under non-stressed conditions. When homogeneously stressing both mother and daughter ramets, N transfer was significantly reduced to 8.5~~-~~–14.6%, independent of the nature of the stress. When only daughters were stressed (heterogeneous stress), saline-alkali stress led to a division of labor where daughters had enhanced photosynthesis, and mother ramets had increased ^{15}N uptake and growth. Clipping only daughters reduced biomass and ^{15}N uptake of both daughter and mother ramets.

***Conclusions*:** Our results demonstrated that clonal integration also occurs in homogeneous favorable environments but is reduced under homogeneous stress. In heterogeneous environments, clonal integration is used to translocate resource after clipping and a division of labor is established to overcome saline-alkali stress. Clonal integration continued even when daughters were severely stressed by the combined treatments. Our findings suggest that these mechanisms are key to the success of *L. chinensis* in the Songnen grassland.

Keywords:

Clonal integration, ^{15}N isotope labeling, Salt stress, Homogeneous and heterogeneous habitat, Resource sharing

~~1.~~1 Introduction

Ramets in clonal fragments, ~~that~~ which are in different habitats or at different developmental stages, can share nutrients, water, secondary metabolites, and hormonal signals through their connected rhizomes or stolons (Yu et al., 2004; Q5 Jónsdóttir and Callaghan, 1990; Liu et al., 2016). This can greatly enhance the fitness of individual ramets and contribute to the growth of the whole plant community (Kelly, 1995). Clonal integration is an important ecological adaptation strategy for clonal plants to utilize heterogeneous resources effectively and enables the community to overcome environmental stresses (e.g. nutrients depletion and drought) (Alpert and Stuefer, 1997; de Kroon et al., 2005 ; Batzer et al., 2017). Typically, the older mother ramet supports its daughter during the initial establishment phase, and the direction of clonal integration is acropetal (Zhang et al., 2001), but the direction can reverse and become basipetal (Q6 Pauliukonis and Gough, 2004; Roiloa and Hutchings, 2013; You et al., 2016). Evolutionary, clonal integration only makes sense if, eventually, the recipient can become a donor, supporting the formation of new clones. The different development stages of the ramets thus play an important role in clonal integration. The connected ramets are usually in different development stages and thereby differ in the ability to acquire resources (de Kroon and van Groenendael, 1997; Roiloa and Hutchings, 2013; Dong et al., 2015).

Reversal of the direction of clonal integration is possible because the direction and intensity (degree) of sharing depend not only on the developmental stages but also on the environmental conditions the respective ramets are exposed to ~~(~~Dong (2011)~~)~~. Local heterogeneity in water, light, and soil nutrient conditions typically intensifies the effect of clonal integration and can reverse the direction (Alpert, 1999; Lechuga-Lago et al., 2016), where the direction of clonal integration follows the resource gradient (Batzer et al., 2017). Under heterogeneous soil nutrients (Huang et al., 2018), water (Lechuga-Lago et al., 2016), or light resource environment (Alpert, 1999), the respective resources are transferred from the ramets in favorable to those with less favorable growth-conditions. For example, an isotopic labeling experiment showed that ^{15}N transfer significantly increased from the ramet with high to the one provided with low N-availability (Saitoh et al., 2006).

Ramets can donate some resources and simultaneously be a recipient of others. Clonal integration can thus be bi-directional. For instance, ramets in a stable high-light environment can be a donor for carbohydrates to lower light ramets and simultaneously be a recipient of water or nitrogen (Alpert, 1999; Saitoh et al., 2006). This leads to a division of labor where the high-light ramet~~'~~'s main task is aboveground, whereas that of the low-light is belowground. Such a division of labor may be enhanced by plasticity responses, by which plant can improve the access to abundant resources under reciprocal patchiness of resources (Yu et al., 2004; Wang et al., 2017b). Magyar et al. (2007) predicted with a model that this strategy is stable and beneficial in most of the simulated environments, and benefits increase with increasing environmental heterogeneity. Thus, the heterogeneity of the environment determines both the direction and intensity of the clonal integration (Roiloa and Hutchings, 2013; Lyu et al., 2016). Examples of how clonal integration intensity depends on environmental heterogeneity are given by He et al. (2011), Zhang et al. (2016), and Wijesinghe and Hutchings (1999). These studies show that homogeneous environments lead to a 'conservative' resource sharing strategy, whereas heterogeneous environments lead to intensive resource sharing and thereby a 'mutually supportive' strategy.

Numerous studies have been carried out on the direction and intensity of clonal integration under heterogeneous soil nitrogen, light and water resource environment which are relatively stable over time. How clonal integration is influenced by temporal stresses is much less understood, in particular saline-alkali stress and clipping. These two stresses can limit clonal plant growth in grasslands considerably and are of particular importance in the Songnen grassland. We thereby asked how clonal integration may help clonal grasses, and, in particular, *Leymus chinensis*, to overcome a combination of saline-alkali stress and grazing pressure.

Soil salinization is an increasing environmental problem restricting plant production in the grasslands of the Songnen grassland, Northeast China (Zhu, 2004). Saline-alkali stress (pH = 8.4~~-~~–10.3, EC = 74~~-~~–1740 µs cm^{-1}) often has patchy distributions at the level of individual plants in this area and is a temporally stable stress environment (Zhu, 2004). As a dominant clonal species in the Songnen grassland of northeast China, *Leymus chinensis* (Poaceae) has a strong ability to tolerate saline-alkali stress as well as resist grazing pressure (Zheng et al., 2019; Ma et al., 2020). Clonal integration is thought to be an important mechanism through which *L. chinensis* can persist under these harsh conditions. The intensity of resource sharing depends on the saline-alkali levels (Salzman and Parker, 1985; Zhang et al., 2015; Wang et al., 2019). Grazing (and more recently mowing) presents additional temporal stress. Poor management has led to over-grazing and a reduction in pasture production (Gao et al., 2014; Zheng et al., 2019). Grazing/clipping affects *L. chinensis* growth through the loss of leaves and associated photosynthesis and modifies the carbon allocation towards regrowth of the lost leaves (Zhang et al., 2020). In response to severe clipping, *L. chinensis* can increase the distribution of resources to the rhizomes, stems, and roots (Ma et al., 2020), helping it tolerate the grazing/clipping disturbance (Zhao et al., 2009).

Although *L. chinensis* usually suffers from both saline-alkali stress and grazing/moving in the field, to our knowledge, no study has considered the interactive effects of saline-alkali and clipping on the clonal integration strategy of *L. chinensis*. We hypothesized that: (1) when both ramets are stressed (homogeneous treatment), independent of the nature of the stresses (e.g. clipping, saline-alkali, and clipping × saline-alkali), there is a low resource sharing between ramet pairs; (2) when daughter ramets are temporarily stressed by clipping of leaves, the unclipped mother ramet will support the regrowth of the daughter through the transfer of resources, particularly carbon; (3) when daughter ramets are treated with saline-alkali stress, the mother and daughter ramets will establish a clonal division of labor in which the saline-alkali stressed daughter will be a supplier of photosynthates, whereas the unstressed mother will provide resources to the ramet network; (4) when the treated daughter ramets face both aboveground (clipping) and belowground (saline-alkali) stresses, the untreated mother ramet will adopt a relatively conservative resource sharing strategy, and thereby quasi 'abandon' its daughter not worth 'saving'. To study the first hypothesis, we conducted a greenhouse experiment in which we grew ramet pairs of *L. chinensis* and treated half of the pairs with clipping, and half of the pairs with saline-alkali, using a factorial design. To study the other hypotheses, the experiment was repeated, but not with homogeneous treatments of both mother and daughter, but with the heterogeneous treatment for which only the daughter was treated.

~~2.~~2 Materials and methods

~~2.1.~~2.1 Species and site description

L. chinensis, strongly depending on vegetative propagation for regeneration, is a dominant clonal grass in the Songnen grassland. The daughter (younger) ramets originate from the mother (older) ramets, and those ramets connect each other through rhizomes and form the *L. chinensis* community (Zhu, 2004). All clonal ramet pairs of *L. chinensis* in our experiment were obtained from Grassland Ecological Research Station of Northeast Normal University, Jilin Province, China (44~~o~~°40~~'~~′ to 44~~o~~°44~~'~~′ N; 123~~o~~°44~~'~~′ to 123~~o~~°47~~'~~′ E). The area has a semi-arid, continental climate with a frost-free period of about 140 days, the annual mean temperature is 4.9 ~~o~~°C, and the annual precipitation is from 400 to 500 mm (67% fall between June to August). The main soil type in the top 30 cm layer was chestnut (i.e. Calcic Kastanozems), which is equivalent to Calcic - Orthi - Caridosol in the United States Soil Taxonomy classification system (Soil Survey Staff, 2014). The vegetation of the region is dominated by *Leymus chinensis*, *Kochia scoparia*, *Chloris virgata*, *Phragmites communis*, *Puccinellia tenuiflora,* and *Artemisia frigida* (Diabate et al., 2015).

~~2.2.~~2.2 Greenhouse experimental design

In May 2014, 100 ramet pairs of *L. chinensis* were selected from the Songnen grassland and labeled as mother ramet and daughter ramets based on their different developmental stage (Dong, 2011). The ramet pairs were only accepted when the connecting rhizome was intact and the stretched out lengths of the individual ramets were within the range, 18~~-~~–22 cm and 10~~-~~–12 cm for mother and daughter ramets respectively. Each ramet was planted in a 5.5~~-~~-liter pot, 20 cm in diameter and 22 cm high. The connecting rhizome ran through a 1 cm hole in the side of the pot at a height of 15 cm. The ramet pairs were carefully put into the two pots through these holes and the connecting rhizome was packaged with black waterproof paper to avoid dehydration (Fig. 1). The growth medium consisted of completely washed sand, and the diameter of sand was between 1.7~~-~~ and 2.2 mm. Each pot was filled with 5 kg of washed sand, pH = 6.04~~-~~–6.31, EC = 108~~-~~–144 μs cm~~-~~$^{-1}$. Ramet pairs of *L. chinensis* were watered every 3 days with 200 ml of Hoagland nutrient solution. Excess water was allowed to drain from the bottom. Plants were grown in a temperature-controlled greenhouse in Northeast Normal University, Changchun, Jilin, China, with a photoperiod of 14/10 h at 28/24 ~~o~~°C (light/darkness), 1200 μmol photons m^{-2} s^{-1} maximum PAR, and 40~~-~~–70% relative humidity.

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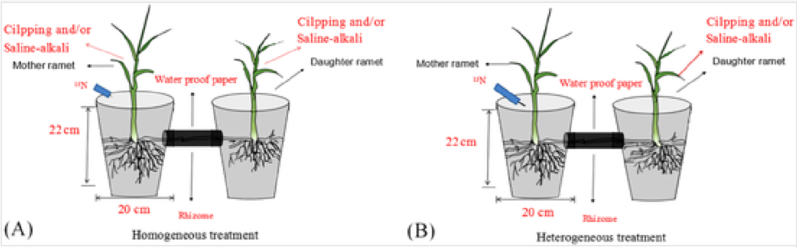
~~Figure 1~~Fig. 1



Schematic representation of the experimental design. Connected ramets of *L. chinensis*, consisting of mother and daughter ramets, were planted in both homogeneous (experiment 1) and heterogeneous (experiment 2) habitats, carefully keeping the connecting rhizomes intact. Each ramet was placed in its own pot. All mother ramets were labeled with ^{15}N. (A) Experiment 1 in which both the mother and daughter ramets were treated with the same treatment. Treatments were clipping stress, saline-alkali stress, clipping × saline-alkali stress or without stress addition; (B) Experiment 2: Identical to experiment 1, but only the daughter ramets were treated and the labeled mother ramets were left untouched.

For the first experiment, with homogeneous treatments of both mother and daughter ramets, 16 ramet pairs were selected from 100 pairs collected in the field. Their size uniformity was established based on three criteria: (1) tiller buds number of mother ramets (± s.e.) 7 ± 0.25 and tiller buds number of daughter ramets 7 ± 0.67, (2) rhizome buds number of mother ramets 7 ± 0.25 and daughter ramets 8 ± 0.25, and (3) fresh biomass of ramet pairs (4.50 ± 0.34 g pair^{-1}; $p \geq 0.05$). We had four treatments according to a full factorial completely randomized design with four replicate blocks (Figs. 1A and S1A). The four treatments were ‘unstressed control’, ‘clipping’, ‘saline-alkali’, and ‘clipping × saline-alkali’. The second experiment, with heterogeneous treatments, was conducted identically to the first. In this experiment, only daughter ramets were treated and not the mother ramets (Figs. 1B; ~~Fig.~~ S1B). Ramets in the second experiment were slightly smaller than in the first, with tiller buds numbers of mother ramets 5 ± 0.25 and daughter ramets 6 ± 0.50, rhizome buds numbers of mother ramets 4 ± 0.25 and daughter ramets 6 ± 0.25, and Q7 biomass of ramet pairs (3.30 ± 0.34 g pair^{-1}; $p \geq 0.05$). Both experiments were conducted at the same time.

~~*2.2.1.*~~*2.2.1* *Treatments*

Previous studies have shown that Na^+, CO_3^{2-}, HCO_3^- and Cl^- are the main ions in Songnen saline-alkali grassland soils (Shi and Wang, 2005; Ma et al., 2020). The salts were added to mimic the natural real situation in the Songnen grassland. We chose one neutral salt (NaCl) and two alkaline salts ($NaHCO_3$ and Na_2CO_3). The saline-alkali stress treatment involved adding 3.45 g of saline-alkali per pot (0.2 mol L^{-1} saline-alkali solution, 393 ml each pot) one month after transplanting, on June 10, 2014. The saline-alkali was added as a composing solution of NaCl, $NaHCO_3$, and Na_2CO_3 at a molar ratio of 2:2:1 with a pH of 10. The clipping stress was performed by removing 70% of the plant height, which is equal to about 60% aboveground biomass removal. Clipping was repeated twice: on June 15, 2014, and June 30, 2014.

~~*2.2.2.*~~*2.2.2* *^{15}N labeling*

To track the N transfer from mother to daughter ramets, mother ramets were labeled with ^{15}N 5 days before the first clipping (Fig. 1A, B). The mother ramets were labeled by injecting a 7.5% $CH_4{}^{15}N_2O$ solution into the soil, 5 cm below the soil surface. The abundance of ^{15}N in the solution was 5.18% and a total amount of 5.8 ml per pot was injected using a syringe. The daughter ramets were injected with an equivalent CH_4N_2O solution with a natural abundance of ^{15}N (0.366%) (Ma et al., 2020).

~~*2.2.3.*~~*2.2.3* *Sampling and measurements*

On July 20, 40 days after the first treatments, the youngest, fully expanded leaves were selected for measurement of net photosynthetic rate (Pn_i) using an LI-6400 portable photosynthetic system (LI-COR Biosciences). After the measurements, soil samples were taken and the plants were harvested. The soil samples were collected randomly from 5 points at a depth of 0–10 cm with a soil drill (3 cm in diameter), then a 300 g soil sample was collected and air-dried to constant weight. The dried soil samples were sieved over a 2 mm soil sieve, and then it was extracted with demineralized water ratio using an 1:5 water to soil ratio, i.e., 50 ml deionized water added to 10 g of soil. After stirring vigorously for 1 h and standing for 2 h, soil pH and electrical conductivity (EC) were measured in the extract by pH meter and conductivity meter. The saline-alkali treatment increased pH by about 3 pH units and the EC nearly tenfold (Table S1), indicating our study created a severely stressful saline-alkali soil environment.

At harvest, first, the rhizome between the pots was cut-off, then the roots of each ramet were dug out carefully. The aboveground and belowground parts were cleaned, and the tiller and rhizome numbers were counted. Then, ramets were divided into leaf, stem, rhizomes, and roots, and stored in paper envelopes. The clipped and final biomass ~~were~~ was collected and oven-dried at 65 ~~o~~°C for 48 h for biomass and ^{15}N measurements. For clipping treated ramets, the final total dry mass and aboveground dry mass included both clipped and the final harvested biomass. Dry samples of *L. chinensis* were ground into a fine powder using a ball mill (Retsch, Germany) and a 100-micrometer mesh sieve. δ^{15}N was determined by ISO-Prime stable isotope mass spectrometer (Isoprime100, Elementar Ltd., UK).

~~2.3.~~2.3 Calculations

We calculated the relative effect of treatments by comparing the change in dry mass (DM) associated with the treatment to that of the control. The percentage of dry mass change (DMC) was calculated using:

$$DMC\,(\%) = 100\% \frac{DM_{treatment} - DM_{control}}{DM_{control}} \qquad (1)$$

with $DM_{control}$ and $DM_{treatment}$ being the dry biomass of the control and the respective treatments: clipping, saline-alkali, and clipping × saline-alkali.

We first calculated the absolute amount of ^{15}N in both ramets (Eq. (2)). From it, we computed the percent of ^{15}N applied that was recovered by the root system of the labeled mother ramet (Eq. (5)) and the percent of ^{15}N transferred to the daughter ramet (Eq. (6)).

The absolute excess amounts of ^{15}N in a ramet w~~as~~ere calculated by multiplying the '^{15}N-Total N ratio' with the total N in the biomass (or in Eq. (5), fertilizer):

$$ {}^{15}N_{absolute}\,(mg) = {}^{15}N_{excess}\,(\text{‰}\blacksquare)\,/1000\text{‰}\blacksquare * total\ N\,(mg) \tag{2} $$

The enrichment, or excess, of ^{15}N was calculated from the ^{14}N and ^{15}N in the samples:

$$ {}^{15}N_{excess}(\text{‰}\Big) = 1000\text{‰} * \frac{{}^{15}N_{excess,sample}}{{}^{15}N_{excess,sample} + {}^{14}N_{excess,sample}} \tag{3} $$

where ${}^{15}N_{excess,\ sample}$ is calculated from the measured $\delta^{15}N_{sample}$ by correcting it for the $R_{standard}$, which is the natural abundance of ^{15}N ($R_{standard} = 3.663\text{‰}$):

$$ {}^{15}N_{excess,sample} = R_{standard} * \left(\frac{\delta^{15}N_{sample}}{1000} + 1\right) * {}^{14}N_{sample} \tag{4} $$

The amount recovered ^{15}N is the sum of ^{15}N in both ramets (mg, Eq. (2)) divided by the amount of ^{15}N applied (mg, Eq. (2)):

$$ {}^{15}N\ uptake\ ratio(\%) = 100\% * \frac{{}^{15}N_{Mother}(\mathrm{mg}) + {}^{15}N_{Daughter}(mg)}{{}^{15}N_{fertilizer}(mg)} \tag{5} $$

To estimate the amount of ^{15}N that was transferred from one ramet to the other, we calculated the absolute excess (more than naturally occurring) amount of ^{15}N (mg) in the daughter ramet and divided it by the total absolute excess amount of ^{15}N (mg) in both ramets (mother and daughter), which means that the total amount of ^{15}N that was taken up by the mother plant:

$$ {}^{15}N\ transfer\ ratio\,(\%) = 100\% \frac{{}^{15}N_{Daughter}(mg)}{{}^{15}N_{Daughter}(mg) + {}^{15}N_{Mother}\,(mg)} \tag{6} $$

~~2.4.~~2.4 Statistical analysis

Statistics have been computed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All data were tested for normal distribution and homogeneity of variance using Shapiro-Wilk and Levene's test respectively. The data that did not meet normality were log-transformed. Since the collected ramets in the homogeneous and heterogeneous experiment were statistically significant from each other ($p \leq 0.05$), one-way ANOVA was used to evaluate the effect of clipping, saline-alkali and dual stresses on biomass, tiller numbers, rhizome numbers, ^{15}N amount, ^{15}N uptake ratios, and ^{15}N transfer ratios in the homogeneous and heterogeneous experiment, separately. Significant differences of means were compared with Tukey multiple-comparison range test. The significance level was set at $p \leq 0.05$.

~~3.~~3 Results

~~3.1.~~3.1 Exp 1: biomass, tiller, and rhizome numbers in response to homogeneous applied stresses

When both ramets were treated, the clipping treatment significantly reduced the total dry mass (~~-~~–62.58%, total biomass includes the clipped biomass), aboveground dry mass (~~-~~–72.1%), and belowground dry mass (roots and rhizomes, ~~-~~–57.8%), but significantly increased root mass fraction (30.2%) (Fig. 2~~-~~A and B, Table 1). Mother and daughter ramets were equally affected in the homogeneous clipping treatment (Fig. 2~~-~~A and B). The saline-alkali treatment did not reduce the biomass or root mass fraction significantly (<~~-~~–15.0%, Fig. 2~~-~~A and B, Table S2). Mother ramets were the smallest when stressed with both clipping and saline-alkali, but the combined treatment had a similar effect on the daughter ramet compared to the clipping without saline-alkali stress treatment (Fig. 2~~-~~A and B). Thus saline-alkali stress, despite the high pH and EC values (Table S1), had only mild effects on biomass.

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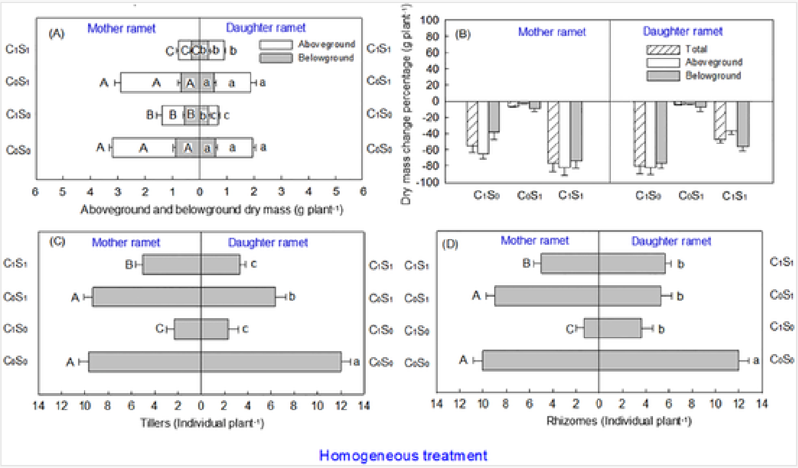
~~Figure 2~~Fig. 2



Effects of four homogeneously applied stresses on dry mass (A), dry mass change (percentage, relative to control) (B), tillers (C), and rhizomes (D) of *L. chinensis* ramet pairs. Values are means ± S.E. (n = 4). Uppercase letters are for comparison biomass, dry mass change percentage, and tillers and rhizomes between four treatments in mother ramet; lowercase letters are for comparison biomass, dry mass change percentage, tillers, and rhizomes between four treatments in daughter ramet; means with the same letter are not significantly different at $p \geq 0.05$. Treatment labels are C_0: No clipping; C_1: Clipping (70% of the plant height); S_0: No saline-alkali stress; S_1: Saline-alkali stress.

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Table 1

The table layout displayed in this section is not how it will appear in the final version. The representation below is solely purposed for providing corrections to the table. To preview the actual presentation of the table, please view the Proof.

Q1 Results of One-way ANOVA on effects of clipping (C), saline-alkali (S) and clipping × saline-alkali (C × S) stress on aboveground dry mass, belowground dry mass, tillers number, rhizomes number and Pn_i of *L. chinensis* under homogeneous and heterogeneous treatments.

Treatment		**Aboveground dry mass**		**Belowground dry mass**		**Tillers number**		**Rhizomes number**		**Pn_i**	
		M	**D**	**M**	**D**	**M**	**D**	**M**	**D**	**M**	**D**
Homogeneous	C	3.23*	2.18*	3.41*	3.22*	4.13*	3.43*	4.59*	7.63**	1.34 ns	1.34 ns
	S	0.81 ns	2.61*	2.39*	1.46 ns	1.03 ns	3.08*	1.07 ns	4.69*	2.43*	3.13*
	C × S	2.19*	4.21*	2.97*	3.13*	2.91*	2.73*	5.32*	4.19*	2.28*	3.96*
Heterogeneous	C	6.97*	6.13*	3.29*	3.39*	3.04*	3.15*	5.97**	6.13**	4.97*	4.03*
	S	1.03 ns	1.03 ns	0.39 ns	0.96 ns	4.62*	1.98 ns	6.29**	6.19**	3.28*	2.76*
	C × S	4.39*	5.03*	3.97*	3.13*	0.11 ns	2.01*	1.06 ns	1.15 ns	1.59 ns	3.29*

NS no significant difference~~,~~.

Table Footnotes

* $p \leq 0.05$.

** $p \leq 0.01$.

Compared to the control, clipped plants had dramatically reduced tiller numbers (mother: ~~-~~–70.2% and daughter: ~~-~~–78.5%, Fig. 2~~-~~C), as well as rhizome numbers (mother: ~~-~~–83.5% and daughter: ~~-~~–68.8%, Fig. 2~~-~~D, Table 1). Saline-alkali stress significantly decreased tiller and rhizome numbers of daughter ramets but not that of mother ramets. When the combined clipping × saline-alkali treatment, both mother and daughter had significantly reduced tiller numbers (mother: ~~-~~–48.3%, daughter: ~~-~~–72.2%) and rhizome numbers (mother: ~~-~~–50.0%, daughter: ~~-~~–52.8%). The combined treatment, however, did not reduce tiller and rhizome numbers compared to the only clipping treatment (Fig. 2~~-~~C and D, Table 1).

~~3.2.~~3.2 Exp 2: biomass, tiller, and rhizome numbers in response to heterogeneous applied stresses

Treatment of only daughter ramets affected the biomass, tiller, and rhizome production of both daughter and mother ramets, but not in the same way (Fig. 3, Table 1). As in the heterogeneous treatments, clipped daughter plants had significantly reduced its biomass and root mass fraction, fewer tiller, fewer rhizomes, whereas saline-alkali stressed daughter plants had the same biomass and tiller counts and had a significantly increased number of rhizomes. Combining clipping and saline-alkali stresses reduced biomass and tiller counts comparably to those for clipping, but tiller numbers were higher than in the daughter plants that were only clipped. Consequently, it was similar to the control treatment. Daughter plants in the heterogeneous environment responded similarly to the treatments as the daughter plants in the homogeneous environment, except that those were saline-alkali stressed daughters seemed to have increased production of rhizomes.

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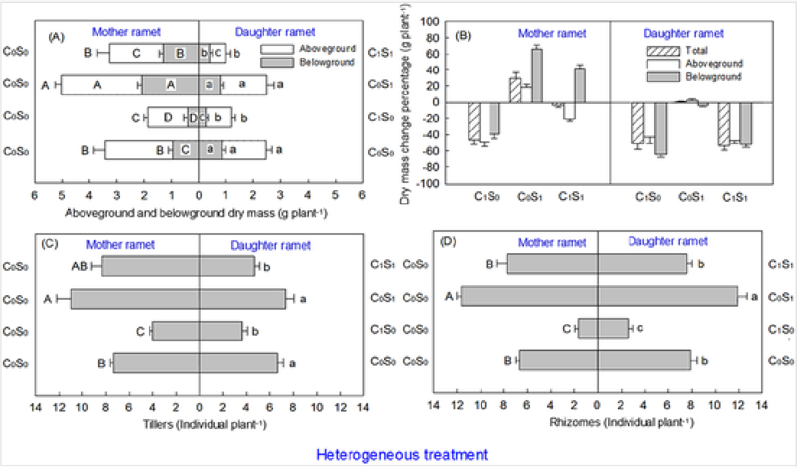
~~Figure 3.~~Fig. 3



As in Fig. 2, but for data from experiment 2 in which only the daughter ramet was treated and not the mother ramet. Note that treatment of the daughter affects the mother ramet.

Clipping the daughter, reduced the biomass of the mother plant, whereas saline-alkali stressing the daughter, increased the biomass of the mother plant (Fig. 3~~-~~A and B, Table 1). Tiller and rhizome numbers in this case follow the biomass pattern. Combined clipping and saline-alkali resulted in an average response, not significantly different from the control. The clipped daughters that had an unclipped mother had less reduction in biomass (~~-~~–50.7%, Fig. 2~~-~~B) than the daughters that had a clipped mother (~~-~~–81.3%, Fig. 3~~-~~B). As these were separate experiments, we can however not establish the statistical significance of these numbers. The reduction in biomass of the mother, however, seems severe compared to the increase in biomass of the daughter (Figs. 2A and 3~~-~~A~~-~~). More surprisingly, saline-alkali not only increased the biomass of the mother, but it did not decrease the biomass of the daughter and even seemed to have stimulated the daughter to produce more rhizomes (Fig. 3~~-~~D).

~~3.3.~~3.3 Effects of saline-alkali and clipping on photosynthesis

We measured the photosynthesis rate of a fully expanded young leaf to understand how stress may affect carbon acquisition rates. In the first experiments, both mother and daughter plants had the same photosynthesis rates after the clipped treatments as in the control plants, and saline-alkali reduced the photosynthesis rate (Fig. 4~~-~~A, Table 2). When only daughters were treated, the response, however, was quite different. The daughters had reduced photosynthesis rates when clipped, and even improved in the rates when only saline-alkali stressed (Fig. 4~~-~~B).

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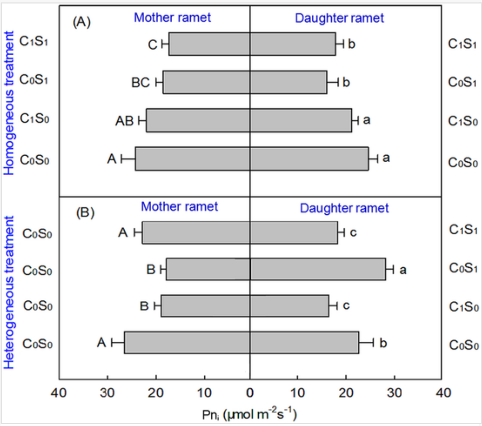
~~Figure 4~~Fig. 4



Effects of four homogeneous and heterogeneous on single leaf net photosynthesis rate (Pn_i). Values are means ± S.E. ($n = 4$). Uppercase letters are for comparison the Pn_i between four treatments in mother ramet; lowercase letters are for comparison the Pn_i of daughter ramet between four treatments; means with the same letter are not significantly different at $p \geq 0.05$. In the ramet pairs, C_0: No clipping; C_1: Clipping (70% of the plant height); S_0: No saline-alkali stress; S_1: Saline-alkali stress.

alt-text: Table 2

Table 2

The table layout displayed in this section is not how it will appear in the final version. The representation below is solely purposed for providing corrections to the table. To preview the actual presentation of the table, please view the Proof.

Results of One-way ANOVA on effects of different clipping (C), saline-alkali (S) and clipping × saline-alkali stress on ^{15}N amount, ^{15}N uptake ratio and ^{15}N transfer ratio of *L. chinensis* under homogeneous and heterogeneous treatments.

Treatment		**^{15}N amount**		**^{15}N uptake ratio**	**^{15}N transfer ratio**
		M	**D**		
Homogeneous	C	2.91*	3.53*	2.87*	3.22*
	S	1.06 ns	3.14*	3.32*	3.14*
	C × S	3.03*	3.23*	3.18*	4.61*
Heterogeneous	C	3.71*	3.35*	3.44*	3.01*
	S	5.01**	1.14 ns	2.62*	3.25*
	C × S	0.93 ns	3.11*	1.78 ns	1.98*

NS no significant difference.

Table Footnotes

* $p \leq 0.05$.

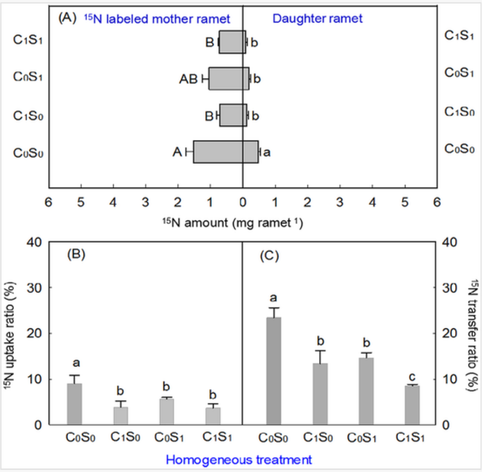
** $p \leq 0.01$.

~~3.4.~~3.4 N acquisition and transfer from mother to daughter using a ^{15}N label~~.~~

To understand how mother plants support their daughter in the face of stress, we labeled the mother ramet's soil with ^{15}N and determined at harvest how much of the ^{15}N was taken up (exp1: 9.0% and exp2: 14.0% recovered, Figs. 5B and 6B, Table 2), and how much of taken up ^{15}N was transferred to the daughter through the connecting rhizome (exp1: 23.2% and exp2: 21.8% transferred, Figs. 5C and 6C). Absolute amounts of ^{15}N in the respective ramets biomass (exp1: mother: 1.5 mg ramet^{-1}, daughter: 0.5 mg ramet^{-1}, exp2: mother: 2.4 mg ramet^{-1}, daughter: 0.7 mg ramet^{-1}) are given in Figs. 5A and 6A. ^{15}N uptake ratios (3.7%, 5.6% and 3.6%, Fig. 5B, Table 2) by the mother ramets were significantly reduced when both ramets were stressed with either clipping, saline-alkali, or both. Transfer of ^{15}N from mother to daughters (^{15}N transfer ratios, 13.3%, 14.6% and 8.3%, Fig. 5C) also significantly decreased with the stress, and the strongest decrease was when both stresses were combined.

alt-text: Fig. 5

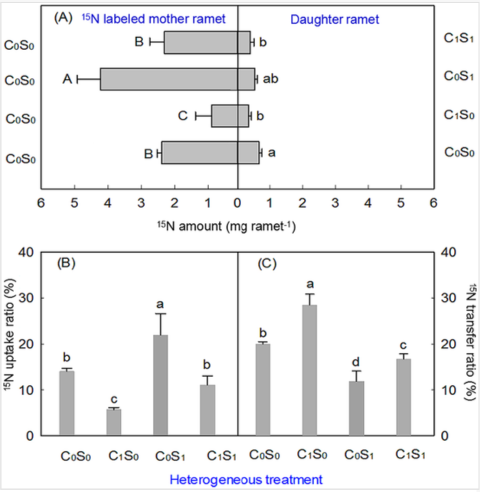
~~Figure 5~~Fig. 5



Effects of four homogeneous on ^{15}N amount in mother and daughter ramets (A), mother of ^{15}N taken up by the labeled mother ramet (uptake ratio, B) and ^{15}N transfer ratio from treated mother to treated daughter ramets (C) in *L. chinensis*. Values are means ± S.E. ($n = 4$). Uppercase letters are for comparison the ^{15}N amount between four treatments in mother ramet; lowercase letters are for comparison the ^{15}N amount of daughter ramet, and ^{15}N uptake ratio and ^{15}N recovery ratio between four treatments; means with the same letter are not significantly different at $p \geq 0.05$. In the ramet pairs, C_0: No clipping; C_1: Clipping (70% of the plant height); S_0: No saline-alkali stress; S_1: Saline-alkali stress.

alt-text: Fig. 6

~~Figure 6~~Fig. 6



Effects of four heterogeneous on ^{15}N amount in mother and daughter ramets (A), mother of ^{15}N taken up by the labeled mother ramet (uptake ratio, B) and ^{15}N transfer ratio from treated mother to treated daughter ramets (C) in *L. chinensis*. Values are means ± S.E. ($n = 4$). Uppercase letters are for comparison the ^{15}N amount between four treatments in mother ramet; lowercase letters are for comparison the ^{15}N amount of daughter ramet, and ^{15}N uptake ratio and ^{15}N recovery ratio between four treatments; means with the same letter are not significantly different at $p \geq 0.05$. In the ramet pairs, C_0: No clipping; C_1: Clipping (70% of the plant height); S_0: No saline-alkali stress; S_1: Saline-alkali stress.

When only the daughter was stressed, the results were quite different. Clipping the daughter reduced the ^{15}N uptake ratio, whereas saline-alkali of the daughter increased the ^{15}N uptake ratio compared to the unstressed control. The combined stress, was in between the single stresses and thereby not different from the control (^{15}N uptake ratio, 14%, 6%, 22%, 11%, Fig. 6B). Although the mother ramets took up more ^{15}N, they transferred relatively less to the saline-alkali stressed daughters (mother: 4.3 mg ramet^{-1}, daughter: 0.6 mg ramet^{-1}; ^{15}N transfer ratio, 13%, Fig. 6A, C). The net result was that saline-alkali stressed daughters received as much ^{15}N as the unstressed daughters (Fig. 6A). ^{15}N transfer amount from mother to daughters also decreased by 42.9% and 36.3% under clipping and both stresses compared with control, saline-alkali stress had no significant effect on ^{15}N transferred amount from mother to daughters. Clipping the daughters had the opposite effect: the mothers recovered less but transfers relatively more to the clipped daughters (mother: 0.8 mg ramet^{-1}, daughter: 0.2 mg ramet^{-1}; ^{15}N transfer ratio, 28%, Fig. 6~~-~~A, C). In absolute amounts, however, they transferred not as much as they did when daughters were not clipped (Fig. 6~~-~~A).

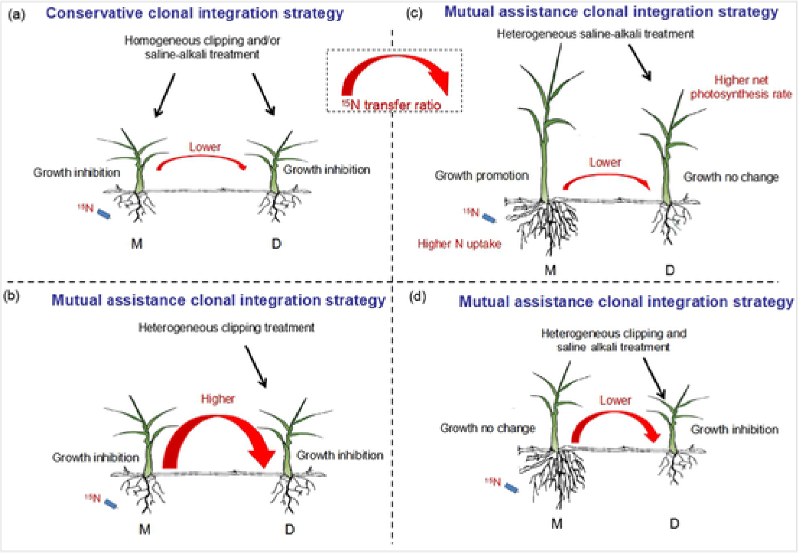
~~4.~~4 Discussion

~~4.1.~~4.1 Clonal integration occurs in homogeneous favorable environments but is reduced under homogeneous stress

Independent of the environment, clonal integration is acropetal during the establishment of the daughter ramet: i.e. the daughter ramet initially lives of the resource supply by the mother ramet (Zhang et al., 2001). It is, however, thought that in homogeneous habitats, the flow of resources from mother ramet to daughter quickly ceases when the daughter has established its own leaf area and root system as there seems to be no reason for the mother to continue its life support (Alpert, 1999; Oborny et al., 2000). Theoretically, the daughter may even start supporting the parent and the direction of transfer changes. More recent studies have questioned this idea and show that the transfer of resources and metabolites continues in homogeneous environments well beyond the establishment stage (Dong et al., 2015; Wang et al., 2021). It seems that there is a continued need to maintain a certain degree of information exchange, signal transmission, and nutrition allocation adjustment (Salzman and Parker, 1985; Gomez et al., 2007, 2008). At the start of the experiment, our ramets in the homogeneous treatment were not similar in size, and at the end of the experiment, mother and daughter ramets had similar responses to the stress. Thus, clonal integration led to size equality among the ramets. However, we observed that 23.2% of ^{15}N taken up by the mother ramet was transferred to the daughter in the control treatment. In agreement with other previous studies (Dong et al., 2015; Wang et al., 2021), our results suggest that indeed a significant amount of resource transfer occurs among ramets under homogeneous and favorable growth conditions. Possibly the transfer of N in unstressed plants functions as a ‘health’ signal telling the daughter ramet that the mother is rich in nitrogen (Liu et al., 2016; Wang et al., 2017a). When stressing both mother and daughters equally, the percent transfer declined significantly to 14.6% when one stress was applied, and only 8.5% when both stresses were applied. The results thereby suggest that the intensity of the clonal integration goes down with increasing homogeneous stress. We further proposed a conceptual model showing clonal integration strategies of *L. chinensis* under homogeneous/~~-~~heterogeneous clipping and/or saline-alkali stress (Fig. 7), we define “conservative clonal integration strategy” as lower resource integration, which means that no division of labor occurs or nitrogen transfer is lower when compared with the control. Furthermore, we define “mutual assistance clonal integration strategy” as higher resource integration, which means that division of labor occurs or nitrogen transfer is higher when compared with the control. Therefore, both mothers and daughters took a ‘conservative’ resource integration strategy under homogeneous saline-alkali and/or clipped stresses in the first experiment (Fig. 7~~-~~a).

alt-text: Fig. 7

~~Figure 7~~Fig. 7



Conceptual model of clonal integration strategies of *L. chinensis* under homogeneous/~~-~~heterogeneous clipping and/or saline-alkali stress. In the ramet pairs, M: mother ramet; D: daughter ramet. Differences in ramet pair~~s~~ size simulate the actual growth of *L. chinensis*. Red arrows represent~~s~~ the ^{15}N transfer ratio from mother to daughter ramets. The conservative strategy of clonal integration was supported through reduced ^{15}N transfer ratio under different homogeneous stresses (a). The mutual assistance strategy of clonal integration was supported through increased ^{15}N transfer ratio under heterogeneous clipping stress (b), and division in labor under heterogeneous saline-alkali stress (c). Under heterogeneous dual stresses, mother did not abandon their severely stressed daughter ramets, still showing a low ‘mutual assistance’ clonal integration strategy (d). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

~~4.2.~~4.2 Heterogeneous saline-alkali stress leads to a division of labor

It is generally thought that clonal integration is beneficial to the clonal network in heterogeneous environments in which the clones can specialize in acquiring the locally most abundant resource (Salzman and Parker, 1985; Liu et al., 2016). We expected this division of labor to be quite different when daughters were saline-alkali stressed or clipped. This is because saline-alkali stress mainly consistently affects plant belowground parts, although saline-alkali concentrations can quickly accumulate in the shoot as well; while clipping (mimicking grazing) is primarily imposed on plant aboveground parts temporarily, as the shoot biomass will be restored with the quick regrowth of leaves.

Clipping the daughter indeed reduced the biomass, tiller numbers, and rhizome numbers of the mother in nearly equal amounts compared to the clipped daughter. It suggests that the clonal network shares ^{15}N resources and possibly photosynthates, thus the unclipped mother supports the clipped daughter. Meanwhile, clipping reduced the underground biomass allocation of the daughter plant, which supposedly reduced the absorptive capacity of the daughter plant to some extent. The lower root mass fraction of the daughter ramet seems to create a greater dependence on nutrient transfer from the mother to the daughter ramet (Fig. S2). The strong transfer of resources from the mother to the daughter is reflected in the increased ^{15}N transfer, which increased compared to the control from 21.8% to 28.4%, indicating a 'mutual assistance' clonal integration strategy between connected ramets under the heterogeneous clipping stress (Fig. 7-b). ^{15}N uptake ratio is decreased under the clipping treatment, which may be surprising as clipping not only deprives the ramets of biomass but also removes a lot of nitrogen, and the regrowth of the shoot requires new nitrogen uptake. However, clipping away 60% of the aboveground biomass in twice is a strong treatment, and the reduction in ^{15}N uptake is consistent with the study of Ma et al. (2020), who found that nutrient uptake increased after moderate clipping, but strongly decreased after heavy clipping.

Although the saline-alkali treatment did not reduce the growth as much as the clipping treatments, the stress is not easily overcome as the soil environment will remain stressful to the plant for a long time (Albacete et al., 2014). In this condition, the saline-alkali stressed daughter may 'specialize' in photosynthesis, living of nutrients, and possibly water, coming from the mother plant. In consistent with our hypotheses, we found that the photosynthesis rates of the saline-alkali stressed daughter ramet increased, and those of the mother ramet decreased compared to the unstressed control. Furthermore, the ^{15}N uptake by the mother ramet increased with a ^{15}N uptake ratio increasing from 14.0% to 21.9%. Although the absolute amount of ^{15}N transferred to daughter ramet had no significant difference with the control, the percentage of N transferred ratio decreased. This is because the uptake by mother ramets increased, instead of the decreased transfer of ^{15}N absolute amount to the daughters. These results are consistent with previous heterogeneous light/nutrient studies from pot experiments of clonal species (Stuefer, 1998; Wang et al., 2011; Roiloa et al., 2016). Clonal growth of *L. chinensis* benefited from a division of labor when heterogeneously saline-alkali stressed, this is because saline-alkali changed resource sharing and coordinated the allocation of nutrients between ramets, thus leading to a change in the biomass of connected proximal ramets under heterogeneous environment. All these strategies can help the plant adapt to the heterogeneous saline-alkali environment to improve soil nutrients absorption through resources sharing (Evans and Whitney, 1992; Wang et al., 2019). Surprisingly, the mother ramets of the saline-alkali stressed daughters had increased biomass production compared to the control. Larger plants usually have smaller root mass fractions, but these mother ramets had a larger root mass fraction (Fig. S2). We conclude that when daughter ramets are facing saline-alkali stress, different plant organs specialize the function: the root system of the mother ramets supply water and nutrients for the daughter ramets to promote its photosynthesis, in return, mother ramets get photosynthates rewards from the saline-alkali stressed daughter ramets (Fig. 7-c).

Hence, we summarize that clipping the daughter ramets led to a relatively intensified clonal integration in which the mother aids the daughter, whereas saline-alkali the daughter ramets led to a division of labor benefiting both the mother and the daughter ramet: the stressed daughter was provided with sufficient nutrients and water that it could increase its photosynthesis rates and thereby increase the mother~~’~~'s growth rate.

~~4.3.~~4.3 The mother ramet helps its daughter under heterogeneous dual stresses

We asked if the mother would "give-up" on its daughter when the daughter was simultaneously stressed both belowground and aboveground. Our data, however, suggests that even under these conditions, clonal integration continues. The result is rather a mixture of the results of both stresses. The mother ramet may continue to support the daughter, transferring 16.7% of its acquired ^{15}N. These mothers had greater biomass and lower shoot mass fraction than when the daughters were only clipped (Fig. S2, Table S2). The data suggest that, although in the combined treatment the clipping presents a loss shared by the whole clonal network, the ramets still try to establish a division of labor just as they did when daughters were only saline-alkali stressed. We thus can reject our hypothesis and conclude that mother did not abandon their severely stressed daughters, still showing a low 'mutual assistance' clonal integration strategy under heterogeneous dual stresses (Fig. 7-d).

~~4.4.~~4.4 Clonal integration is likely beneficial to the success of *Leymus chinensis* in the Songnen grasslands

L. chinensis is a very successful grass in the Songnen grassland, where it is characterized by patchy saline-alkali stress and heavy grazing pressure (Zheng et al., 2017; Wang et al., 2019). We asked if the clonal integration strategy of this grass is key to its success. Although we can~~-~~not answer this question at a grassland community level, we did show that *L. chinensis* responds to clipping by reallocating resources and that it overcomes saline-alkali stress through a division of labor. Somewhat surprisingly, the biomass production of the whole clonal network under heterogeneous saline-alkali stress was greater than under non-stressed control conditions, as if the saline-alkali stress was a necessary signal for establishing a beneficial division of labor.

~~5.~~5 Conclusions

We demonstrated that clonal integration occurs in *L. chinensis* under all testing conditions, but more strongly so under heterogeneous stress, and least so under severe homogeneous stress. Heterogeneous clipping resulted in a sharing of resources among ramets such that ramets were at harvest nearly equally affected by the simulated grazing. Heterogeneous saline-alkali stress led to a division of labor where the saline-alkali stressed ramet had increased photosynthesis rates and the unstressed ramet increased N uptake and growth. The division was so effective, that the total biomass production of both ramets was greater when one ramet was saline-alkali stressed than under the unstressed control treatment. We conclude that clonal integration is key in the response of *L. chinensis* to the heterogeneous saline-alkali stress.

CRediT authorship contribution statement

YZG conceived and designed the experiments. YZG and HS performed the experiments. CCZ, YZG and HS analyzed the data. HS, CCZ and YZG wrote the first draft; other authors provided reviewing and editing advice.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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~~Appendix A.~~Appendix A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149675.

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Graphical abstract

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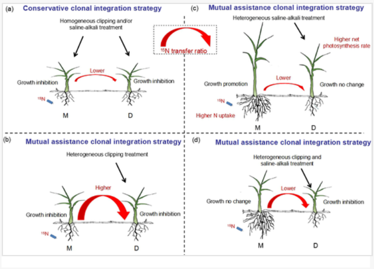

Highlights

- Clonal integration strategies of *Leymus chinensis* differ in response to homogeneous and heterogeneous stress.
- Conservative resource integration strategy was supported under homogeneous conditions.

- *L. chinensis* takes resource sharing or division of labor strategy under heterogeneous saline-alkali or clipping stress.
- These strategies help to explain the success of *L. chinensis* in the Songen grasslands.

~~Appendix A.~~Appendix A Supplementary data

Multimedia Component 1

Supplementary material

alt-text: Image 1

Queries and Answers

Q1

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