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THE EFFECTS OF ALKALINE STRESS AND DEVELOPING STAGE ON THE MAGNITUDE OF CLONAL INTEGRATION IN LEYMUS CHINENSIS: AN ISOTOPIC (^{15}N) ASSESSMENT

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Abstract

Clonal integration has been shown in many clonal plants to be influenced by stress and growth stage, but few studies have examined the magnitude of such influence. To quantify the transport of resources, glasshouse experiments were conducted by tracing the movement of ^{15}N label between interconnected ramets to compare the effects of soil alkalinity and developing stages of *Leymus chinensis* apical ramets on clonal integration. The apical ramets were referred to as "pre-rooting" (those that have not rooted in the soil) and "post-rooting" (those that have rooted). We found that a low amount of ^{15}N was transported from basal to post-rooting apical ramets, and ^{15}N translocation significantly increased from basal to pre-rooting apical ramets. However, there were significant increases in ^{15}N for both pre- and post-rooting stages when apical ramets were exposed to stress conditions. Moreover, severing their connections reduced apical ramet growth under stress conditions. Therefore, the magnitude of clonal integration is affected by the developing stage and environmental condition. In addition, clonal integration enhances the growth of apical ramets under pre-rooting stage and alkaline stress, but has relatively little effect on post-rooting apical ramets under no stress conditions. Alkaline stress also had a greater impact than the developing stage. Our study indicates that clonal integration might ameliorate negative effects of alkaline stress and growth stage through source-sink feedback regulation such as nitrogen. This may contribute greatly to the species to withstand local alkaline stress and successfully reproduce from pre- to post-rooting stages.

Key words: Clonal integration magnitude, Alkaline stress, *Leymus chinensis*, Developing stage, Stable isotopes, Nitrogen transport.

Introduction

Clonal integration in clonal plants as a response to stressful conditions (Charpentier *et al*., 2012; Herben, 2004; Oborny *et al*., 2000) and developmental stage (Gao *et al*., 2013; Matlaga & Sternberg, 2009) is well documented. Integration involves the sharing of resources, such as mineral nutrients, water, and photosynthates between "basal" and "apical" ramets through the interconnected rhizome (Alpert, 1999; Xiao *et al*., 2011; Yu *et al*., 2002). Many studies have shown that clonal plants are able to transport resources obtained from non-stressful environments to those located in stressful ones (Jonsdottir & Watson, 1997; Hartnett & Bazzaz, 1983), and in most cases, such resource transfer did not significantly reduce the growth of non-stressed donating ramets (Li *et al*., 2011; Chen *et al*., 2010). This advantage of clonal integration can alleviate local stress caused by inadequate nutrition (Roiloa & Retuerto, 2006; Slade & Hutchings, 1987), drought (Chen *et al*., 2010; Dong & Alaten, 1999), and salinity (Evans & Whitney, 1992; Xiao *et al*., 2011). In a previous study, we demonstrated that clonal integration enhances the performance and survival of apical ramets experiencing severe alkaline stress due to water transport from basal to apical ramets which greatly mitigated the negative effects of alkalinity stress (Zhang *et al*., 2015). Alkaline stress can affect the performance and survival of alkaline habitats and influences plant morphology and growth. For example, high pH associated with alkaline conditions can directly affect clonal bulbil reproduction (Lv *et al*., 2013).

Similarly, the developmental stage also influences clonal integration among clonal plants. Many studies have demonstrated that a high degree of integration in clonal species may occur at early establishment, and as juvenile ramets age, the resource support from basal ramets may end (Alpert & Mooney, 1986; Xiao *et al*., 2010). Clonal plants produce clonal offspring that pass through distinct pre- and post-rooting stages (Matlaga & Sternberg, 2009). Pre-rooting offspring receive a small amount of water from their parents, and severing their connections reduces their growth. This indicated that the offspring at this growth stage still require support from the mother ramet to overcome more stressful conditions; post-rooting offspring, however, receive no water from their parent, and severing the connection has little effect on their performance. This occurs because the apical ramet constantly improves and develops as the dependence on basal ramets gradually weakens (Matlaga & Sternberg, 2009; Xiao *et al*., 2010). Clonal plants can optimize the efficiency of their resource utilization by translocating resources between interconnected ramets (Stuefer & Hutchings, 1994). The intensity of resource transport depends on the source-sink relationship between ramets (Marshall, 1990; Hartnett & Bazzaz, 1983), which can change over time and stress environment (Marshall, 1968; Li *et al*., 2011). However, studies focusing on clonal integration under stress conditions and the developmental stage are limited.

Clonal integration has been investigated by tracing the movement of resources between ramets or through severing interconnected rhizomes, both of which test ramet interdependence in terms of growth and survival

(Pitelka & Ashmun, 1985). However, these methods have rarely been combined (DeKroon *et al*., 1996; Matlaga & Sternberg, 2009). More commonly, stable isotopes, such as ^{15}N (Saitoh *et al*., 2006; Welker *et al*., 1987), ^{14}C (Jonsdottir & Watson, 1997), acid fuchsin (D'Hertefeldt & Jonsdottir, 1999), and deuterated water (DeKroon *et al*., 1996 ; Matlaga & Sternberg, 2009), are used as tracers in clonal plant studies. Isotope experiments can be used to quantify the transport of resources between labeled and recipient (unlabeled) ramets at a specific point in time, but not to assess the consequences of resource sharing. On the other hand, the importance of clonal integration can be evaluated by comparing the performance between ramets connected and disconnected with superior ramets.

Leymus chinensis (Trin.) is a perennial rhizomatous clonal plant that is distributed over a wide range, from Russia to eastern parts of the People's Republic of Mongolia to Northeast of China (Zhu *et al*., 1981; Zhou *et al*., 2014). In the grassland in northeast China, alkalization is a serious problem and low topographic is one major cause. *L. chinensis* across drainage basins in arid northeast China parallels gradients of decreasing soil fertility and increasing alkaline, pH, and toxic ion concentrations (e.g., Na, CO_3^{-2}) at lower positions (Zhu *et al*., 1981; Wang *et al*., 2004). Thus, at lower topographic positions, productivity and diversity decline and, eventually, monospecific stands develop at the most stressful sites. Availability of soil N and other nutrients limited growth at the alkaline stress site for adult and juvenile life stages. Increasing alkalinity reduces N availability through volatilization of mineralized NH_4^+ and decreased other nutrients solubility (James *et al*., 2005).

During the growing season, *L. chinensis* begins its reproductive phenology by producing new buds from a reproductive rhizome and emerging from the ground with slender culms and leaves. On average, the offspring increases in leaf area and develops roots in the soil by 4 days post-leaving, so offspring growth mainly depends on the nutrients from local soil through newly formed roots as well as the basal ramets from clonal integration (Zhang *et al*., 2015). Clones of *L. chinensis* also show a high degree of clonal integration, including both basal and apical translocation of resources between ramets along a rhizome (Zhou *et al*., 2014).

In this study, we examined the effect of clonal integration for apical ramets growing in stressful conditions (alkaline stress vs. no stress) for two developing stages (rooted vs. no rooted) and two rhizome treatments (connected vs. severed). The translocation of resources along rhizome systems was investigated using the stable isotope ^{15}N in a glasshouse. The following two questions were addressed: (1) Does ^{15}N translocation increase to unrooted ramets compared to rooted ramets? Based on source-sink feedback regulation, we hypothesized that assimilated demand from basal ramets would enhance nitrogen efficiency in unrooted plants; (2) if so, we specially expected the magnitude of clonal integration significantly increase in rooted ramets under stressful conditions in comparison with rooted ramets in no stressed conditions. As a result, clonal integration would significantly enhance the growth of the apical ramets under stressful environment and without any cost to basal ramets.

Materials and Methods

Plant species: *L. chinensis* is usually a "guerilla" clonal species with long, strong rhizomes and vigorous vegetative propagation, giving rise to extensively spreading clones that often form monodominant plant communities in the Eurasian Steppe. Rhizomes of *L. chinensis* lie horizontally at approximately 10 cm below the soil surface to form fairly large clonal systems, with guerilla rhizomes that are likely to experience different environmental stresses (Wang *et al*., 2004; Zhang *et al*., 2002). In order to eliminate the genotype limitation, all material used in the study was reproduced from rhizomes and seeds. *Leymus chinensis* seeds were collected from institute of Pratacutural Sciences, Heilongjiang Academy of Agricultural Sciences in China (46°32′17″ N, 125°28′24″ E) and pre-grown in the greenhouse. After sixty days, when enough ramets were produced, we collected 72 apical ramets, each connected with its basal ramet, through in-between rhizome segment of roughly 4 cm long, with a mean height of 6 cm and mean root length of 3 cm. To avoid possible confounding effects of genotypes, the plants were assigned randomly to the treatments.

Experimental design: The experimental design was comprised of three factors: developing stage, alkaline stress, and rhizome connection. To examine ^{15}N transport between basal and apical ramets, we traced ^{15}N translocation between the basal (provided with enriched nitrogen) and apical (not provided with nitrogen) ramet. We cut off the roots of apical ramets for half of the 72 ramet pairs to simulate the unrooted developmental stage (hereafter referred to as pre-rooted), while the roots of the remaining apical ramets were left intact (hereafter referred to as rooted). We remove roots as necessary with a sharp blade, with minimal disturbance to the apical ramets in order to avoid an immediate negative impact. Both half of the pre-rooted and rooted ramet pairs were then disconnected by severing the rhizome connection within each ramet pair. This resulted in four types of ramet pairs: pre-rooted & connected, pre-rooted & disconnected, rooted & connected, and rooted & disconnected. Furthermore, alkaline stress was applied to apical ramets within ramet pairs of half of each of the four categories. Consequently, we assessed 8 treatment groups, each with 9 replicates (Fig. 1).

The ramet pairs were grown in 72 pairs of round plastic cups (each 0.45 L) and were watered with modified Hoagland's solution (Alpert & Mooney, 1986). We provided labeled ^{15}N (5.77 g $^{15}NH_2CO^{15}NH_2$) to the basal ramets by dissolving it in Hoagland solution. The apical ramets did not receive ^{15}N. The culture solution was replaced every 4 days, and the process was repeated three times. Alkalinity was introduced to the apical ramet through a mixture of Na_2CO_3 and $NaHCO_3$ (sodium ions in a 1:1 ratio), and alkaline solution concentrations were 80 mmol L^{-1}. Basal ramets were always grown in the absence of alkaline conditions. To avoid floating, the plant's stem were fixed in the corresponding hole on the two side of foam sheet. There was a small slit in the plastic cups for the rhizome to run through.

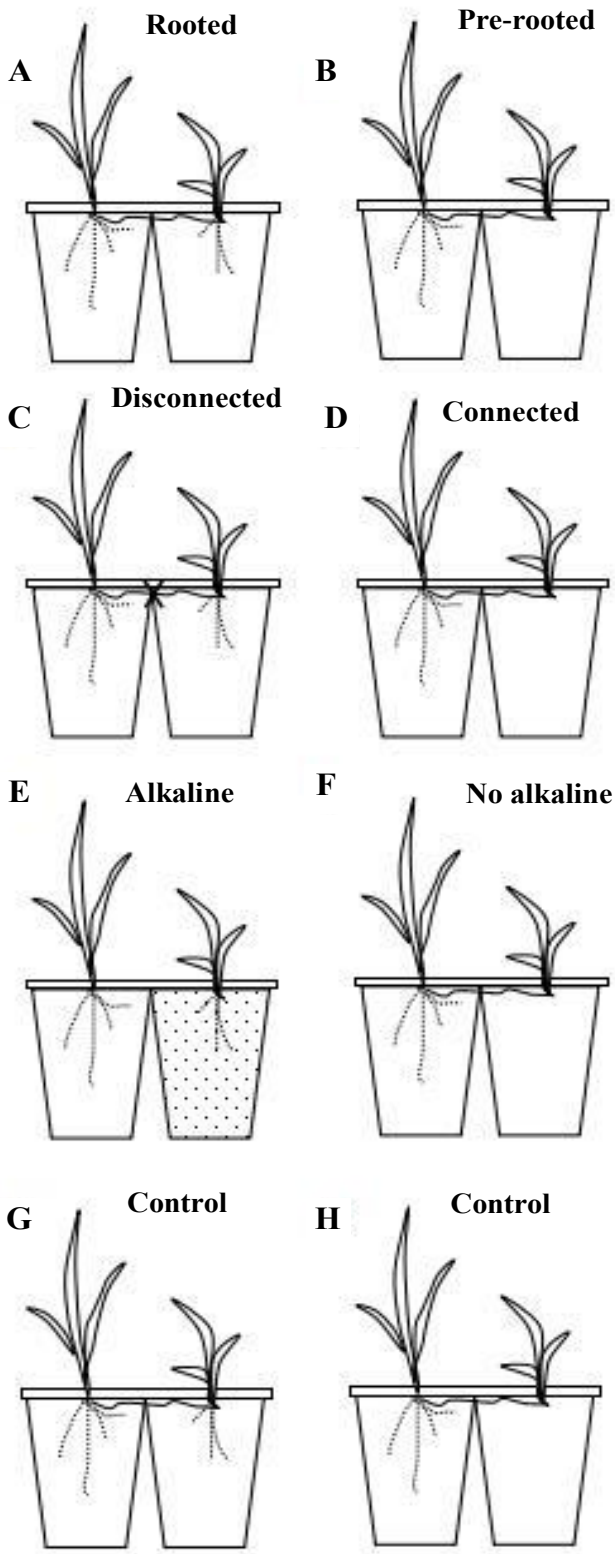

Fig. 1. Effects of rooting stage, rhizome connection, and alkaline stress on ^{15}N transport between basal and apical ramets of *Leymus chinensis*. Rooted (A), pre-rooted (B), disconnected (C), connected (D), alkaline (E), no alkaline (F), control (G), control (H).

Measurements: Vegetative growth was measured each week for the duration of the experiment. All plants were harvested on September 5, 2017. Rhizomes were cut at the mid-point between basal and apical ramets, and samples were harvested, dried at 60°C for 72 h to determine the dry matter, and ground to < 0.3 mm for isotopic analysis. Total nitrogen content (total N) and percentage of ^{15}N in plant samples were determined using a MAT-251 elemental analyzer interfaced with a VGI mass spectrometer (Isotope Services Inc., Los Alamos, NM, USA). The percentage of ^{15}N was calculated according to a previous method (HÖGberg, 1997). Since the biomass of roots was too small, we pooled the root biomass and shoot biomass together. The percentage of ^{15}N was defined as the ratio of the total amount of ^{15}N translocated from labelled (basal) to unlabelled (apical) ramets.

Data analysis: A three-way analysis of variance (ANOVA) was used to investigate the effects of rhizomal connection, rooting stage, alkaline stress, and their interactions on biomass, height, total N, and percentage of translocated ^{15}N of apical ramets. To test whether translocation of ^{15}N between ramets was related to rooting stage with respect to alkaline stress, the effects of rhizome severing on the percentage of translocated ^{15}N was analyzed by two-way ANOVA in each experiment. The two-way ANOVA was followed by a Student's *t*-test in order to detect any differences between connected and disconnected, pre- and post-rooting stages, with and without alkaline stress, while Steel-Dwass test was used to compare the significance of these effects under alkaline and non-alkaline treatment. Nonparametric tests were also performed to determine whether the data assume particular distribution. Finally, all datasets were checked for normality before analysis, and none of them were found significantly deviated from a normal distribution. SAS version 9.1 was used for all analyses (SAS Institute, Cary, NC, USA).

Results

Nitrogen translocation: The percentage of ^{15}N and total N content of apical ramets were significantly higher in connected than disconnected treatments (Table 1 and Fig. 2A, B). The translocation of ^{15}N to pre-rooting apical ramets was significantly higher (3.17%) than to post-rooting ones (0.89%) non-alkaline treatment (Table 1 and Fig. 2A; $F = 303.70$, $p<0.001$). However, under alkaline treatment, such differences were not observed (Table 1 and Fig. 2A; $F = 5.75$, $p>0.05$). The total N content of apical ramets followed the same pattern with translocated ^{15}N, i.e., higher in pre-rooting apical ramets under non-alkaline treatment, but no difference was found between pre- and post-rooting apical ramets under alkaline treatment (Table 1 and Fig. 2B). The percentages of ^{15}N and total N content of basal ramets were slightly lower in connected than in disconnected treatments, but the effect was not statistically significant (Table 1 and Fig. 2C, D). However, the percentage of ^{15}N in basal ramets was marginally higher in post-rooting treatment (4.32%) than in pre-rooting treatment (3.78%) under non-alkaline treatment (Table 1 and Fig. 2C; $F = 8.66$, $p<0.05$).

Table 1. Percentage of ^{15}N transported and total N content between basal and apical ramets and pre- and post-rooted in *Leymus chinensis*.

Rooting stage	Apical ramet		Basal ramet	
	Percentage of ^{15}N (%)	Total N (mg/kg)	Percentage of ^{15}N (%)	Total N (mg/kg)
No alkaline				
Pre-rooted	3.178 ± 0.130[a]	2.575 ± 0.066[a]	3.785 ± 0.181[b]	3.066 ± 0.025[a]
Post-rooted	0.890 ± 0.018[b]	1.599 ± 0.082[b]	4.322 ± 0.032[a]	3.261 ± 0.071[a]
Alkaline				
Pre-rooted	4.086 ± 0.041[a]	3.221 ± 0.062[a]	3.600 ± 0.079[a]	2.581 ± 0.091[a]
Post-rooted	3.845 ± 0.092[a]	3.027 ± 0.060[a]	3.762 ± 0.112[a]	2.599 ± 0.082[a]

[a,b]indicate significant differences among different treatments (p<0.05, n=9)

Table 2. Analysis of variance for the effects of alkaline stress, rhizomal connection, and rooting stage on basal and apical ramets of *Leymus chinensis*.

Source	d.f.	Apical ramet		Basal ramet	
		Biomass	Height	Biomass	Height
Alkaline	1	16^{***}	16.98^{***}	0.09^{ns}	0.32^{ns}
Severing	1	17.04^{***}	30.41^{***}	3.51^{ns}	0.29^{ns}
Rooting stage	1	19.97^{***}	148.05^{***}	0.81^{ns}	5.4^{*}
Alkaline × Rooting stage	1	0.11^{ns}	1.26^{ns}	1.82^{ns}	0.95^{ns}
Alkaline × Severing	1	9.75^{**}	14.54^{***}	0.01^{ns}	1.33^{ns}
Rooting stage × Severing	1	0.41^{ns}	0.19^{ns}	0.89^{ns}	0.01^{ns}
Alkaline × Severing × Rooting stage	1	1.68^{ns}	0.47^{ns}	0.01^{ns}	0.01^{ns}

F-values are shown for each variable followed by their respective significance levels; *p<0.05; **p<0.01; ***p<0.001; ns, p>0.05

Biomass and height: The biomass and height of apical ramets were significantly higher in the connected than in the disconnected treatment (Table 2 and Fig. 3A and B; biomass: F = 17.04, p<0.001; height: F = 30.41, p<0.001). However, disconnection did not affect the biomass or height of apical ramets that had already rooted in the absence of alkaline stress (Fig. 3A and B). The growth of apical ramets was influenced by whether they had already rooted, whether they were connected to their basal ramets had been severed, whether they were subject to alkaline stress, and interaction between disconnection and alkalinity (Table 2). In addition, the biomass and height of basal ramets did not differ significantly between the connected and severed treatment (Table 2 and Fig. 3C, D). There was a slight increase in severed ramets compared to connected ramets in post-rooting stage without alkaline treatment, but the results were not statistically significant (p>0.05; Table 2 and Fig. 3C).

Discussion

The effects of the developing stage on clonal integration: This ^{15}N-labelling study clearly revealed that the rhizomatous, clonal plant *Leymus chinensis* readily translocates nitrogen between basal and apical ramets along the rhizome system, particularly during the pre-rooting stage. These results support the hypothesis that clonal integration increases nitrogen translocation in unrooted ramets compared to rooted ramets, suggesting that clonal integration is influenced by growth stage (Gao *et al*., 2013; Matlaga & Sternberg, 2009). This finding is consistent with results from *Calathea marantifolia* in wet forest, whereby the pre-rooting apical ramets received more deuterium water from their basal ramets than post-rooting ones. Moreover, severing their connections to the basal ramets reduced their performance; however, after the apical ramets were rooted in soil, they received no deuterium water from their basal ramets and severing the connections has little effect on their performance (Matlaga & Sternberg, 2009).

Clonal integration plays a more important role in juvenile ramets because they are more vulnerable to environmental stress compared to adult ones. Therefore, for juvenile ramets, more support is required from the basal ramet to overcome stressful conditions. Previous work has shown that support from basal ramets decreases as the connected apical ramets age. The decreased support occurs due to increased tiller size, root area, and leaf surface area of the apical ramets when they were gradually becoming mature (Matlaga & Sternberg, 2009; Xiao *et al*., 2011). Our experimental results showed that post-rooting ramets had less translocated ^{15}N than pre-rooting ramets, which could be due to source-sink dynamics (Hartnett & Bazzaz, 1983). Nitrogen transport depends on the strength of the nitrogen potential gradient between source and sink. Since the nitrogen potential gradient between donating and recipient ramets is due to the difference in the uptaking capacity of their roots, little ^{15}N would be translocated from the basal ramet if the apical ramet is as efficient in nitrogen uptake. Therefore, in our study, ^{15}N may not have been transported between basal and rooted apical ramets which can independently take up nutrients. Our results suggest that apical ramets of *L. chinensis* are weaned from their basal ramets as they progress from pre- to post-rooting stages.

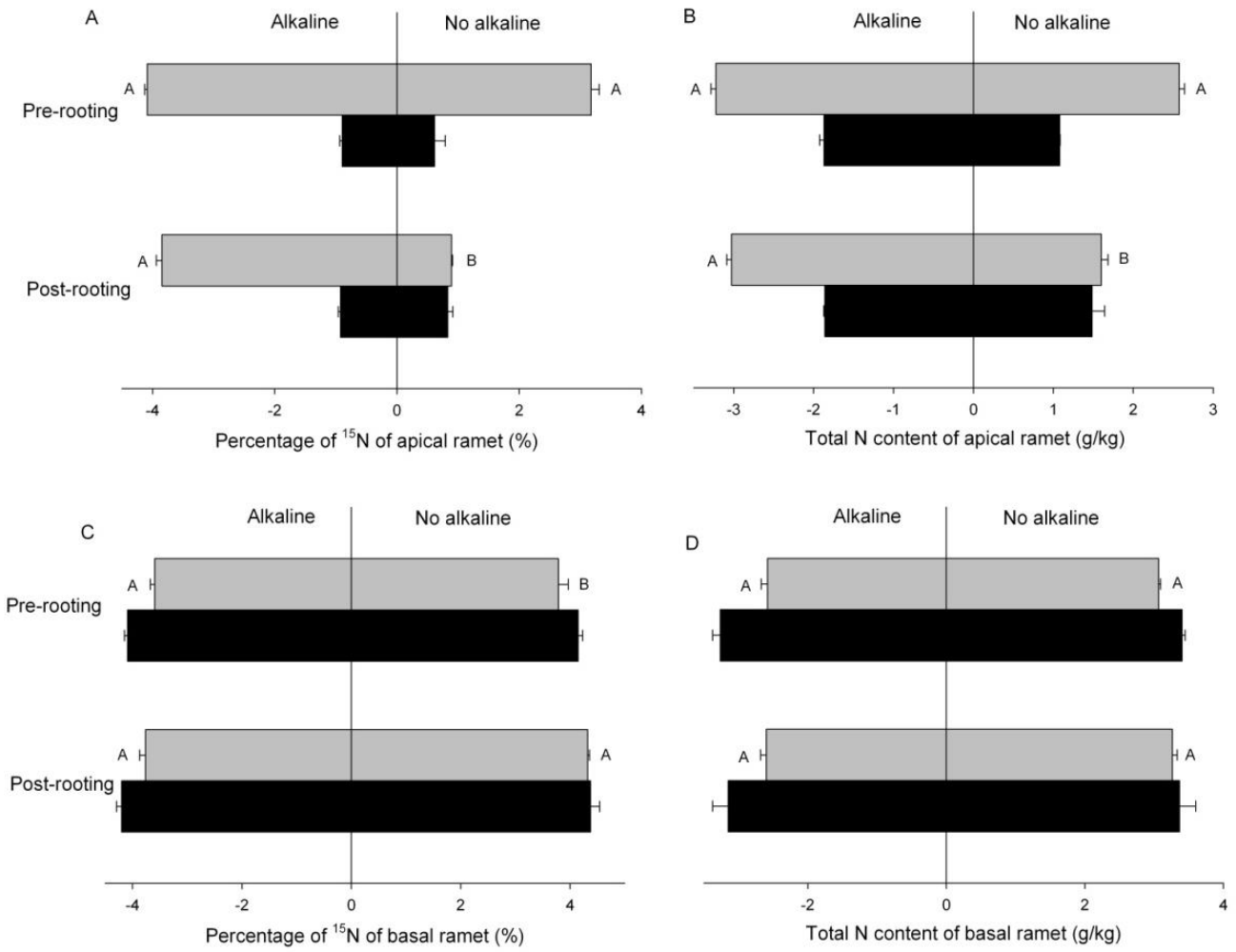

Fig. 2. Percentage of ^{15}N (A), total N content of apical ramets (B), percentage of ^{15}N (C), and total N content of basal ramets (D) in *Leymus chinensis* under combinations of connected, developing stages, and alkaline treatments (Open bars, connected ramets; black bars, severed ramets).

Change in magnitude of clonal integration under alkaline stress conditions: Previous studies, ever found that clonal integration is affected by genetically-based physiological factors, developing stage and morphological structures as well as environmental condition (Pan & Clay, 2004; Matlaga & Sternberg, 2009; Wang *et al*., 2004). In our experiments, the percentage of ^{15}N transferred from labelled to unlabeled post-rooting ramets was higher when the latter were alkaline stressed than not stressed. However, ^{15}N transportation from the basal to apical ramets was significantly increased when the apical ramets experienced alkaline stress. Our results suggest that alkaline stress is a powerful force and mat increase clonal integration. Therefore, magnitude of clonal integration could be changed by external environmental stress.

Source-sink feedback regulation plays an important role in fulfilling the resource translocation between the interconnected ramets (Hartnett & Bazzaz, 1983). Under heterogeneous conditions, non-stressed ramets will serve as "donors" that supply resources, while stressed ramets will function as "recipients" of the resources (Li *et al*., 2011). In the present study, alkaline stress caused nitrogen regulation in internal systems. Thus, independent ramets can receive nutrients from other ramets when they suffer soil stress, and the increased ^{15}N translocation in stressed ramets was most likely a result of clonal integration. These results are in agreement with previous studies showing that dependent ramets can receive nutrients from other ramets when they suffer leaf removal or damage (Watson & Casper, 1984).

Clonal integration affects the growth of interconnected ramets: As expected, clonal integration markedly increased the biomass and height of apical ramets under alkaline stress conditions and increased those of pre-rooting *L. chinensis* under non-alkaline conditions. These results are in agreement with previous studies that suggest clonal integration ameliorates the negative effects caused by inadequate nutrition (Roiloa & Retuerto, 2006), salinity (Xiao *et al*., 2011), drought (Chen *et al*., 2010; Liang *et al*., 2020) and developing stage (Matlaga & Sternberg, 2009). One possible reason is that the nitrogen resource acquisition of entire clonal fragments could be increased by improving the

photosynthetic capacity or resource uptake capacity in stress environments (Roiloa & Retuerto, 2006; Saitoh *et al*., 2006). Under post-rooting in non-alkaline conditions, however, the rhizome connection did not increase the performance of *L. chinensis*, suggesting that integration contributed little to the growth of this species under such conditions. Similarly, clonal integration did not occur to *L. chinensis* ramets even when they were clipped to a height of 10-15 cm (< 70% shoot removal)(Wang *et al*., 2004) This is likely because for the post-rooting ramets, nutrients taken up by their own roots were sufficient for their growth, and therefore clonal integration was not necessary. In this experiment, translocation of resources to apical ramets did not negatively affect the growth of interconnected basal ramets, suggesting that there is little net cost of clonal integration at the clonal level. This is consistent with previous studies, which reported that apical ramets gain increased benefits without any cost to their basal ramets (Yu *et al*., 2002; Chen *et al*., 2010).

As a "guerilla" clonal species, *L. chinensis* clone is very likely to grow in a heterogeneous habitat, and apical ramets are usually benefit from clonal integration especially when they are exposed to environmental stress, but such benefit depends on the developmental age of the recipient ramets. The pre-rooting ramets usually benefit more than post-rooting ramets and can increase growth with the nitrogen integration of the connected basal parts. However, post-rooting ramets are gradually cut off from the basal ramet resources as inter-ramet connections age. On the other hand, basal ramets may provide increased support when apical ramets (both pre- and post-rooting stage) experience alkaline stress. Thus, clonal integration may greatly improve the ability of the rhizomatous species to tolerate alkaline stress and such effect may change with the developing stage and environments. The molecular mechanisms responsible for the induced nitrogen integration adjustments among ramets during different developing stages and/or under alkaline stress conditions need to be ascertained in future studies.

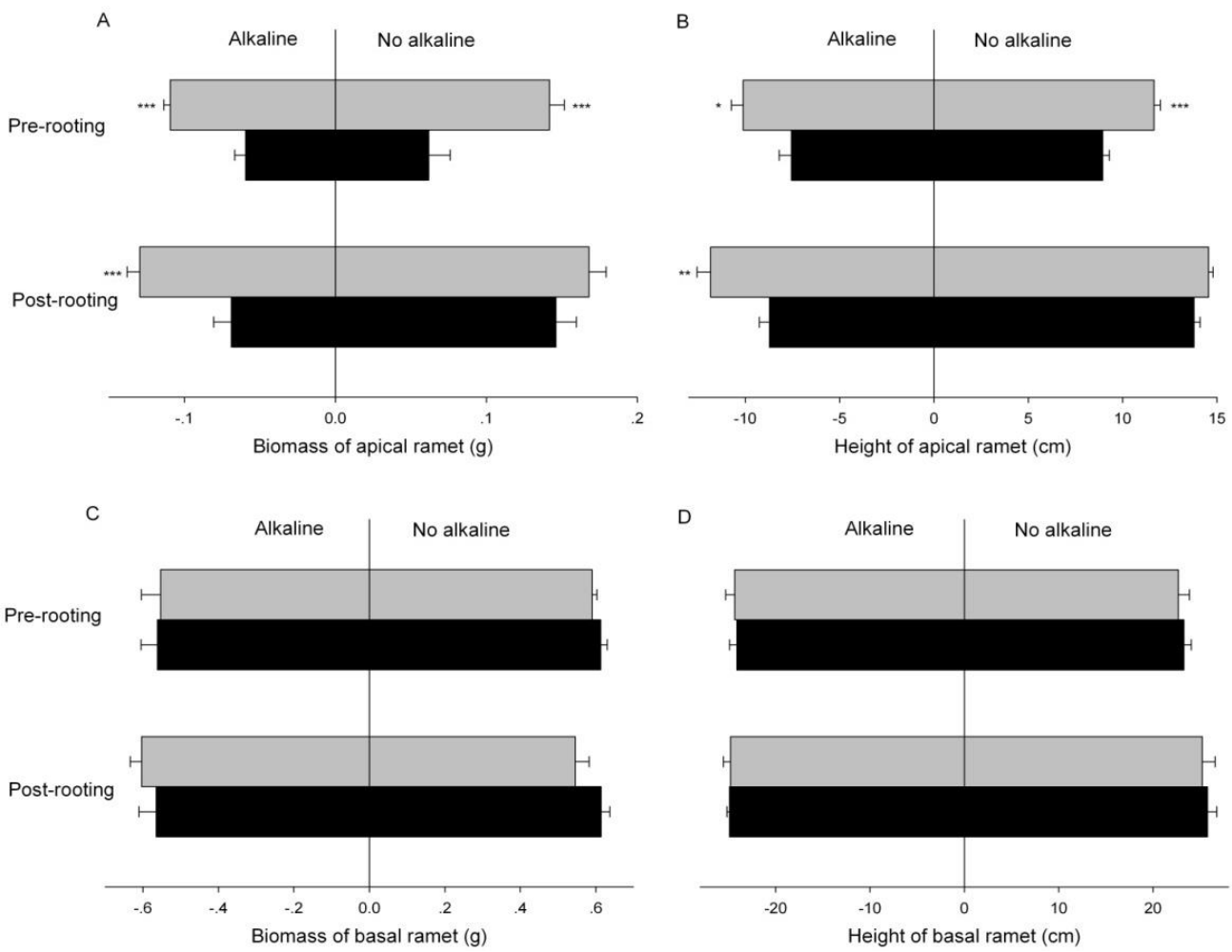

Fig. 3. Biomass (A), height of apical ramets (B), biomass (C) and height of basal ramets (D) in *Leymus chinensis* under combinations of connected, developing stages and alkaline treatments (Open bars, connected ramets; black bars, severed ramets).

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