Linking ethylene to nitrogen-dependent leaf longevity of grass species in a temperate steppe

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INTRODUCTION

Leaf longevity is an important plant functional trait that often varies with soil nitrogen supply. Ethylene is a classical plant hormone involved in the control of senescence and abscission, but its role in nitrogen-dependent leaf longevity is largely unknown.

Methods Pot and field experiments were performed to examine the effects of nitrogen addition on leaf longevity and ethylene production in two dominant plant species, Agropyron cristatum and Stipa krylovii, in a temperate steppe in northern China.

Key Results Nitrogen addition increased leaf ethylene production and nitrogen concentration but shortened leaf longevity; the addition of cobalt chloride, an ethylene biosynthesis inhibitor, reduced leaf nitrogen concentration and increased leaf longevity. Path analysis indicated that nitrogen addition reduced leaf longevity mainly through altering leaf ethylene production.

Conclusions These findings provide the first experimental evidence in support of the involvement of ethylene in nitrogen-induced decrease in leaf longevity.

Key words: Agropyron cristatum, Stipa krylovii, ethylene production, Inner Mongolia, leaf life span, leaf nitrogen, grassland, nitrogen addition.
China is reported to be sensitive to environmental change (Christensen et al., 2004; Kang et al., 2007; Xu et al., 2012). As N is one of the key limiting factors for plant growth in this area (Bai et al., 2010), projected increases in N deposition (Galloway et al., 2004) would presumably impact plant physiological processes. Recent work in this area revealed that leaf longevities of two dominant species, Agropyron cristatum and Stipa krylovii, significantly decreased with increasing soil N supply (Ren et al., 2011). Since ethylene is an important plant hormone relating to leaf senescence (Abeles et al., 1992) and improved N could stimulate ethylene production from plants (Tian et al., 2009), we hypothesized that N-dependent leaf longevity of grass species is mediated by ethylene. We evaluated the role of ethylene via both pot and field experiments, to explore the mechanisms underlying how N addition decreases leaf longevity. The main objectives of this study were to determine (1) whether ethylene production is responsive to N addition, (2) whether leaf longevity is related to ethylene production, and (3) whether leaf N influences leaf longevity directly or indirectly via changing ethylene production.

MATERIALS AND METHODS

Study site

Two complementary experiments, one in the field and another in pots, were conducted in a typical steppe in Duolun county (116°17′ E, 42°02′ N, elevation 1324 m a.s.l.), Inner Mongolia, China. The climate is temperate and semiarid with cold winters and warm summers (mean January and July temperatures are −17.5 and 18.9 °C, respectively). Mean annual temperature is 2.1 °C and mean annual precipitation is about 380 mm. The major soil type is chestnut soil. Dominant vegetation at the study site consists of two C3 grasses (Stipa krylovii and Agropyron cristatum) and a forb (Artemisia frigida).

Field experiment

In our previous field experiment, we found that leaf longevity of S. krylovii and A. cristatum was reduced by N addition in 2007 and 2008 (Ren et al., 2011). In the present field study, we used leaf longevity data for 2008 to determine the relationships between leaf longevity and leaf ethylene and N concentrations, because the annual precipitations in 2010 (329 mm) and 2008 (357 mm) were very close.

The field experiment was deployed in a long-term nutrient manipulation experiment conducted since 2005. It is a randomized block design with five replicate blocks, each containing four 8 × 8 m² plots. The four plots in each block randomly received 0, 5, 10 and 15 g N m⁻² year⁻¹, respectively, in the form of urea applied on two occasions, early May and July (half the amount applied each time). Addition of N occurred in the early and peak stages of the growing season, in order to alleviate N limitation of plant growth. Ethylene and leaf N concentrations of A. cristatum and S. krylovii were determined from July to October in 2010. The main objective of the field experiment was to determine the responses of leaf longevity, ethylene production and N concentration to N addition in situ and to compare the results with those of a pot experiment.

Pot experiment

We conducted the pot experiment to manipulate ethylene production by adding cobalt chloride (CoCl₂, an ethylene biosynthesis inhibitor) and to explore the linkage between ethylene production and N-dependent leaf longevity. For the pot experiment, four treatments, comprising no additions (control), added N, added CoCl₂ and combined N and CoCl₂ addition (N + CoCl₂) were applied. Each of the four treatments had five replicates and each replicate consisted of 20 pots. In the added N treatment, 0.45 g N (equivalent to 10 g N m⁻² year⁻¹) was added in the form of urea applied twice, on 27 July and 11 August 2010. The N addition rate was chosen based on our goal of alleviating potential N limitation. We applied CoCl₂ to soils at 10 μM on 27 July and this addition was repeated approximately every 4 days. Equal amounts of solution without CoCl₂ were also added at the same time to the control pots. One dominant species, A. cristatum, was used in the pot experiment. Seedlings, which were generally of identical growth status, of similar size and collected from a natural steppe site close to our field experiment site, were transplanted to plastic pots on 27 June. Each pot was planted with five seedlings. The plastic pots were 30 cm in diameter and 25 cm in height and filled with 4 kg in situ soil. The background values of total organic carbon, total N and total phosphorus in the pot soil were 29.9, 2.6 and 0.6 g kg⁻¹, respectively. Experimental treatments were applied to the seedlings ~20 days following transplantation. Thereafter, leaf longevity, ethylene production and N concentrations were measured from July to October 2010.

Measurements of leaf longevity

For the pot experiment, a leaf census of A. cristatum was made every 10 days from July to October in the pot experiment in 2010. Protocols modified from Craine et al. (1999) and Craine and Reich (2001) were used to determine the average leaf longevity of plants. Briefly, leaf longevity was estimated by dividing the accumulated length of those leaves that were born and senesced over the census period based upon the following model (Ren et al., 2011):

\[
\text{Leaf longevity} = \frac{(A - B - C - D)}{(S_j - L_{A_j})}
\]

\[
\sum_{n=0}^{i} \left( \frac{L_{A_n} + L_{A_{n+1}}}{2} \right) \times K_n
\]

\[
\sum_{n=0}^{i} \left( \frac{S_n + S_{n+1}}{2} \right) \times K_n
\]

\[
\left[ (n_s - n_i) \times L_{A_j} \right] - \left[ \sum_{n=i}^{n_s} \left( \frac{S_n + S_{n+1}}{2} \right) \times K_n \right]
\]

\[
\sum_{n=i}^{n_s} \left( \frac{L_{A_n} + L_{A_{n+1}}}{2} \right) \times K_n - \left[ (n_s - n_i) \times L_{A_j} \right]
\]

where \(i\) is the starting census date, \(j\) is the last census date, \(L_{A_n}\) and \(S_n\) are cumulative length or area of leaves produced and senesced, respectively.
by census date \( n \), respectively. \( K_n \) is time interval between census \( n \) and \( n + 1 \), \( n_s \) is the date at which the amount of leaf length or leaf area senesced is equivalent to the amount of leaf length or leaf area at the first census \( (S = LA_s) \) and \( n_l \) is the date at which the cumulative leaf length or leaf area produced is equal to the amount of leaf length or leaf area senesced by the last census date \( (LA = S_n) \). Values of \( n_s \) and \( n_l \) were calculated by the linear interpolation method.

Equations (1A) and (1B) represent the cumulative amount of leaf-days for both green and senesced leaves and for senesced leaves, respectively, and their difference is the total unsenesced leaf-days over the entire census period. Equation (1C) represents the amount of leaf-days for leaves present at the beginning of the census period, and eqn (1D) represents the leaf-days of leaves that had not senesced by the end of the census period.

This method of calculating leaf longevity considered the leaves from various ages, including leaves with birth date unknown, leaves with death date unknown, and leaves that were born and senesced within the experimental period (Craine et al., 1999). Therefore, the leaf longevity calculations were not affected by leaf age variations, if any.

**Measurements of leaf ethylene production and nitrogen concentration**

For both the pot and the field experiment, measurements of leaf ethylene production and nitrogen concentration were carried out in 2010. Leaves were sampled approximately once every 4 days from late July to mid-September to determine leaf ethylene production and N concentration in the pot experiment, and weekly or every 2 weeks from early July to mid-September in the field experiment. For each species at each sampling time, 5–10 representative leaves were harvested from at least five individual plants in each plot. The leaves were sampled generally from the third node from the top, ensuring that all leaves collected had relatively similar age. N concentrations in senesced leaves were determined when the whole individual plant wilted in mid-October.

Leaves were collected from individual plants under different treatments and placed in open vials (5 ml for *S. krylovii* in the field experiment and 10 ml for *A. cristatum* in both the pot and the field experiment) for 30 min, and these vials were sealed with gas-tight stoppers. One millilitre of gas was collected from the vials after 1 h under dark conditions and analysed using a gas chromatograph with a photoionization detector and a packed Teflon column (GC-4400; East & West Analytical Company, Beijing, China). Thereafter, leaf samples were collected and dried at 65 °C to determine leaf N concentration with the Auto-Kjeldahl method (Kjeltec System 1026 distilling unit; Kjeltec Systems, Sweden).

**Data analysis**

Two-way analysis of variance (ANOVA) was performed to test the effects of N and CoCl₂ addition on leaf longevity. The effects of N and CoCl₂ addition on leaf ethylene production and N concentration were examined using repeated measures ANOVA. One-way ANOVA with Duncan’s multiple range test was used to evaluate the differences among the experimental treatments. Pearson’s correlation coefficients were employed to examine bivariate relationships between leaf longevity, ethylene production and leaf N. These three variables were entered into a path analysis model, which estimates the strength of direct and indirect relationships among variables (Shipley, 2000), to disentangle their interrelationships. Of particular interest was whether leaf N affected leaf longevity directly or indirectly by altering ethylene production. We restricted path analysis to data from the pot experiment, as limited leaf longevity data from the field (measurements done for the control and 10 g m⁻² year⁻¹ N plots only) reduces the power of the analysis. All statistical analyses were performed using SPSS 18-0 (SPSS, Inc., Chicago, IL, USA).

**RESULTS**

**Leaf longevity responded to N and CoCl₂ addition**

Leaf longevity of *A. cristatum* was significantly shortened, from 61.5 ± 0.5 (mean ± SE) days in the control to 57.4 ± 0.4 days in the N-added plots in the pot experiment \((P < 0.001, \text{Fig. 1})\). Adding the ethylene synthesis inhibitor CoCl₂ significantly prolonged leaf longevity of *A. cristatum* (from 61.5 ± 0.5 to 64.5 ± 0.3 days, \(P < 0.001, \text{Fig. 1}\)). The reduction in leaf longevity induced by N addition was alleviated by CoCl₂, increasing leaf longevity from 57.4 ± 0.4 to 59.9 ± 0.3 days \((P < 0.001, \text{Fig. 1})\).

**Addition of N evoked leaf ethylene production**

In the pot experiment, ethylene production from the leaves of *A. cristatum* reached peaks on the 5th day after the first N addition and the 3rd day after the second N addition, and gradually declined thereafter (Fig. 2A). Overall ethylene production from leaves in the N-addition pots was significantly higher \((P < 0.001)\) than that in the control pots throughout the experimental period (Fig. 2A, Supplementary Data Table S1). Adding CoCl₂ led to a significant reduction in ethylene production from leaves in the absence of N addition (Fig. 2A, Supplementary Data Table S1). Ethylene production induced by N addition was markedly suppressed by CoCl₂ throughout the experimental period. For instance, the peak of ethylene production after N

\[
\begin{align*}
\text{Leaf longevity} & = \text{leaf days at census } S \text{ for both green and senesced leaves} \\
& - \text{leaf days for senesced leaves} \\
& = \text{leaf days at census } S_n \text{ for both green and senesced leaves} \\
& - \text{leaf days for senesced leaves} \\
& = \text{leaf days at census } S_n \text{ for both green and senesced leaves} \\
& - \text{leaf days for senesced leaves}
\end{align*}
\]

**Fig. 1.** Average leaf longevity ± SE (days) of the dominant species, *Agropyron cristatum*, in the pot experiment for control, added N (N), added CoCl₂ (CoCl₂) and combined N and CoCl₂ addition (N + CoCl₂) treatments. Different letters above bars indicate significant differences according to Duncan’s multiple range test \((P < 0.001)\); \(n = 5\) for all treatments.
Addition was reduced from 3.8 ± 0.3 to 1.7 ± 0.2 ppm g⁻¹ FW h⁻¹ by CoCl₂ (P < 0.001, Fig. 2A, Supplementary Data Table S1).

As in the pot experiment, a significant increase in ethylene production in response to N addition was observed in the field experiment for both *S. krylovii* and *A. cristatum* (Fig. 3A, B, Supplementary Data Table S2). The magnitude of increase in ethylene production was dependent on the amount of N added for *A. cristatum* (P < 0.01), but not for *S. krylovii*. For instance, ethylene production from leaves of *A. cristatum* under N addition of 10 and 15 g N m⁻² year⁻¹ was significantly higher than under addition of 5 g N m⁻² year⁻¹ (P < 0.01, Fig. 3b, Supplementary Data Table S2). The ethylene production from leaves of *S. krylovii* increased by 62.9, 74.7 and 75.3 % on application of 5, 10 and 15 g N m⁻² year⁻¹, respectively (Fig. 3A, Supplementary Data Table S2); however, there were no significant differences in ethylene production among the three levels of N addition (Supplementary Data Table S2).

**Addition of N increased leaf nitrogen concentration**

In the pot experiment, N addition led to a significant increase in leaf N concentration (P < 0.001, Fig. 2B, Supplementary Data Table S3). The effect of CoCl₂ addition, however, depended on N levels. Adding CoCl₂ had little effect on N concentration in the presence of N addition, but caused a small decrease (2.8 %) in leaf N concentration in the absence of N addition (Fig. 2B inset, Supplementary Data Table S3).

**Leaf N concentration of *S. krylovii* also increased in response to N addition in the field experiment** (P < 0.001, Fig. 3C, Supplementary Data Table S4). Compared with the controls, leaf N concentration increased by 7.7, 15.6 and 16.4 % in the N5, N10 and N15 treatments, respectively (Supplementary Data Table S4). A similar increase in leaf N concentration in response to N addition was also observed for *A. cristatum* in the field experiment (P < 0.001, Fig. 3D, Supplementary Data Table S4).

**Contributions of leaf ethylene production and leaf N to leaf longevity**

Leaf longevities of the two dominant species correlated negatively with both leaf ethylene production and leaf N (P < 0.001,
DISCUSSION

We set out to explore N-dependent variation in leaf longevity via both pot and field experiments. Three main results emerged: (1) N addition enhanced leaf ethylene production; (2) increased leaf ethylene production was associated with decreased leaf longevity; and (3) N addition reduced leaf longevity mainly through altering leaf ethylene production.

Leaf longevity in relation to N addition

Our results showed that N addition shortened leaf longevity of the two dominant species, *A. cristatum* and *S. krylovii*, in a typical steppe. Many other studies have reported similar findings (Shaver, 1981; Lajtha and Whitford, 1989; Cordell et al., 2001; Craine and Reich, 2001). Variations in leaf longevity are linked to trade-offs between leaf persistence and productivity, such that leaves with thicker lamina, higher tissue density and longer longevity tend to have lower nutrient concentration and photosynthetic rate (Reich et al., 1991, 1992; Diemer, 1998; Wright et al., 2004; He et al., 2009). Carbon cost–benefit analysis suggests that leaf longevity should decrease as leaf photosynthetic rate increases and/or construction cost decreases, maximizing plant carbon gain (Chabot and Hicks, 1982; Kikuzawa, 1991, 1995; Shipley et al., 2006). The observed negative response of leaf longevity to increased N availability, which increases photosynthesis rate and reduces construction cost, supports this prediction. However, some physiological studies have also found a reduction in leaf longevity under N shortage in an annual herb, the sunflower (*Helianthus annuus*) (Ono et al., 1996) and *Arabidopsis* (Hanaoka et al., 2002), which may be attributed to the source–sink N dynamics within plants (Ono et al., 2001; Hikosaka, 2005). Specifically, under N deficiency new leaves (sink organs) may accelerate retranslocation from old leaves (source organs) to satisfy their N demand, resulting in accelerating senescence in old leaves (Hikosaka, 2005).

Regardless, leaf dynamics are regulated to maximize carbon gain and resource-use efficiency of plants. The contradictory results of physiological studies and ecological studies may be attributed to different N deficiency status, i.e. physiological experiments often subject plants to a sudden change in N supply, while ecologists usually use field plants, in which nitrogen turnover is nearly at a steady state (Hikosaka, 2003).

Besides, whether N addition decreases leaf longevity might be dependent on growth conditions and species. For instance, if the amount of N added is sufficient for plant growth and other factors are unaltered, the plant may not need to re-translocate N within itself and their leaf longevity may not change. On the other hand, if older leaves are progressively over-shaded by new leaves with the acceleration of growth, leaf longevity may decrease with N addition. But these conditions did not occur in our study. Plant growth was stimulated by addition of 10 g N m⁻² and may continue to increase when more N could be available, which was verified by a multi-level N addition gradient experiment in the same region (Huang et al., 2008). Additionally, shading rarely occurs in the two studied species because of their tiny leaves and loose canopy structure.

Ethylene in relation to nutrient status

In our study, ethylene production from leaves of the two dominant species increased rapidly with N addition. Such positive responses of ethylene production to high N supply are known to occur in plant roots (Feng and Barker, 1993; Leblanc et al., 2008; Tian et al., 2009). This arises presumably because a high N concentration stimulates the activities of enzymes associated with ethylene synthesis (Tian et al., 2009). In addition, increased ethylene may also be involved in modulating nitrate transporters (Tian et al., 2009) and nitrate metabolism (Leblanc et al., 2008) under high nitrate conditions.

Ethylene in relation to N-dependent changes in leaf longevity

Leaf senescence and longevity are sensitive to many factors: not only N and ethylene, but also light level, leaf sugar content, competition and other hormones, such as cytokinin. It is well established, however, that ethylene is the most key plant hormone associated with leaf senescence (Jackson and Osborne, 1970; Grbic and Bleecker, 1995).

In addition, although numerous studies have demonstrated the linkage between nitrogen and leaf longevity, none have investigated the potential role of ethylene in this context. By applying CoCl₂, which has been widely used to inhibit ethylene biosynthesis, we were able to confirm the involvement of ethylene in leaf senescence and longevity. Most importantly, the result of our path analysis suggests that N-induced change in ethylene production is behind the N-dependent leaf longevity. Given the status of ethylene as a common plant hormone, our findings have important implications for ecosystems experiencing elevated N deposition; this is especially so as N deposition has been predicted to increase globally (Vitousek et al., 1997), and the projected rates of increase are particularly high in the Eurasia area (Galloway et al., 2004). Nevertheless, several caveats are worth noting here. First, our main results, particularly those of the path analysis, were based on the pot experiment. The
generality of these results would need to be assessed in field experiments. Second, our experiment focused on examining leaves of similar ages, and did not investigate the responses of leaves of different ages to experimental treatments. A natural extension of our experiment would thus be to examine whether leaves at different locations of the plant respond to experimental treatments in the same way. Third, the ethylene biosynthesis inhibitor CoCl₂ was used to alter ethylene production in our experiment. An alternative approach would be to manipulate the concentration of exogenous ethylene directly. This direct approach, however, would be much more costly.

In summary, this study has produced the first experimental evidence that ethylene modulates the N-induced decrease in leaf longevity, providing a novel yet intuitive explanation for a widely observed ecological phenomenon. Indeed, a decrease in leaf longevity after N addition has been consistently observed for other common plant species in the northern China temperate steppe (Ren et al., 2011), suggesting that N deposition-induced reduction in leaf lifespan may be a common feature of plants in this region. Nevertheless, this reduction in leaf lifespan is insufficient to offset the overall increase in productivity in response to increased N deposition that has been observed across various ecosystems (Elser et al., 2007; Yahdjian et al., 2011).

SUPPLEMENTARY DATA
Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: multiple comparison (Duncan test) results of leaf ethylene production for Agropyron cristatum in the pot experiment. Table S2: Multiple comparison (Duncan test) results of leaf N concentration of exogenous ethylene directly. This direct approach, however, would be much more costly.

LITERATURE CITED


