Effects of Pollination-prevention on Leaf Senescence and Post-silking Nitrogen Accumulation and Remobilization in Maize Hybrids Released in the Past Four Decades in China

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Abstract

In order to understand the regulation of source-sink relationship on leaf senescence in maize, we investigated the effect of pollination prevention on leaf senescence, post-silking dry matter and nitrogen accumulation and remobilization in different vegetative organs in one landrace and ten representative maize hybrids released between 1973 and 2000 in China. When pollination was prevented by covering silk with paper bags at silking stage, leaf senescence of Nongda 60, Yedan 13, Shendan 7 and Zhengdan 958 was delayed as shown by the remaining green leaf area per plant at maturity. However, pollination-prevention did not have obvious effect on the leaf senescence of Baimaya, Danyu 6, Yedan 2 and Xianyu 335. By contrast, pollination-prevention accelerated the leaf senescence of maize hybrids Zhongdan 2, Danyu13 and Yedan 4. It was found that leaf senescence of the late-released (since 1985) Chinese hybrids tended to be delayed by pollination-prevention, except XY335. From silking to physiological maturity, nitrogen content increased in the stem (plus the sheath, cob, husk, and tassel) and root of the non-pollinated plants. However, there was still a net reduction in leaf nitrogen content. We also found that pollination-prevention reduced leaf nitrogen remobilization efficiency, with genotypic difference and variation between the two years of testing. The results suggested that the response of leaf senescence to pollination-prevention is at least partially due to the change of leaf nitrogen remobilization efficiency. Leaf senescence tended to be delayed if leaf nitrogen remobilization efficiency is highly reduced by pollination-prevention.

Key words: leaf senescence, genotypic difference, maize, non-pollination, N remobilization

Introduction

In maize, it is believed that longer leaf duration (“stay-green”) is one of the key traits in modern breeding for high grain yield (Lee and Tolleneear, 2006, Mueller and Vyn, 2016). Correspondingly, post-silking nitrogen (N) uptake has been increased significantly (Pommel *et al.*, 2006; Irena Rajcan, 1999; Bender *et al.*, 2013). However, the stay-green trait was found to have negative effect on vegetative N remobilization and grain N concentration (Martin *et al.*, 2005; Gallais *et al.*, 2007; Coque *et al.*, 2008; Thomas and Ougham, 2014). Grain N concentration has been greatly decreased in modern maize hybrids compared with the old ones (Cassman, 1999; Chen *et al.*, 2013). Because leaf N is a major contributor to grain N, there has been growing interest in adjusting leaf senescence and improving N remobilization efficiency in maize to increase N use efficiency and grain N concentration (Schippers *et al.*, 2015). For example, the activity of glutamine synthetase 1 (GS1) was found to play a role in efficient N remobilization. In GS1 mutants Gln1.3 and Gln1.4, less N is re-translocated from the leaf to the grains (Martin *et al.*, 2006). Asparagine synthases (ZmAS3 and ZmAS4) are also likely to play an important role in N remobilization (Cañas *et al.*, 2010). These studies have been mostly focused on the process of protein turnover, N metabolism within the leaf and N export from the leaf.

Nitrogen remobilization from the leaves is related to the formation of reproductive organs. As the major sink for N deposition during grain filling phase, the size and existence of the grain sink in maize may have great feedback effect on the remobilization of N from the vegetative organs. In soybean, it was found that the leaves of the de-podded plants remained green and did not exhibit the normal senescing pattern (Mondal, 1978). Similarly, some researches indicated that the leaves in the earless (no grain and cob) maize plants remained green for a longer time (Moss, 1962; Christensen *et al.* 1981; Pan, 1986). Nevertheless, in other studies, prevention of pollination or removal of the ears after flowering caused premature senescence of the leaves above the ear (Allison and Weinmann, 1970; Below *et al.*, 1981; Christensen *et al.*, 1981; Crafts-Brandner, 1984). Christensen *et al.* (1981) found that the remobilization of N from the leaf does not depend on the presences of an ear. Crafts-Brandner *et al.* (1984) suggest that ear *per se* does not dictate the rate or completion of the senescence.

The different responses of leaf senescence to sink-removal treatments can be genotype

dependent (Crafts-Brandner *et al.*, 1984). Modern breeding activities during the past four decades have greatly changed the characteristics of N uptake and remobilization (Chen *et al.*, 2013; Ciampitti and Vyn, 2013; Chen *et al.*, 2014). It would be interesting to see if modern breeding has any effect on the response of leaf senescence to grain sink removal. In the present study, one landrace and 10 maize hybrids released from 1973 to 2000 in China have been used to evaluate the regulatory role of grain sink removal on leaf senescence and N remobilization from different vegetative organs. The aims are (1) to determine if the response of leaf senescence to grain sink removal is related to breeding process during the past decades; (2) to understand if the differential response of leaf senescence to grain sink removal among different hybrids is related to their difference in stay-green ability, N partitioning characters and/or leaf N remobilization potential during grain filling phase.

Materials and methods

Soil and Plant materials

The experiments were conducted in 2013 and 2014 at Shangzhuang Experimental Station of China Agricultural University in Beijing. The soil type is a typical Ustochrept soil with silty loam texture. The chemical properties of the soil in 2013 (0-30cm) and 2014 (0-40cm) were as follows: extracted mineral N ($N_{\min}$) 3.1 and 3.2 mg kg⁻¹, pH 7.3 and 7.2, Olsen-P 29.8 and 17.1 mg kg⁻¹, NH₄OAc extracted K 82.7 and 65.7 mg kg⁻¹, organic matter 8.5 and 9.5 g kg⁻¹, respectively. The field was irrigated with a sprinkler ten days before sowing and was plowed just before sowing.

A land race (Baimaya, BMY) and ten major maize hybrids released in the past four decades in China were used for the experiment (Table 1). The hybrids were Danyu 6 (DY6), Zhongdan 2 (ZD2), Yedan 2 (YD2), Danyu 13 (DY13), Shendan 7 (SD7), Nongda 60 (ND60), Yedan 4 (YD4), Yedan 13 (YD13), Zhengdan 958 (ZD958) and Xianyu 335 (XY335). Xianyu 335 is bred by Pioneer USA and its parents have no relationship with the other Chinese hybrids. These hybrids were the dominant hybrids grown in Northeast China at the time of their release, and had been ever accumulatively used more than 2.8 million ha in popularly cultivated period in China. ZD958 and XY335 are still the most popular commercial hybrids across North and Northeast of China

(Zhao *et al.*, 2011; Chen *et al.*, 2013; Ma *et al.*, 2014).

Seeds were sown on May 4, 2013 and April 29, 2014, respectively. Before sowing, plots were fertilized with 72 kg N ha⁻¹ (urea), 90 kg P₂O₅ ha⁻¹ (super phosphate), and 100 kg K₂O ha⁻¹ (potassium chloride). Then 168 kg N ha⁻¹ were banded as top dressing at V6 stage (33 days after sowing in 2013, and 37 days after sowing in 2014). Weed growth in plots was controlled by herbicides before seedling emergence and hand cultivation during the growth season. Plots were kept free of insects and diseases by using fungicide and pesticide when necessary.

In 2013, the dates of silking for the hybrids are: July 6 for BMY; July 10 for XY335 and YD4; July 11 for ZD958 and YD2; July 13 for DY6, ND60, and SD7; July 15 for DY13, YD13, and ZD2. In 2014, the dates of silking for the hybrids are: July 1 for BMY; July 6 for YD2, YD4, XY335, and ZD958; July 8 for DY6, ZD2, ND60, and SD7; July 15 for DY13 and YD13. The plants were harvested at physiological maturity when the black layer formed in the base of the kernels. The dates in 2013 are: August 26 for BMY, DY6, YD2, and XY335; August 29 for DY13, YD4, and YD13; August 31 for ND60, SD7, and ZD2; September 6 for ZD958. The dates in 2014 are: August 20 for BMY and DY6; August 21 for YD2 and XY335; August 26 for DY13, YD4, and YD13; August 29 for ND60, SD7, and ZD2; September 4 for ZD958. During maize growth period, the sunshine hours in 2013 were 9.7% lower than in 2014 (Fig. S1). The precipitation in 2013 (470mm) was 58.6% higher than in 2014 (296mm). No irrigation was conducted in 2013. At silking stage in 2014, the plots were irrigated to relieve drought stress.

Experimental design

The experiments were a randomized block design with four replicates. Each plot was 4 m long and 4.8 m wide. The distance between rows and plants was 60 cm and 28 cm, separately. The planting density was 60,000 plants per hectare. At silking stage when the silks were shown in 50% of the plants, two treatments were conducted: 1) Pollination: pollen was collected and applied to the silks when at silking stage; 2) Non-pollination: the silks were covered upon appearing with paper bags to prevent any pollination. As a result, no grains were formed on the cob. Sixty plants were treated in each plot and these plants were marked for sampling.

Plant sampling

At silking and physiological maturity stage, three healthy and uniform plants from each plot were cut at the soil surface and separated into the leaves, the stem (plus the sheath, cob, husk, and tassel) and the grains (only at physiological maturity). Roots were excavated within a soil volume of 60 cm (length) $\times$ 28 cm (width) $\times$ 40 cm (depth) for each plant. Roots were gently washed free of soil using a banister brush, dried at 70°C to a constant weight (Ning *et al.*, 2012). At maturity two rows from each plot were harvested for yield measurement. The non-pollinated plants were harvested at the same time as the control plants.

At both sampling dates, green leaf area (GLA) was determined using the formula $GLA = \text{leaf length} \times \text{maximum leaf width} \times 0.75$ (Chen *et al.*, 2014). Leaf chlorophyll index was estimated between 09:00 AM and 12:00 AM using a SPAD-502 Plus chlorophyll meter (Tokyo, Japan). The mean of eight readings taken along the ear blade (four on each side of the lamina) was recorded. A linear relationship between leaf chlorophyll content and SPAD meter readings has been previously determined (Dwyer *et al.*, 1991). All samples were heated at 105°C for 30 min, dried at 70°C to constant weight. After weighed to obtain dry matter (DM) weight, the samples were ground into fine powder for N measurement. Nitrogen concentration was measured using a standard Kjeldahl procedure (Chen *et al.*, 2015). Harvest index (HI), N harvest index (NHI), N remobilization, N remobilization efficiency % (NRE) in vegetative organs, as well as post-silking N uptake were calculated based on single plant as described below (Chen *et al.*, 2015):

$$HI = \text{Grain yield} / \text{Total plant DM at maturity}$$

$$NHI = \text{Grain N} / \text{Total plant N at maturity}$$

$$\text{N remobilization amount} = \text{vegetative N content at silking} - \text{vegetative N at maturity}$$

$$\text{N remobilization efficiency \%} = \text{N remobilization amount} / \text{vegetative N content at silking}$$

$$\text{Post-silking N uptake} = \text{Total plant N content at maturity} - \text{Total plant N content at silking}$$

Statistical analysis

Variance of the data was analyzed by two- or three-factor variance analysis program using ANOVA procedure of the IBM SPSS Statistics 20.0. Pollination and year were considered to be

fixed. Means were tested by least significant difference at $P < 0.05$ (LSD0.05). Figures were constructed using GraphPad Prism 6.0, and regression analyses were conducted using Curve Expert 1.3.

Result

Genotypic differences in grain yield and related traits

Grain yield (GY) was significantly affected by year and cultivar, but not year x cultivar interaction (Table 2). Grain yield in 2013 was 20% more than that in 2014 (Table 2), possibly because of more rains in 2014 (Fig. 1S). In general, GY tended to increase with increasing years of hybrid release, although there were exceptions. Grain N concentration (GNC) was the same in the two-year's experiment, but was affected by cultivar and year x cultivar interaction. In general, GNC tended to decrease with increasing years of hybrid release. GNC was the highest in the landrace BMY, possibly due to its low GY. Its harvest index (HI) and N harvest index (NHI) were much lower compared that of the other hybrids. Because of the dry weather at silking in 2014, as a landrace which had low ability of resistance to drought, the yield of BMY had been affected heavily. The HI and NHI were different among the hybrids. However, this difference does not seem to be related to the years of hybrid release.

Effects of pollination prevention on leaf senescence and leaf N concentration

In 2013 and 2014, prevention of pollination increased green leaf area (GLA) per plant in SD7, ND60, YD13 and ZD958, and reduced GLA in ZD2 (only in 2014), DY13 and YD4 at maturity (Fig. 1). For the remaining hybrids, prevention of pollination did not show effect on the leaf senescence. Prevention of pollination increased leaf N concentration in almost all the genotypes (Fig. 2). However, this effect was not significant in BMY, YD4 and ZD958 in 2013 and in ZD2 in 2014.

Effects of pollination prevention on post-silking DM accumulation and partitioning

Year, cultivar, pollination treatment, and their interactions had significant effect on post-silking

DM accumulation in different organs (Table 1S, 2S). Except for BMY and ZD2 in 2013, there was a net accumulation of DM in the vegetative organs in both pollination treatments (Table 3), suggesting post-silking DM production exceeds the requirement of grain yield formation. In pollination prevention treatment, post-silking DM in the whole vegetative organs increased or kept unchanged in comparison to that in the control plants (Table 3). Among different vegetative organs, this increase in DM was mainly shown in the stem. Across genotypes, pollination prevention increased the accumulation of DM by 19.6 % and 30.1 % in the stem, 41.6% and 40.6% in the roots, in 2013 and 2014, respectively.

In 2013, leaf DM showed a net decrease from silking to maturity, regardless of pollination treatments. In 2014, leaf DM kept almost unchanged in both pollination treatments. This difference in leaf DM accumulation might be explained by the higher grain yield in 2013 (Table 2), which led to more DM mobilization from the leaves, and resulted in higher HI (Table 2). Although some genotypic differences, pollination prevention reduced leaf DM export by 2% in 2013, and increased leaf DM accumulation by 7.4% in 2014.

Effects of pollination prevention on Pre-silking N remobilization and post-silking N accumulation

In control plants, N content in almost all the organs showed a net reduction, suggesting N was readily remobilized into the grains during post-silking stage (Table 4). In non-pollination plants, N content in the total vegetative organs increased, indicating that post-silking N uptake continues even though there was no ear. The net accumulation of N was shown in the stem and the roots, but not the leaves. Across the genotypes, prevention of pollination increased N accumulation by 1.9 and 1.6 fold in the stem, 95% and 76% in the roots, in 2013 and 2014 respectively. In the leaves, however, prevention of pollination reduced N export in some hybrids (SD7, YD13, ND60, ZD958), but had little effect on the others (ZD2, YD4) (Table 4). This data suggests that leaf N remobilization do not depend on the existence of an ear, or grain formation.

The remobilization efficiency of N in the leaves was further analyzed (Fig. 3). Across years and cultivars, pollination prevention reduced N remobilization efficiency (NRE) of the leaves. However, the responses were different among the cultivars and between the two years. In 2013,

the leaf NRE of BMY, DY6, YD2, YD4, and ZD958 did not response to pollination prevention. In 2014, the NRE of ZD2, SD7, YD4, and XY335 did not response to pollination prevention.

The relationship between non-pollination-induced leaf senescence and leaf N remobilization

The relationship between non-pollination-induced leaf senescence and all the tested physiological parameters, HI, NHI, leaf N concentration, leaf NRE, were analyzed. A close relationship between the change of leaf NRE and the change of GLA has been identified (Fig. 4). That is, when pollination prevention led to a lower reduction of leaf NRE (higher relative leaf NRE value), non-pollination treatment tends to decrease GLA per plant, indicating enhanced leaf senescence. On the contrary, when the pollination prevention led to a higher reduction of leaf NRE (lower relative leaf NRE value), non-pollination treatment tend to increase GLA per plant, indicating delayed leaf senescence. Therefore, the change of leaf NRE at least partially regulated the response of leaf senescence to pollination prevention.

Discussion

Previously it was found that the effect of pollination prevention on the DM of different organs varied among different genotypes (Ceppi *et al.*, 1987). In the present study, non-pollination treatment increased root and stalk DM accumulation during post-silking stage (Table 3), suggesting these organs remained the growth ability and/or had a great potential for DM deposition during post-silking stage. In fact, stalk is taken as a temporary or buffer sink for photo-assimilates (Daynard, 1969; Sekhon *et al.*, 2016). When pollination was prevented, the root became bigger and more husks were formed (data not shown). Similarly, Yan *et al.* (2011) and Ning *et al.* (2012) found that root length was increased when the ear was removed. However, leaf DM kept decreased or remain rather stable when grain sink was removed in 2013 (Table 3), suggesting the leaf is always the source of photo-assimilates.

With leaf senescence, N in the leaves is remobilized into the grains to fulfill the requirement of protein synthesis in the grain (Pan, 1995; Borrás *et al.*, 2003; Jing and Nam, 2012). Therefore, it is expected that removal of the grain sink by preventing pollination have a negative effect on

leaf N remobilization and hence delay leaf senescence. It was true that leaf N concentration was increased in almost all the genotypes in pollination prevention treatment (Fig. 1). Correspondingly, leaf senescence of ND60, SD7, YD13, and ZD958 was delayed as shown by the remaining GLA per plant at maturity. However, in BMY, DY6, YD2, and XY335, prevention of pollination did not have an effect on leaf senescence. Even more, in hybrids ZD2, DY13, and YD4, prevention of pollination resulted in faster leaf senescence as indicated by reduced GLA at maturity. Sink-removal induced leaf senescence was also shown in Christensen *et al.* (1981), Crafts-Brandner *et al.* (1984), Rajcan and Tollenaar (1999). Besides, a study found that a decreased N and accelerated senescence in earless leaf (Steven *et al.*, 1984). Christensen *et al.* (1981) suggested that the rapid leaf senescence in earless plant may be explained by the cessation of N uptake into the leaves. However, in this study, leaf N concentration in almost all other hybrids was increased by sink removal (Fig.2). Hence, loss of nitrogen might not be the reason for faster leaf senescence. Another possible explanation is that more sugar or starch accumulation in the leaves can result in leaf senescence (Wingler and Roitsch, 2008; Seebauer *et al.*, 2010). It is possible that prevention of pollination inhibited carbohydrate export from the leaves, resulting sugar-induced early leaf senescence. Indeed, it has been reported that sugar and starch concentration increased significantly in the leaf of the earless plants (Allison and Weinmann, 1970; Below *et al.*, 1981). In the hybrids which leaf senescence was enhanced by pollination prevention, the color of the leaves (especially the upper leaves which exposed to greater light density) became purplish red before they died, suggesting more anthocyanin was synthesized in these leaves due to surplus carbohydrate accumulation (Allison and Weinmann, 1970). Pollination prevention might also change the level of plant hormones related to leaf senescence such as cytokinin (Brugière *et al.*, 2008; DécimaOneto *et al.*, 2016). More physiological research is required to elucidate the mechanism.

The different response of leaf senescence to pollination prevention among maize hybrids could not be simply explained by the year of hybrids release (Table 1, Fig.1). Nevertheless, among the seven old hybrids released before 1985, three hybrids (ZD2, DY13, and YD4) showed enhanced leaf senescence after prevention-pollination, other three hybrid (BMY, DY6, and YD2) did not response to non-pollination treatment. Only leaf senescence in SD7 was delayed by sink

removal. Among the four hybrids released after 1985, three (ND60, YD13, and ZD958) showed delayed leaf senescence after non-pollination treatment. The only exception is XY335. This hybrid was bred by Pioneer USA and its parent inbred lines has nothing to do with the germplasm in China (Chen *et al.*, 2013; Ma *et al.*, 2014). Therefore, it might be safely to say that leaf senescence of the late-released (since 1985) Chinese hybrids tended to be delayed by pollination-prevented.

The genetic information of a maize hybrid is determined by its parents. Therefore, the response of leaf senescence in a hybrid to grain sink removal could be related to the leaf senescence characteristic of its parent inbred lines. Previous research (Allison and Weinmann (1970), Christensen *et al.* (1981), Crafts-Brandner *et al.* (1984) and Below *et al.* (1985)) showed that hybrid B73 x Mo17 enhanced pre-mature senescence after ear removal or pollination prevention. Among the parents of the hybrids (ZD2, DY13, and YD4) which showed premature senescence in pollination prevention treatment, Mo17 was also the male parent for ZD2 and DY13 (Table 1). It is possible that some genes in Mo17 control the leaf senescence in response to source-sink regulation. It would be interesting to check if Mo17 also presents premature senescence after grain sink removal.

Physiologically, the genotypic difference in N partitioning characters during grain filling phase may have an effect on the response of leaf senescence to grain sink removal. It is found that, among different hybrids, leaf senescence indicated by the reduction of GLA induced by pollination prevention was highly related to N remobilization in leaves. These results suggest that whether prevention of pollination delays leaf senescence or not, largely depends on the change of N remobilization efficiency in the leaves (Fig. 4). In pollination prevention treatment, if leaf NRE was highly reduced, leaf senescence tended to be delayed. On the contrary, if prevention of pollination had less effect on leaf NRE, leaf senescence might not respond, or even show enhanced leaf senescence. Therefore, the characteristic of leaf senescence induced by sink-removal might be taken as an indicator to predict a hybrid's sensitivity of leaf N remobilization efficiency to grain sink regulation. For some hybrids (ND60, YD13, and SD7), their leaf N remobilization efficiency was highly sensitive to sink removal, suggesting that grain N accumulation in these hybrids depends on leaf N remobilization to a larger extent compared to the other hybrids. This would not favor grain N accumulation because grain N concentration is more

likely increased if more N is absorbed and transported into the grains (Yang *et al.*, 2016). Interestingly, grain N concentrations in these hybrids were relatively low among the tested hybrids (Table 2). Nevertheless, it should be noticed that the correlation efficient of the fitted model in Fig. 4 was only 0.72, suggesting that some other physiological factors also affect the response of leaf senescence to pollination prevention. This may explain the inconsistency between the change of leaf NRE (Fig. 4) and the change of GLA (Fig. 1).

Plant DM and N accumulation and grain yield formation are closely related to source-sink relationship (Borras, 2002; 2003; Tanaka and Maddonni, 2009; Seebauer *et al.*, 2010; Shekoofa *et al.*, 2013). When sink was removed by prevention of pollination, DM and N content in the vegetative organs continued to increase in some cultivars, or kept unchanged in others (Table 4). Similarly, Crafts-Brandner *et al.* (1984) showed that DM and N in the stover of earless plants increased in B73 x Mo17 and FS854, but did not changed in P3382, compared with respective controls. These genotypic differences were not related to the response of leaf senescence to pollination prevention. The DM and N were mainly allocated into the stem and less into the roots (Table 3 and 4), suggesting the stem can be a major temporary sink for both DM and N storage. The accumulation of N seemed closely related to DM in the stem (Fig. 2S), suggesting that DM accumulation was the primary driving force for N storage. Since N in the stem is readily remobilized for grain filling (Gallais, 2006), it is possible that plants can increase pre-silking N uptake, store it temporarily in the stem and use it for grain filling during post-silking stage (Incoll, 1992). By this way, nitrate leaching loss during grain filling stage may be potentially reduced.

Conclusion

From silking to physiological maturity, N content increased in the stem (plus the sheath, cob, husk, and tassel) and the root in the non-pollinated plants. However, there was still a net reduction of leaf N content. There was a genotypic difference in the response of maize leaf senescence to grain sink removal. Pollination prevention reduced leaf N remobilization efficiency, with genotypic difference and variation between the two years of testing. Leaf senescence of the late-released (since 1985) Chinese hybrids tended to be delayed after pollination prevention. A correlation was

found between the response of leaf senescence and the change of leaf NRE to pollination prevention. Leaf senescence tended to be delayed if leaf NRE is highly reduced by pollination prevention.

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Supporting Information

Table S1. Variance analysis on the effect of pollination prevention and hybrid on DM, N concentration, N content in vegetative organs in of maize

Table S2. Variance analysis on the effect of pollination prevention and hybrids on post-silking DM and N accumulation of different organs.

Fig S1. Weather conditions in 2013 and 2014.

Fig S2. Correlation between DM and N content in the stem of maize at maturity. Data of the two years are pooled for the analysis.

Fig S3. Effect of preventing pollination on soluble sugar concentration.

Figure legends

Fig 1. Effects of preventing pollination on green leaf area per plant at maturity in 2013 and 2014.*, significant differences between pollination and non-pollination treatment ($P < 0.05$).

Fig 2. Effect of preventing pollination on leaf N concentration at maturity in 2013 and 2014.*, significant differences between pollination and non-pollination treatment ($P < 0.05$).

Fig 3. Effect of preventing pollination on leaf N remobilization efficiency in 2013 and 2014.*, significant differences between pollination and non-pollination treatment ($P < 0.05$).

Fig4. Correlations between the relative nitrogen remobilization efficiency (NRE) and the relative green leaf area (GLA) under non-pollination treatment. Data of the mean value of each genotype were fitted using Power fit $y = a * x^b$. Relative GLA was calculated as: relative GLA = GLA at non-pollination treatment / GLA at pollination treatment. Relative leaf NRE was calculated the same way as relative GLA. In each hybrid, data of the two years are averaged for the analysis.

Table 1 Details of maize cultivars used (source: Zhao *et al.*, 2011)

Cultivars	Parental combination	Year of hybrid release	Breeding institution
Baimaya	Landrace		
Zhongdan 2	MO17×Zi330	1973	Chinese Academy of Agricultural Sciences, Beijing
Danyu 6	Lv28×Zi330	1975	Academy of Agricultural Sciences in Dandong, Liaoning province
Danyu 13	MO17×E28	1979	Academy of Agricultural Sciences in Dandong, Liaoning province
Shendan 7	5003×E28	1982	Academy of Agricultural Sciences in Shenyang, Liaoning province
Yedan 2	Ye107×Huangzao4	1983	Maize research institute in Laizhou, Shandong province
Yedan 4	Ye8112×Huangzao4	1984	Academy of Agricultural Sciences in Laizhou, Shandong province
Nongda 60	5003×Zong31	1985	China Agricultural University, Beijing
Yedan 13	Ye478×Dan340	1989	Academy of Agricultural Sciences in Laizhou, Shandong province
Zhengdan 958	Zheng58×Chang7-2	1996	Henan Academy of Agricultural Science, Henan province
Xianyu 335	PH6WC×PH4CV	2000	Pioneer Co., Ltd at Tieling, Jilin province

Table 2. Grain N and grain components of different cultivars.

Treatments	Grain yield (g ear ⁻¹)	Grain N concentration (mg g ⁻¹)	HI	NHI
Year				
2013	185a	16.3a	0.51a	0.66a
2014	154b	16.4a	0.45b	0.56b
Cultivar				
BMV	93e	18.4a	0.37e	0.48d
ZD2	149d	16.5de	0.48bcd	0.63bc
DY6	157cd	17.2bc	0.44d	0.58c
DY13	185bc	17.7ab	0.50abc	0.69a
SD7	174bcd	15.3fg	0.52ab	0.63bc
YD2	158cd	16.7cd	0.46cd	0.59c
YD4	162cd	16.5de	0.48bcd	0.58c
ND60	229a	15.7ef	0.54a	0.69ab
YD13	165cd	14.8g	0.51abc	0.64c
ZD958	190bc	15.1fg	0.51abc	0.60c
XY335	205ab	15.7fg	0.50abcd	0.63bc
Source of variation				
Year	***	NS	***	***
Cultivar	***	***	***	***
Year × Cultivar	NS	**	NS	NS

Within cultivar or year, numbers followed by different letters indicate significant difference ($P < 0.05$). NS: non-significant ($P > 0.05$).

*Significant at $P < 0.05$. **Significant at $P < 0.01$. ***Significant at $P < 0.001$.

Table 3. Effects of prevent pollination manipulation on post-silking DM accumulation in vegetative organs in two years.

Culti var	Treatment	2013			2014				
		Root (g plant ⁻¹)	Stem (g plant ⁻¹)	Leaf (g plant ⁻¹)	Vegetati ve organs (g plant ⁻¹)	Root (g plant ⁻¹)	Stem (g plant ⁻¹)	Leaf (g plant ⁻¹)	Vegetati ve organs (g plant ⁻¹)
BMY	Pollination	4.36a	15.21a	-18.71a	0.86a	2.22b	56.33a	4.86a	63.41a
	Non-pollination	6.10a	19.94a	-25.17a	0.87a	4.08a	69.53a	3.82a	77.42a
ZD2	Pollination	-6.47b	9.82b	-23.17a	-19.82b	-3.22b	55.19b	1.42a	53.39b
	Non-pollination	3.02a	40.35a	-19.13a	24.24a	-0.59a	88.11a	-4.42b	83.09a
DY6	Pollination	6.77b	56.54a	-8.63a	54.68a	1.26b	66.14b	-0.68b	66.73b
	Non-pollination	9.89a	51.54a	-11.70a	49.73a	6.73a	102.98a	9.38a	119.08a
DY1 3	Pollination	-3.54b	34.63b	-26.76a	4.34b	-1.34b	52.77b	1.49a	52.92b
	Non-pollination	3.54a	54.73a	-22.04a	36.22a	0.99a	119.95a	1.30a	122.24a
SD7	Pollination	3.76b	39.19b	-3.71b	39.23b	1.46b	47.05b	0.29b	48.80b
	Non-pollination	13.78a	106.88a	2.51a	123.17a	4.36a	73.83a	2.18a	80.37a
YD2	Pollination	2.53b	37.60b	-3.75a	36.38b	-1.36b	41.75b	-3.66b	36.73b
	Non-pollination	5.21a	64.79a	-14.93b	55.08a	2.62a	89.11a	0.43a	92.15a
YD4	Pollination	-2.48b	40.65b	-4.70a	33.47b	-6.29b	44.97b	7.22a	45.89b
	Non-pollination	-0.96a	57.37a	-7.73b	48.68a	0.87a	84.21a	4.64b	89.71a
ND6 0	Pollination	5.35a	68.44a	-4.03b	69.76a	-0.58b	50.38b	1.59b	51.39b
	Non-pollination	6.40a	58.77b	-1.22a	63.95a	2.78a	79.03a	6.82a	88.63a
YD1 3	Pollination	3.08b	25.08b	-7.26b	20.90b	1.52b	45.73b	-2.55b	44.70b
	Non-pollination	24.16a	81.30a	6.09a	111.55a	15.65a	123.68a	20.25a	159.57a
ZD95 8	Pollination	-0.60b	16.23b	-2.00a	13.63b	-0.77b	64.25b	0.93a	64.41b
	Non-pollination	5.47a	55.72a	-6.72b	54.47a	1.38a	118.12a	1.14a	120.63a
XY3	Pollination	5.16b	47.70a	-3.03b	49.83b	1.56b	79.28b	6.42a	87.25b

Non-pollin								
ation	11.94a	57.95a	0.00a	69.90a	7.16a	116.53a	7.69a	131.38a

Numbers within a cultivar followed by different letters indicate significant difference between pollination and non-pollination treatment ($P < 0.05$). Negative number indicates net export of DM during post-silking stage.

Table 4. Effects of prevent pollination manipulation on post-silking N accumulation in vegetative organs in two years.

Cultivar	Treatment	2013				2014			
		Root (mg plant ⁻¹)	Stem (mg plant ⁻¹)	Leaf (mg plant ⁻¹)	Vegetative organs (mg plant ⁻¹)	Root (mg plant ⁻¹)	Stem (mg plant ⁻¹)	Leaf (mg plant ⁻¹)	Vegetative organs (mg plant ⁻¹)
BMY	Pollination	-20.9b	-128.9b	-758.8a	-908.6b	10.5b	218.4b	-312.4b	-83.5b
	Non-pollination	42.3a -157.2b	907.1a	-831.5a	117.9a	69.7a	1154.2a	-132.2a	1091.8a
ZD2	Pollination	b	-476.7b	-847.5a	-1481.3b	-59.6b	-526.0b	-503.6a	-1089.2b
	Non-pollination	-5.7a	822.3a	-737.3a	79.4a	8.8a	1264.9a	-535.3a	738.4a
DY6	Pollination	36.9b	-73.0b	-553.4a	-589.5b	5.0b	-222.5b	-389.0b	-606.6b
	Non-pollination	151.1a	1193.8a	-509.3a	835.6a	186.3a	1544.2a	-40.3a	1690.2a
DY13	Pollination	-93.9b	-595.1b	-932.4b	-1621.4b	-22.7b	-643.8b	-584.4a	-1250.9b
	Non-pollination	69.8a	1248.4a	-774.9a	543.4a	65.7a	1956.8a	-509.5a	1523.0a
SD7	Pollination	-28.5b	-641.5b	-455.1b	-1125.1b	5.9b	-433.0b	-405.7b	-832.8b
	Non-pollination	191.1a	2215.8a	-130.2a	2276.7a	125.4a	1483.3a	-138.5a	1470.2a
YD2	Pollination	-40.3b	-257.5b	-484.9a	-782.7b	-19.4b	-141.0b	-511.0b	-671.4b
	Non-pollination	15.7a	1427.5a	-577.2a	865.9a	65.9a	1626.3a	-282.6a	1409.5a
YD4	Pollination	-95.3b	72.2b	-436.7a	-459.7b	-92.0b	-97.1b	-294.4a	-483.6b
	Non-pollination	-30.8a	1288.4a	-444.9a	812.7a	46.0a	1556.2a	-286.7a	1315.5a
ND60	Pollination	-56.8b	-231.0b	-421.4b	-709.2b	-47.4b	-532.5b	-519.3b	-1099.2b
	Non-pollination	131.4a	1599.1a	-224.7a	1505.8a	86.9a	1449.5a	-38.7a	1497.6a
YD13	Pollination	-60.4b	-442.7b	-615.2b	-1118.3b	-15.6b	-467.7b	-520.4b	-1003.7b
	Non-pollination	255.7a	1373.1a	-232.9a	1395.9a	273.2a	1991.0a	87.8a	2352.0a
ZD958	Pollination	-68.2b	-609.0b	-341.2b	-1018.4b	-15.3b	9.3b	-325.9b	-331.9b
	Non-pollination	51.9a	865.5a	-414.8a	502.5a	38.8a	2211.2a	-52.9a	2197.1a
XY33									
5	Pollination	-23.2b	-213.1b	-572.5b	-808.7b	-23.5b	134.5b	-342.7b	-231.7b
	Non-pollination	114.8a	1026.9a	-237.2a	904.5a	70.7a	1782.4a	-236.8a	1616.3a

Numbers within a cultivar followed by different letters indicate significant difference

between pollination and non-pollination treatment ($P < 0.05$). Negative number indicates net export of N during post-silking stage.

Figure 1. Effects of preventing pollination on green leaf area per plant at maturity in 2013 and 2014. *, significant differences between pollination and non-pollination treatment ($P < 0.05$).

Figure 2. Effect of preventing pollination on leaf N concentration at maturity in 2013 and 2014. *, significant differences between pollination and non-pollination treatment ($P < 0.05$).

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