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RESEARCH ARTICLE

Top-dressing nitrogen fertilizer rate contributes to decrease culm physical strength by reducing structural carbohydrate content in *japonica* rice

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Abstract

Lodging is an important factor limiting rice yield and quality by bending or breaking stem in *japonica* rice (*Oryza sativa* L.) production. The objectives of this study were to determine the mechanism of lodging resistance in *japonica* rice as affected by carbohydrate components, especially its related arrangement in culm tissue and response to top-dressing nitrogen (N) fertilizer. Field experiments were conducted in Danyang County, Jiangsu Province, China, by using two *japonica* rice varieties Wuyunjing 23 (lodging-resistance variety) and W3668 (lodging-susceptible variety) with three top-dressing N fertilizer rates (0, 135 and 270 kg N ha^{-1}) in 2013 and 2014. Lodging related physical parameters, morphological characteristics and stem carbohydrate components were investigated at 30 d after full heading stage. Results showed that with increasing N fertilizer rates, the lodging rate and lodging index increased rapidly primarily due to significant reduction of breaking strength in two *japonica* rice varieties. Correlation analysis revealed that breaking strength was significantly and positively correlated with bending stress, but negatively correlated with section modulus, except for significant correlation at W3668 in 2014. Higher stem plumpness status and structural carbohydrate contents significantly enhanced stem stiffness, despite of lower non-structural carbohydrate. With higher N fertilizer rate, the culm wall thickness was almost identical, and culm diameter increased slightly. The structural carbohydrates, especially for lignin content in culm, reduced significantly under high N rate. Further histochemical staining analysis revealed that high N treatments decreased the lignin deposition rapidly in the sclerenchyma cells of mechanical tissue, large vascular bundle and small vascular bundle region, which were consistent with reduction of bending stress, especially for W3668 and thus, resulted in poor stem strength and higher lodging index. These results suggested that structural carbohydrate plays a vital role for improving stem strength in *japonica* rice. N rate decreased lodging resistance primarily due to poor stem stiffness, by reducing structural carbohydrate content and lignin deposition in the secondary cell wall of lower internode culm tissue.

Keywords: *japonica* rice, lodging resistance, nitrogen, stem strength, structural carbohydrate

1. Introduction

Japonica rice (*Oryza sativa* L.) is one of the major food crops for the world's population, and in Asia, 95% of the population

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depended on rice for food. The need for increased rice production is particularly urgent because the population of traditional rice producing countries will require 70% more rice by year 2025 (IRRI 1995). To ensure food security, rice production must be largely produced by elevating yield per unit area (Horie *et al*. 2005). Lodging, the permanent displacement of the vertical rice stem due to loss of balance within the body of the plants, is the most important constraint factor to achieving high grain yield (Setter *et al*. 1997; Wu *et al*. 2012). Reduction of plant height has been the major target improving lodging resistance in cereals crops. Since the 1960s, semi-dwarf rice varieties by using dwarfing gene, *sd-1*, has been very effective tools for reducing lodging risks and increasing grain yield, named the "Green Revolution" (Khush 1999). However, the height of semi-dwarf rice plant may reduce the photosynthetic capacity of a canopy and biomass production, thereby, limiting further improvement of grain yield (Wu *et al*. 2002).

Recently, stem strength, the bending or breaking strength of the culm, is identified as the new target for improving lodging resistance (Islam *et al*. 2007; Zhang *et al*. 2010). Nutrient availability, particularly nitrogen (N), has been widely shown to reduce plant lodging resistance primarily due to stem strength (Crook and Ennos1995; Tripathi *et al*. 2003; Wei *et al*. 2008; Li *et al*. 2013). Among the morphological traits, culm diameter was directly correlated with breaking strength of the rice (Zuber *et al*. 1999; Ma *et al*. 2004; Ookawa *et al*. 2010b). However, Zhang *et al*. (2013) reported that culm wall thickness and dry weight cm^{-1} of culm and leaf sheath are the important traits that determine lodging resistance of rice plant, in spite of the smaller culm diameter. Especially, higher leaf sheaths wrapping can rapidly enhance stem strength due to its reinforcement to the culms (Ookawa *et al*. 1992; Quang Duy *et al*. 2004).

The stem strength can be further separated into two parts: the section modulus, which is directly affected by the culm morphological traits (e.g., culm diameter and culm wall thickness), and bending stress, which is an indicator of culm stiffness (Ookawa *et al*. 2010b). In previous study, starch content is closely related to the culm rigidity of rice plant (Ishimaru *et al*. 2008). Zhang *et al*. (2010) showed that increasing non-structural carbohydrate (NSC) content of stem at the early filling stage can be beneficial to improving lodging resistance of rice. In addition, *prl5* locus, a quantitative trait loci on chromosome 5, has been identified that are responsible for the higher NSC re-accumulation after grain filling, consequently, for the higher lodging resistance during typhoon in the lower rice stem, which show that the stronger stem primarily depends on a high NSC re-accumulation in the lower stem (Kashiwagi and Ishimaru 2004; Kashiwagi *et al*. 2006, 2010). Several genes associated with lodging resistance also have been identified, e.g., *DEP1* and *IPA1*, which shows more vascular bundles, sclerenchyma cells and spikelets per panicle, thus, increasing mechanical strength and grain yield (Huang *et al*. 2009; Jiao *et al*. 2010). As we all know, the *japonica* rice is grown under a wide range of environment conditions. NSC content in stem tends to large translocation from stem to grain during filling stage under following environment conditions, low temperature (Slewinski 2012), low solar radiation (Okawa *et al*. 2003) and drought stress (Gupta *et al*. 2011), because rice yield under these environment conditions is often attributed to high efficient translocation of NSC from stem to grain, thereby, lodging risk may increase. Pan *et al*. (2014) suggested that optimum distribution ratio between structural carbohydrates (cellulose, hemicelluloses and lignin) and NSC in stem determines stem stiffness and lodging resistance of rice. Guo *et al*. (2003) reported that starch content is not correlated with stem stiffness among different rice varieties. Therefore, there may be another factors also determining stem stiffness and lodging resistance in *japonica* rice.

N fertilizer rates significantly affect lodging resistance in rice plants. Lodging index reduced first, and then increased with N fertilizer rates, that means, lodging risks of rice plants are the smallest in middling N fertilizer rate (Shi *et al*. 2008). Rice with sparse planting density accompanied with small amount of N fertilizer in the early growth stage can effectively increase lodging resistance, especially for long-culm varieties (Quang Duy *et al*. 2004). However, excessive N fertilizer increase tiller numbers, length of lower internodes and gravity center height, reduce dry weight cm^{-1} of lower internodes and breaking strength, and it then led to higher lodging risks in rice and wheat plants (Wei *et al*. 2008; Yang *et al*. 2009; Li *et al*. 2014).

As mentioned above, lodging resistance is one of important agricultural quantitative traits, which is determined by the interaction between genetic and environmental factors. Previously, the morphological traits related to lodging resistance were compared by using different *japonica* rice varieties (Zhang *et al*. 2009; Li *et al*. 2012). To our knowledge, information about the carbohydrate components (e.g., NSC, cellulose and lignin) as affected by top-dressing N fertilizer rates and their relationships to lodging resistance are limited in *japonica* rice. The objectives of this study were to determine the key physiological traits associated with lodging resistance and its response to top-dressing N fertilizer rates in *japonica* rice. A greater understanding of that information would provide a theoretical basis to increase lodging resistance combinations with improved grain yield.

2. Results

2.1. Yield

Grain yield of Wuyunjing 23 (11.5 t ha^{-1}, mean value in two years) was 22.3% higher than that of W3668 (9.4 t ha^{-1}, mean value in two years) (Table 1). Mean value of sink size (spikelets m^{-2}) of Wuyunjing 23 was 7.1% smaller than that of W3668 in two years, which were due to 17.4% lower panicles m^{-2} in both years. There were significant differences between two varieties in spikelets per panicle, spikelet fertility and grain weight, which were 12.6, 4.0 and 26.5% greater in Wuyunjing 23 than in W3668, respectively. With increasing N application, grain yield of Wuyunjing 23 increased by 17.4% with no significant difference in two years, but decreased slightly in W3668 in both years. Higher N increased the spikelets per panicle of Wuyunjing 23 by 24.7% with significant difference in 2014, but decreased spikelet fertility and grain weight by 7.26 and 5.0% (Wuyunjing 23), and 12.2 and 8.6% (W3668) in both two years, respectively.

2.2. Lodging rate (LR)

The LR of Wuyunjing 23 (8.3%, mean value in two years) was significant lower than that of W3668 (71.6%, mean value in two years) in both two years (Fig. 1). With increased N rates, the average value of LR increased from 0 to 19.6% in Wuyunjing 23 and from 36.6 to 94.2% in W3668 across two years, respectively. In addition, it was at mature stage (BBCH 89) that lodging happened slightly for Wuyunjing 23 in 2014 under higher N rates, but lodging was not occurred in 2013. And time of lodging happened for W3668 was at the mature stage at LN (low nitrogen top-dressing fertilizer rate of 0 kg N ha^{-1}) in 2014 and at the soft dough stage (BBCH 85) at MN (moderate nitrogen top-dressing fertilizer rate of 135 kg N ha^{-1}) and HN (high nitrogen top-dressing fertilizer rate of 270 kg N ha^{-1}) in two years (data not shown).

2.3. Lodging index (LI) and its related physical parameters

The average LI value of Wuyunjing 23 was 46.4% lower than that of W3668 in both two years (Table 2). In this paper, average bending moment by whole plant (WP) of Wuyunjing 23 was 23.6% higher compared with W3668 owing to its larger fresh weight (FW) from the broken point to the panicle top (F=298.1**). Average breaking strength (M) of Wuyunjing 23 showed 1.3-fold greater values relative to W3668 in across two years, which resulted from higher bending stress (F=59.9**) and section modulus (F=425.4**). Thereby, the increasing magnitude of the M was greater than

Table 1 Yield and yield components in two *japonica* rice varieties under different N fertilizer levels in 2013 and 2014

Treatments[1)]	Yield (t ha^{-1})	Panicles m^{-2}	Spikelets per panicle	Spikelets (×10^3 m^{-2})	Spikelet fertility (%)	1 000-weight (g)
2013						
Wuyunjing 23						
LN	10.5 a	354.6 a	104.6 a	37.1 b	93.5 a	30.3 a
MN	11.6 a	371.7 a	121.6 a	45.2 ab	87.7 b	29.2 b
HN	11.8 a	394.4 a	121.9 a	48.2 a	86.2 b	28.4 c
W3668						
LN	9.8 a	403.4 b	110.4 a	44.5 a	91.8 a	24.1 a
MN	9.2 a	447.6 a	105.7 a	47.5 a	86.0 b	22.5 b
HN	8.9 a	441.7 a	110.7 a	48.9 a	83.4 b	21.9 b
2014						
Wuyunjing 23						
LN	10.3 a	341.5 a	106.6 b	36.5 b	93.9 a	30.0 a
MN	12.0 a	343.2 a	126.4 ab	43.2 ab	92.4 a	30.0 a
HN	12.6 a	349.4 a	141.4 a	49.4 a	87.7 a	29.1 a
W3668						
LN	9.7 a	427.5 b	98.3 b	42.1 b	92.4 a	24.8 a
MN	9.6 a	428.1 b	106.6 ab	45.6 ab	88.8 a	23.8 a
HN	9.0 a	461.3 a	110.3 a	50.8 a	78.4 b	22.6 a
Analysis of variance						
Year (Y)	0.3	2.5	0.7	0.2	0.6	7.9**
Varity (V)	28.7**	133.9**	20.9**	5.6*	9.7*	684.3**
Nitrogen (N)	0.8	6.9**	10.2**	14.7**	22.5**	18.8**
Y×V	0.1	7.9**	4.6*	0.1	2.1	1.0
Y×N	0.3	1.7	2.0	0.6	2.1	1.1
V×N	3.9*	0.4	4.3*	1.4	1.5	1.1
Y×V×N	0.1	1.5	0.6	0.1	0.8	0.4

[1)] LN, low nitrogen top-dressing fertilizer rate of 0 kg N ha^{-1}; MN, moderate nitrogen top-dressing fertilizer rate of 135 kg N ha^{-1}; HN, high nitrogen top-dressing fertilizer rate of 270 kg N ha^{-1}.

Different lower case letters represent significant difference at the 0.05 level. *, F-value significant at the 0.05 probability level; **, F-value significant at the 0.01 probability level. The same as below.

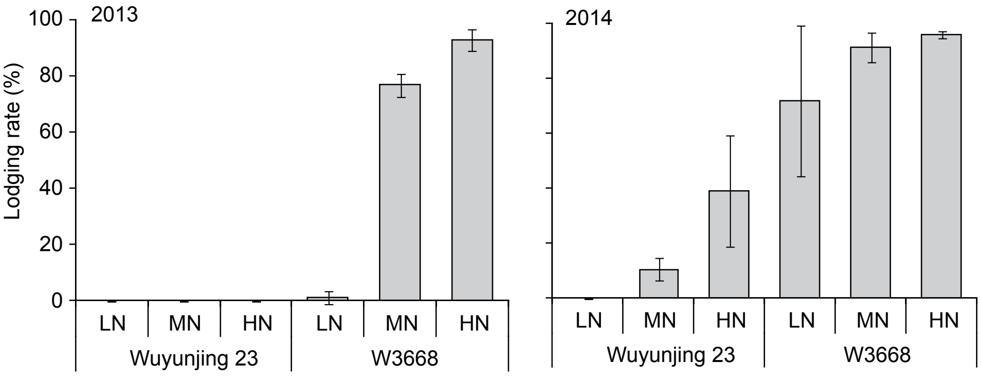

Fig. 1 Lodging rate in two *japonica* rice varieties under different N fertilizer treatments in 2013 and 2014. LN, low nitrogen top-dressing fertilizer rate of 0 kg N ha^{-1}; MN, moderate nitrogen top-dressing fertilizer rate of 135 kg N ha^{-1}; HN, high nitrogen top-dressing fertilizer rate of 270 kg N ha^{-1}. Bars indicate the SD values. The same as below.

Table 2 Lodging index (LI) and its related parameters of the fourth internode from the top (N4) in two *japonica* rice varieties under different N fertilizer treatments1)

Treatments	M (g cm)	SM (mm^3)	BS (g mm^{-2})	WP (g cm)	SL (cm)	FW (g)	LI (%)
2013							
Wuyunjing 23							
LN	2 171.3 a	8.2 ab	2 647.5 a	1 418.2 a	80.3 b	17.7 a	65.4 b
MN	1 956.9 b	8.4 a	2 320.8 b	1 508.7 a	82.7 a	18.2 a	77.3 ab
HN	1 832.0 b	8.1 b	2 251.4 b	1 524.7 a	82.2 ab	18.6 a	83.3 a
W3668							
LN	1 028.8 a	4.6 b	2 239.9 a	1 261.1 a	92.6 b	13.6 a	122.8 b
MN	827.5 b	4.9 ab	1 672.4 b	1 342.9 a	93.9 ab	14.2 a	162.4 ab
HN	762.0 b	5.3 a	1 448.9 b	1 368.0 a	95.3 a	14.3 a	179.5 a
2014							
Wuyunjing 23							
LN	1 980.7 a	7.4 ab	2 672.1 a	1 786.0 a	99.1 a	18.0 a	90.6 b
MN	1 576.3 b	7.1 b	2 220.7 b	1 891.7 a	99.8 a	18.9 a	120.8 a
HN	1 287.6 b	8.7 a	1 486.2 b	1 798.9 a	98.6 a	18.2 a	140.2 a
W3668							
LN	905.7 a	4.3 a	2 185.8 a	1 360.4 a	106.5 a	12.7 a	152.3 c
MN	643.5 b	4.5 a	1 443.8 b	1 320.9 a	107.1 a	12.3 a	205.0 b
HN	539.6 b	5.2 a	1 043.9 b	1 379.3 a	106.2 a	12.9 a	257.0 a
Analysis of variance							
Year (Y)	63.9^{**}	6.4^{*}	11.0^{**}	39.1^{**}	829.8^{**}	4.1	103.0^{**}
Variety (V)	878.3^{**}	425.4^{**}	59.9^{**}	113.3^{**}	355.1^{**}	298.1^{**}	341.4^{**}
Nitrogen (N)	50.6^{**}	7.3^{*}	44.3^{**}	1.8	2.1	1.3	53.9^{**}
Y×V	8.1^{**}	0.5	0.1	27.6^{**}	20.8^{**}	8.2^{**}	0.8
Y×N	3.7^{*}	4.9^{*}	4.9^{*}	0.8	2.3	0.4	6.6^{**}
V×N	2.8	0.3	1.0	0.8	0.4	0.5	9.0^{**}
Y×V×N	1.1	1.9	1.0	0.6	0.2	0.8	0.5

1) M, breaking strength; SM, section modulus; BS, bending stress; WP, bending moment of the whole plant; SL, the distance from the broken point to the panicle top; FW, the fresh weight from the broken point to the panicle top. The same as below.

the WP, resulting in a lower LI for Wuyunjing 23.

With increasing N rates, the LI value significantly increased in both varieties (*F*=53.9^{**}). Compared with LN treatments, the WP value increased slightly in only 2013, but the M value reduced with significant difference in two years. The M could be further divided into the following two parameters: the section modulus (SM) and the bending stress (BS). With higher N rates, the mean BS value was decreased by 29.7 and 43.7% for Wuyunjing 23 and W3668, respectively, but the SM value of W3668 increased with significant difference in 2013. Compared with 2013, LI of Wuyunjing 23 was 55.6% higher than that in 2014, which primarily attributed to lower M (*F*=63.9^{**}) and larger WP (*F*=39.1^{**}). The interaction between the varieties and

N rates was significant for LI in two years.

Correlation matrices for the LI and lodging related parameters in both years were presented in Table 3. In general, the LI was significantly correlated with LR across varieties and years. The LI was strongly and negatively correlated with M, with correlation coefficient (*r*) of –0.893 and –0.987 for Wuyunjing 23, –0.905 and –0.938 for W3668, respectively. Of the relationships among M, SM and BS, the M was significantly and positively correlated with BS, with *r* more than 0.9 in two year experiments, but negatively correlated with SM, except for W3668 in 2014 with significant difference. Also, the LI was significantly and positively correlated with WP, SL and plant height across two varieties in 2013, but no significant relationship among them was observed in 2014.

2.4. Culm diameter, culm wall thickness, plant height and dry weight per unit length

Compared with W3668, the average culm diameter and culm wall thickness of Wuyunjing 23 were increased significantly by 17.5 and 21.5%, but the average plant height was significantly lowered in two years. There were significant differences between two varieties in dry weight cm^{-1} of N4 internode culms and leaf sheath, which were 12.1 and 65.5% higher in Wuyunjing 23 than in W3668, respectively (Table 4).

Table 3 Correlation analysis between LI and lodging related parameters

Indexes$^{1)}$	Wuyunjing 23		W3668	
	LI	M	LI	M
2013				
LR (%)	NA	NA	0.885**	–0.949**
M (g cm)	–0.893**		–0.905**	
SM (mm^3)	0.308	–0.160	0.723*	–0.61
BS (g mm^{-2})	–0.912**	0.975**	–0.924**	0.954**
WP (g cm)	0.815**	–0.472	0.729*	–0.378
SL (cm)	0.685*	–0.729*	0.874**	–0.626
FW (g)	0.694*	–0.309	0.665	–0.299
PH (cm)	0.814**	–0.773*	0.966**	–0.837**
2014				
LR (%)	0.841**	–0.827**	0.729*	–0.852**
M (g cm)	–0.987**		–0.938**	
SM (mm^3)	0.373	–0.365	0.812**	–0.680*
BS (g mm^{-2})	–0.924**	0.926**	–0.936**	0.962**
WP (g cm)	0.084	0.009	0.249	0.004
SL (cm)	–0.044	0.097	0.177	–0.07
FW (g)	0.13	–0.032	0.237	0.024
PH (cm)	0.456	–0.448	0.003	0.062

[1)] LR, lodging rate; PH, plant height.
NA, no lodged rice plants were obtained for Wuyunjing 23 in 2013.

Compared with LN treatments, dry weight per cm of N4 internode culms at MN and HN were reduced by 13.7 and

Table 4 Culm diameter, culm wall thickness, the dry weight per cm of the culms, and leaf sheaths of the N4 and plant height in two *japonica* rice varieties under different N fertilizer treatments

Treatments	Culm diameter (mm)	Culm wall thickness (mm)	Dry weight per cm of culm (mg cm^{-1})	Dry weight per cm of leaf sheath (mg cm^{-1})	Plant height (cm)
2013					
Wuyunjing 23					
LN	4.96 a	0.77 a	21.1 a	24.8 a	81.6 b
MN	5.01 a	0.82 a	17.8 b	20.9 b	87.5 a
HN	4.96 a	0.73 a	16.2 c	16.9 c	88.6 a
W3668					
LN	4.25 a	0.65 a	20.1 a	16.2 a	94.7 b
MN	4.25 a	0.64 a	17.7 b	13.2 ab	99.2 a
HN	4.31 a	0.65 a	15.9 c	9.14 b	101.4 a
2014					
Wuyunjing 23					
LN	4.74 a	0.78 a	16.9 a	25.8 a	100.3 a
MN	4.85 a	0.82 a	15.0 ab	21.8 b	104.4 a
HN	4.94 a	0.81 a	13.6 b	20.6 b	101.9 ab
W3668					
LN	4.02 a	0.66 a	13.8 a	16.7 a	110.1 a
MN	4.03 a	0.65 a	11.3 b	12.1 b	110.9 a
HN	4.19 a	0.65 a	11.1 b	11.8 b	109.9 a
Analysis of variance					
Year (Y)	25.4**	1.3	235.4**	5.7*	759.1**
Variety (V)	513.5**	113.3**	28.4**	257.9**	409.3**
Nitrogen (N)	3.5*	1.3	57.5**	46.3**	24.9**
Y×V	0.4	0.8	18.8**	1.2	19.1**
Y×N	2.3	0.6	2.2	3.3	12.3**
V×N	0.8	1.7	0.4	0.1	1.8
Y×V×N	0.2	1.2	0.8	0.2	0.3

21.5% for Wuyunjing 23, and 14.4 and 20.5% for W3668, respectively; and the dry weight cm^{-1} of leaf sheath N4 internode also decreased with significant difference. The culm wall thickness of two varieties was almost identical with increasing N application, but the clum diameter slightly increased under higher N rates. The plant height was increased significantly in 2013 but varied little in 2014. The culm diameter, dry weight cm^{-1} of N4 internode culms and leaf sheath were significantly lower in 2014 than in 2013, but the plant height was rapidly higher in 2014.

2.5. Carbohydrates in basal stems

The carbohydrate components, including the non-structural carbohydrate (NSC), cellulose and lignin of N4 internodes culm and leaf sheath in two varieties were summarized in Table 5. In culms, the NSC of Wuyunjing 23 was significantly lower than that of W3668, but the lignin and cellulose increased by 44.0 and 31.1%, respectively. In leaf sheath, the NSC, cellulose and lignin content of Wuyunjing 23 were 32.2, 72.1 and 61.9% higher than that of W3668. The proportions of NSC, cellulose and lignin in the culm and leaf sheath were also exhibited as shown in Fig. 2. The proportions of structural carbohydrates (lignin and cellulose) in culm of Wuyunjing 23 were 88.9 and 89.1% in 2013 and 2014, and they increased significantly by 32.5 and 20.0% as compared with W3668. As for the leaf sheath, the proportions of structural carbohydrates were almost identical in two varieties.

Compared with LN treatments, the lignin content at MN and HN in culms was reduced by 20.0 and 32.5% for Wuyunjing 23, and 22.0 and 41.5% for W3668, respectively. Cellulose content in culm was decreased under higher N rate in two varieties ($F=62.6^{**}$). Also, NSC content in culm exhibited significant reduction except for W3668 in 2014. In leaf sheath, higher N rate reduced NSC contents by 53.5 and 56.2% in Wuyunjing 23 and W3668, respectively. The lignin content reduced slightly and with significant difference ($P<0.05$). In addition, with increasing N rate, proportions of lignin in culm were varied with no significant difference, and proportion of cellulose in culm of two varieties always increased rapidly, whereas NSC reduced with significant difference. The NSC, cellulose and lignin contents in culms and leaf sheath in 2013 were significant higher than in 2014, except for cellulose content in leaf sheath.

2.6. N altered lignin arrangement in culm tissue

To further investigate whether the alteration of lignin was localized in particular cells, we examined histochemical

Table 5 Contents of non-structural carbohydrates (NSC), cellulose and lignin of the N4 in two *japonica* rice varieties under different N fertilizer treatments

N treatments	Culm (mg cm^{-1})			Leaf sheath (mg cm^{-1})		
	NSC	Cellulose	Lignin	NSC	Cellulose	Lignin
2013						
Wuyunjing 23						
LN	2.01 a	7.08 a	3.42 a	1.79 a	7.96 a	3.21 a
MN	0.74 b	5.48 b	2.63 b	0.77 b	6.63 b	3.90 a
HN	0.50 c	5.25 b	2.19 c	0.59 b	5.44 b	3.11 a
W3668						
LN	4.14 a	5.57 a	2.67 a	1.40 a	4.59 a	2.98 a
MN	3.76 b	4.49 b	2.04 b	0.66 b	4.37 ab	2.43 ab
HN	1.96 b	3.84 b	1.53 c	0.54 b	2.54 b	1.72 b
2014						
Wuyunjing 23						
LN	1.41 a	5.62 a	3.10 a	1.06 a	8.26 a	6.71 a
MN	0.74 b	4.50 b	2.57 b	0.90 b	6.61 b	4.88 b
HN	0.53 b	4.05 b	2.20 b	0.73 c	5.85 b	4.64 b
W3668						
LN	2.37 a	4.08 a	2.06 a	0.78 a	5.14 a	3.96 a
MN	1.10 a	3.35 b	1.64 b	0.63 b	3.73 b	2.69 b
HN	1.71 a	3.06 b	1.23 c	0.41 c	3.29 b	2.54 b
Analysis of variance						
Year (Y)	30.6^{**}	101.9^{**}	26.4^{**}	26.9^{**}	1.0	20.0^{**}
Variety (V)	92.4^{**}	118.5^{**}	229.8^{**}	35.5^{**}	161.7^{**}	31.6^{**}
Nitrogen (N)	23.8^{**}	62.6^{**}	119.3^{**}	107.6^{**}	32.3^{**}	5.4^{*}
Y×V	19.1^{**}	0.1	8.0^{**}	1.7	0.0	4.7^{*}
Y×N	6.0^{**}	1.6	3.1	34.4^{**}	1.6	2.5
V×N	0.5	1.3	0.5	1.5	0.8	0.1
Y×V×N	4.8^{*}	0.6	0.3	1.9	0.5	1.0

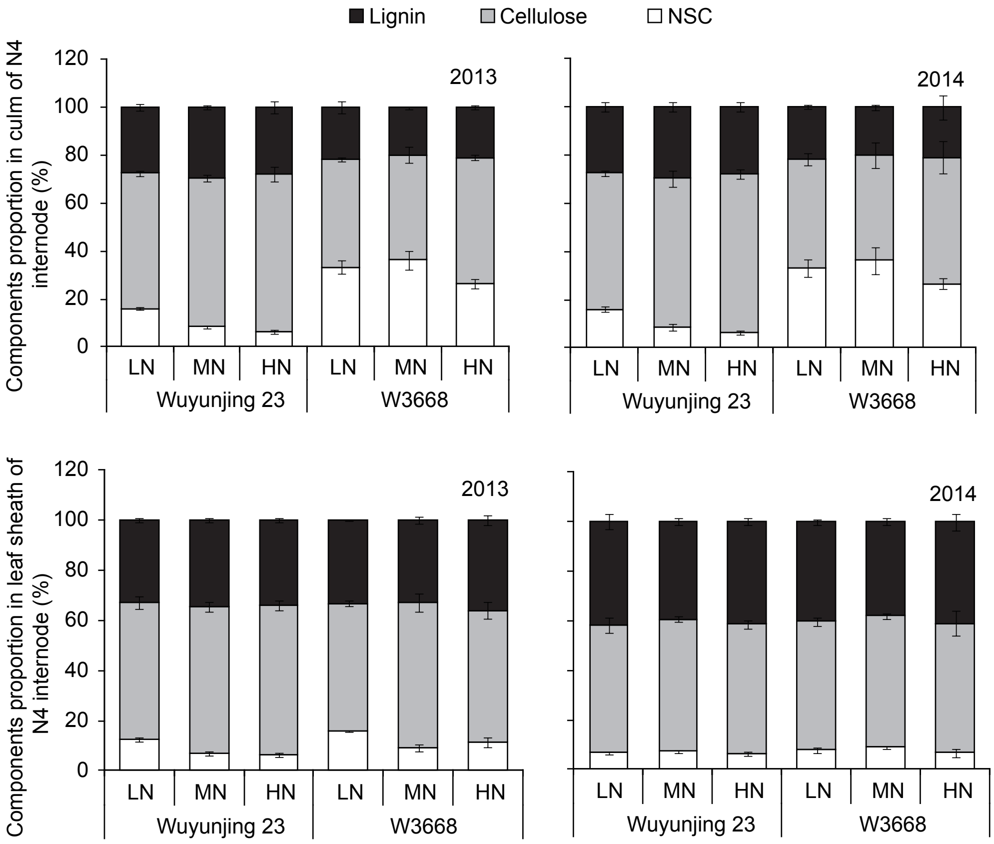

Fig. 2 The proportions of different carbohydrate components of the fourth internode from the top (N4) internodes culm and leaf sheath in two *japonica* rice varieties under different N treatments. The proportions of each component were calculated by each component content divided the total carbohydrates content. NSC, non-structural carbohydrates.

transverse sections of the N4 internode culms with phloroglucinol reagent (Fig. 3). Wiesner stain is known to react with cinnamaldehyde residues in lignin, and the color intensity consistent with the total lignin content. In present study, the N application affected the lignin arrangement in the sclerenchyma cells below the epidermis and vascular bundles sheath (see black arrow), which differed distinctly in genotypes. The mechanical tissues and vascular bundle regions were stained dark red color in Wuyunjing 23, the red color in these region decreased slightly under high N rate. Some cells in parenchyma tissue near the air chamber were also stained with deep red color (see white arrow), but high N reduced them conspicuously (Fig. 3-B). As for the W3668, the mechanical tissues, large vascular bundle and small vascular bundle sheath exhibited light red color and, decreased sharply with increasing N rate, even with light pink color in these regions (Fig. 3-D).

3. Discussion

Stem lodging induces loss of grain yield and reduction of rice quality due to poor grain filling, and decreases mechanical harvesting efficiency (Setter *et al*. 1997). In this study, LI of Wuyunjing 23 was increased with increasing N rate (Table 2), but the lodging occurred slightly at mature stage (BBCH 89) in 2014, and the damage to the crop was minimal. Thus, grain yield of Wuyunjing 23 increased due to larger spikelets per panicle (Table 1). However, time of lodging happened seriously for W3668 was the soft dough stage (BBCH 85) at MN and HN in both years, which induced grain yield loss owing to poor spikelet fertility and 1 000-weight.

Recent studies reported that higher lodging risk of rice population was primarily due to larger upper part of plants, such as heavier panicle weight, higher plant height and gravity center height (Ma *et al*. 2004; Li *et al*. 2013; Zhang J *et al*. 2014). In this study, the LI of Wuyunjing 23 was significantly lower than that of W3668 (Fig. 1, Table 2), but the M and WP of Wuyunjing 23 increased significantly and, the increase magnitude in M was greater than WP (Table 2), thus, the LI decreased significantly. These results indicated that M was the key factor determining the lodging risk of *japonica* rice. In addition, M was always strongly and positively correlated with BS, but negatively correlated

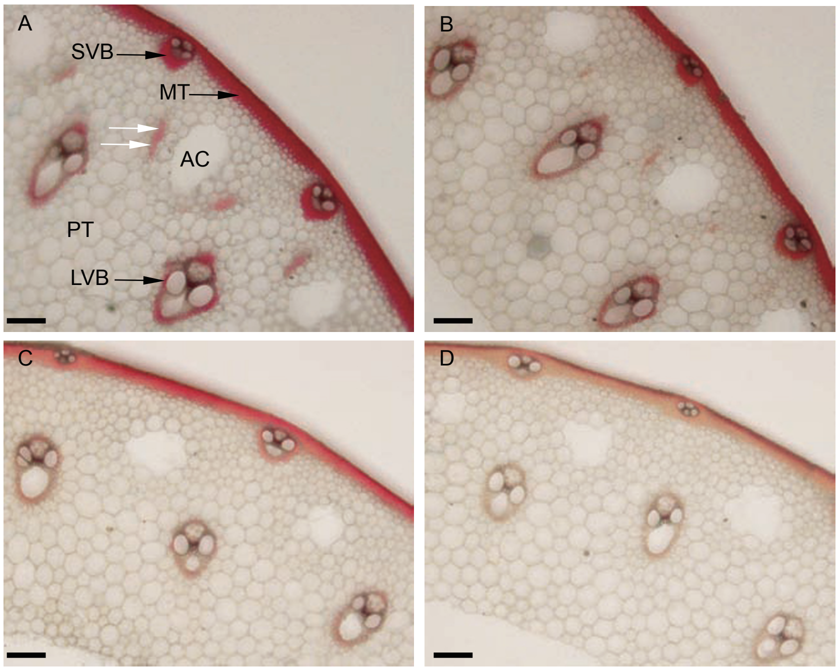

Fig. 3 Histochemical staining of transverse sections of the fourth internode from the top (N4) culm in two *japonica* rice varieties under different N fertilizer treatments. A and B, transverse section at the N4 internodes of Wuyunjing 23 with LN and HN treatments, respectively. C and D, transverse section at the N4 internodes of W3668 with LN and HN treatments respectively. SVB, small vascular bundles; MT, mechanical tissue; AC, air chamber; PT, parenchyma tissue; LVB, large vascular bundles. Bar=100 μm.

with SM, despite with significant correlation for W3668 in 2014 (Table 3), which revealed that M of *japonica* rice was primarily enhanced by BS.

Higher N fertilizer application rate, one of the important environmental factors, improves biomass production and grain yield, but also increases lodging risk due to poor physical strength of the lower internodes (Wu *et al*. 2012; Zhang W J *et al*. 2014). In this study, culm wall thickness, which were closely related to M (Kashiwagi and Ishimaru 2004; Kashiwagi *et al*. 2008; Ookawa *et al*. 2010a), did not show a reduction tendency with increasing N fertilizer rate while the culm diameter increased slightly (Table 4) and LI increased significantly (Table 2) in both years, which was consistent with previous report of Li *et al*. (2013). It was reasonable that high N rate primarily induced significant reduction of dry weight cm^{-1} of N4 internode culm and leaf sheath (Table 4) which resulted in lower BS (Table 2), and consequently, reduced stem physical strength of *japonica* rice.

The stem stiffness of rice plants is a product of the cellulose, lignin and NSC contents at lower internode culm and leaf sheath (Ookawa *et al*. 1993; Ishimaru *et al*. 2008; Zhang J *et al*. 2014). Previously, NSC content of the stem can be partly responsible for stem stiffness, because rice plant tends to lodge when the starch content of the stem parenchymatous cells is very low (Sato 1957). In this paper, the NSC content of Wuyunjing 23 was significantly lower in relation to W3668, but the cellulose and lignin content were 31.1 and 44.0% higher (Table 5), and the proportion of structural carbohydrate (e.g., cellulose and lignin) in culms increased significantly (Fig. 2). The difference in the quantities of these biochemical components in the stem suggested that higher structural carbohydrates might play a more important role for stronger stem strength in *japonica* rice. These results were consistent with the reports by Zhang J *et al*. (2014). Also, higher N application induced significant reduction in the contents of cellulose and lignin which were consistent with the difference of BS, in spite of the lower NSC content. In fact, structural carbohydrate, especially for lignin, primarily accumulated in the secondary cell wall of the sclerenchyma cells and vascular bundles sheath cells, which makes the plant tissue's mechanical intensity and supportable ability (Li *et al*. 2003; Li *et al*. 2009). Thus, we determined the lignin arrangement in the culm tissue by using histochemical staining method. Strong straining was observed primarily in the mechanical tissue and vascular bundles under low N, but the straining color decreased under high N, especially for W3668 (Fig. 3). Thereby, we inferred that lower lignin accumulation in the sclerenchyma cells of mechanical tissue and vascular bundles sheath under high N treatments might be the key factor causing the reduction of the thickness of the secondary cell wall and, consequently, resulting in lower BS and poor lodging resistance of *japonica* rice.

In addition, changes in lodging resistance between the years were also obtained (Table 2). The daily average solar radiation and temperature, especially from panicle initiation stage (PI, BBCH30) to heading stage (HS, BBCH58) in 2014, were conspicuously lower than in 2013 (Table 6). Compared with 2013, the LI increased significantly in 2014 owing to higher WP and lower M. Low solar radiation in 2014 promoted the vertical elongation of plants, such as longer N4, N5 internode length (data not shown) and higher plant height, which resulted in larger WP (Yang *et al*. 2009; Zhang *et al*. 2013). In terms of M, both the culm diameter and carbohydrate components (e.g., cellulose, lignin) were decreased due to reduction of crop growth rate (CGR) under low solar radiation and temperature condition (Ookawa *et al*. 1993), thereby weakening stem stiffness in 2014.

4. Conclusion

Top-dressing N application significantly increased LI of *japonica* rice primarily due to reduction of stem stiffness. The structural carbohydrate (e.g., cellulose and lignin) of N4 internodes were responsible for higher stem stiffness, which showed that a high accumulation of structural carbohydrate in stem instead of NSC, exhibited the superior role for lodging resistance in *japonica* rice. Top-dressing N decreased stem physical strength due to reduction of structural carbohydrate, especially, reducing lignin accumulation and its related arrangement in the culm tissue and thus, resulting in poor lodging resistance.

5. Materials and methods

5.1. Experimental site and soil properties

Field experiments were conducted in 2013 and 2014 in Baolin Farm, Danyang County, Jiangsu Province, China (32°00´N, 119°32´E, 7 m altitude) during the rice growing season from late May to late October. Soil properties of the field are as follows: alluvial loam, with total N 0.973 g kg^{-1}, available phosphorus 13.60 mg kg^{-1}, available potassium 93.50 mg kg^{-1}, organic matter 17.15 g kg^{-1}, and pH 6.9.

5.2. Experimental design and culture practices

Two *japonica* rice varieties Wuyunjing 23 (lodging-resistance variety) and W3668 (lodging-susceptible variety) were arranged in a split-plot block design with three replications. The N fertilizer rates were designed as main plot and varieties were designed as sub-plot. The plot sizes were 24 m^2 (6 m×4 m) for each treatment. Rice plants were transplanted at a planting density of 25 hills m^{-2} (13.2 cm×30 cm) with three seedlings per hill.

In each year, seeds of these varieties were sown in late May, and then, the seedlings around the fifth leaf stage were transplanted in late June. A N rate of 135 kg N ha^{-1} was applied as urea two times (basal fertilizer was applied 1 d before transplanting, tillering fertilizer was applied 7 days after transplanting) using 50 and 50% at each stage. Three topdressing N fertilizer rates including 0 (LN), 135 (MN), 270 (HN) kg N ha^{-1} were applied as urea two times (panicle initiation stage and when the 2nd leaf from top was fully extended) using 60 and 40% at each stage. Phosphorus at 90 kg P_2O_5 ha^{-1} as single superphosphate and potassium at 60 kg K_2O ha^{-1} as potassium chloride were applied at 1 d before transplanting in each plot. Additional potassium at 60 kg K_2O ha^{-1} was applied at the panicle initiation stage in each plot. Each plot between different fertilizer levels was surrounded by 30 cm-wide ridges, which were covered with

Table 6 Daily average solar radiation, average relative humidity, average, the minimum and maximum temperature at Danyang in 2013 and 2014(1)

Growth stage(2)	2013	2014
Daily average solar radiation (MJ m^{-2} d^{-1})		
SS to MS	18.9	13.9
SS to PI	20.3	15.8
PI to HS	21.5	10.7
HS to MS	13.9	13.0
Daily avergae relative humidity (%)		
SS to MS	74.6	77.6
SS to PI	73.3	73.7
PI to HS	71.5	83.3
HS to MS	77.8	78.8
Daily average temperature (°C)		
SS to MS	25.4	23.9
SS to PI	28.1	26.5
PI to HS	29.0	24.8
HS to MS	20.4	19.9
Daily average maximum temperature (°C)		
SS to MS	30.0	28.1
SS to PI	32.1	30.5
PI to HS	33.7	28.3
HS to MS	25.5	24.9
Daily average minimum temperature (°C)		
SS to MS	21.5	20.5
SS to PI	24.5	23.1
PI to HS	25.1	22.3
HS to MS	16.0	15.8

(1) Each value shows the averaged value for each day during the period.
(2) SS, sowing stage (BBCH 01); PI, panicle initiation (BBCH 30); HS, heading stage (BBCH 58); MS, maturity stage (BBCH 89).

plastic films. The plastic films were installed to a depth of 15 cm below the soil surface 2 d before transplanting. Water, weeds, insects and disease were controlled as required to avoid yield loss.

5.3. Meteorological conditions

Seasonal changes in daily average solar radiation, temperature and relative humidity are shown in Table 6. The different growth stages of rice including sowing stage (SS), panicle initiation (PI), heading stage (HS), and maturity stage (MS) were consistent with BBCH-scale (Lancashire *et al*. 1991) BBCH01, BBCH30, BBCH58, and BBCH89, respectively. The daily average solar radiation from sowing stage (BBCH 01) to maturity stage (BBCH 89) in 2013 (18.9 MJ m^{-2} d^{-1}) was greater than in 2014 (13.9 MJ m^{-2} d^{-1}), and it was the greatest difference from panicle initiation stage (BBCH 30) to heading stage (BBCH 58) during the rice growth stages. The daily average temperature throughout the growing season was 25.4°C in 2013 and 23.9°C in 2014, respectively. Maximum temperature in 2013 was generally higher than in 2014, with average maximum temperature from sowing stage (BBCH 01) to maturity stage (BBCH 89) of 30.0°C in 2013 and 28.1°C in 2014. Average minimum temperature throughout the growing seasons in 2013 and 2014 was 21.5 and 20.5°C, respectively. The relative humidity in 2013 was generally lower than in 2014, and the average relative humidity of the whole growing stages was 74.6% in 2013 and 77.6% in 2014.

5.4. Measurements and data analysis

For samples taken at maturity stage (BBCH 89), 5 hills were harvested according to the mean tillers per hill from the each plot to measure the yield. Panicles were hand-threshed and filled spikelets were separated by submerging them in tap water. The filled spikelets were then over-dried at 70°C to constant weight for determining individual grain weight. Spikelets per panicle, grain-filling percentage and spikelets m^{-2} were calculated. The grain yield was adjusted to a moisture content of 13.5% by using a moisture detector (GAC2100AGRI, DICKEY-john, Minneapolis City, Minnesota, USA).

Lodging rate was measured at mature grain stage with the formula: Lodging rate (%)=The lodging area in plot/The plot area×100, as reported by Peng *et al*. (2014). Ten representative main stems were sampled from each plot to measure lodging-related traits at 30 d after heading. There were five elongated internodes for main stem in both two varieties. The five elongated internodes were the first (N1), second (N2), third (N3), fourth (N4), and fifth (N5) internodes from the top, respectively. Stem lodging usually occurred at the N4 or N5 internodes, especially at N4 internodes, where lodging-related characters were determined (Islam *et al*. 2007). Morphological parameters including culm wall thickness and culm diameter of the N4 internodes were measured. The breaking load at the N4 internodes with leaf sheath was measured at a distance of 8 cm between two supporting points by the Ookawa *et al*. (1992) and Guo *et al*. (2003) method with a universal testing machine (Tensilon RTM-50, Orientec). Physical parameters were calculated as follows: (1) Breaking strength (M, g cm), $M=BL\times L\times 1/4\times 10^3$, where, BL is the force applied to break stem segment (kg), L is the distance between two points (cm); (2) bending moment of the whole plant (WP, g cm), $WP=SL\times FW$, where, SL is the length from breaking basal internode to top (cm), FW is the fresh weight from breaking basal internode to top (g); (3) cross section modulus (SM, mm^3), $SM=\prod/32\times(a_1^3b_1-a_2^3b_2)/a_1$, where, a_1 and a_2 represent the outer and inner diameter of the minor axis in an oval cross-section, respectively, while b_1 and b_2 represent those of the major axis in an oval cross section, respectively; (4) bending stress (BS, g mm^{-2}), $BS=M/SM$; (5) lodging index (LI, %), $LI\ (\%)=WP/M\times 100$

The N4 internodes samples were collected and then, the samples were separated into culms and leaf sheaths. Dry weight of each sample was determined after oven-drying at 70°C to constant weight. Samples were then ground into fine powder. Non-structural carbohydrate (NSC) content was determined by using anthrone colorimetric as described in Yoshida *et al*. (1972). In brief, the dried samples (0.1 g) were boiled in distilled water for 2 h and then extracted with 9.2 mol L^{-1} perchloric acid for 3 times, the extract was used to analyze the NSC content after adding anthrone reagent. The absorbance was then measured at 620 nm in a spectrophotometer. Cellulose content was measured according to a modified procedure by Updegraff (1969). We pretreated 100 mg of powdered samples with 5 mL of acetic/nitric reagent (100 mL of 80% acetic acid mixed with 10 mL of concentrated nitric acid) in a tube. The tubes were placed in a boiling water bath for 30 min. The tubes were moved to cool at the room temperature, and then, the supernatant was poured out after centrifuging at 5000 r min^{-1} for 15 min. We repeated the above treatment steps with acetic/nitric reagent more than three times until the residue become white. We then added 5.0 mL of 72% sulphuric acid reagent and let them stand at room temperature more than 12 h. The tubes were added 8 mL of distilled water and were cleaned three times, and then, each wash was poured into the volumetric flask together to reach 50 mL. Finally, we analyzed the cellulose (as a pure cellulose, Sigma) using the anthrone reagent according to the NSC method.

The lignin content was measured as following a procedure by Ishimaru *et al*. (2008). In brief, after extracting of the sample with benzene-ethanol reagent (100 mL of benzene mixed with 50 mL of 90% ethanol) in a soxhlet extractor for 4 h, the air-dried sample (0.5 g) was placed in a small beaker, and 72% sulphuric acid (7.5 mL) was added slowly with stirring. Then, the sample was allowed to stand for 4 h at room temperature with frequent stirring. Then, the material was washed completely into a 500-mL flask by adding distilled water (280 mL) for dilution to a 3% sulphuric acid concentration. The mixture was boiled for 4 h under reflux. The solution was filtered with a glass filter P16 and the insoluble lignin was washed free of acid with hot water. The filter and lignin were dried at 105°C for 2 h until the weight was constant, and the weight was then recorded. The lignin content was calculated as follows:

Lignin (%)=W/S×100

Where, W is the insoluble lignin weight (g) and S is the dried sample weight (g).

For histochemical localization of the lignin accumulation, phloroglucinol staining was carried out according to the standard protocol described in Li *et al*. (2009). Fresh hand-cut sections (~20 μm thick) in the fourth internode culm from the top of rice at the heading stage were incubated for 10 min in phloroglucinol solution (1% in ethanol:water (70:30, v/v); Sigma, USA), the phloroglucinol was removed and treated with 18% HCl for 5 min, then photographed under a light microscope (model DM4000B; Leica, Germany).

5.5. Statistical analysis

To test the differences among the treatments across two years, the variance analysis was performed by using SPSS 20.0 statistical software. The means of treatments were compared based on the significant difference test (LSD) at the 0.05 probability level. The standard deviation of the means was calculated by using Microsoft Excel 2007 software for Windows.

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