

Effects of exogenous cytokinin on photosynthesis, senescence, and yield performance of inferior rice tillers grown under different nitrogen regimes

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Abstract

Inferior tillers contribute negligibly to rice grain yields as their late occurrence or prematurity curtails growth. However, 6-benzylaminopurine (6-BA, a synthetic cytokinin) can delay tiller senescence. Our results indicate that foliar application of 6-BA enhanced the distribution and allocation of light energy for the photochemical reactions of PSII, under appropriate N supply. The percentage of thermal energy dissipation also decreased following the application of 6-BA as a consequence of the reduced risk of chloroplast oxidative damage through excess excitations. Moreover, leaf peroxidase, catalase, and ascorbate peroxidase activities were augmented by adding 6-BA, which significantly inhibited the accumulation of malondialdehyde and H₂O₂. The application of 6-BA increased the grain numbers of the inferior tillers and further enhanced tiller yields under 82.5 and 165 kg(N) ha⁻¹ supply. The effect of 6-BA was negligible for the untreated or excessive N-treated plants.

Additional key words: antioxidant enzyme; distribution of light energy; nitrogen harvest index; yield potential.

Introduction

In order to feed the growing global population, the world's crop yields per unit area must increase. However, population growth and agricultural development remain unbalanced in many areas (Paarlberg 1996). In recent years, arable regions worldwide have faced immense challenges, in the form of urbanization, land degradation, and environmental pollution (Deng *et al.* 2015). In view of the increasingly tense situation of cultivated land resources, the further improvement of grain yields will be critical toward ensuring food security for all (Peng *et al.* 2008).

Rice is the most important food crop in Asia and yields are closely related to the use of fertilizers, particularly nitrogen (N). Farmers use N fertilizers to increase rice tiller numbers, thereby improving rice yields (Zhang *et al.* 2013), but there is an upper threshold, and yields may decline at higher N supply (Jian *et al.* 2014). Although early emerging tillers can attain their maximum grain production with optimal N input, there is still great room for yield improvement in late-emerging tillers (Wang *et al.* 2016).

Tillering is an important biological trait of rice, directly

related to the panicle per unit area in the determination of rice yields (Li *et al.* 2003). The application of N has been shown to enhance the endogenous cytokinin content in rice plants; axillary buds are involved in the regulation of cytokinin production. Although N does not activate the regulation of axillary buds in rice, it does provide critical nutrients for their growth into tillers (Liu *et al.* 2011). Among tillers, the pattern of panicle development is hierarchical – grain yields become poorer for each productive tiller (Sahu *et al.* 2004). Generally, the panicles of late-emerging tillers do not significantly contribute to grain yields, as tiller maturation is synchronous but tiller initiation and development are asynchronous (Wang *et al.* 2007, Mohapatra and Kariali 2008). Kariali *et al.* (2012) measured the degradation rates of soluble proteins and chlorophyll (Chl), as well as the rate of malondialdehyde (MDA) accumulation, and found that they were fundamentally consistent among tillers during the late growth stage. The poor yields of late-emerging tillers might also be due to a low N harvest index. Prematurity was thought to be the primary limiting factor that complicated the transfer of large volumes of N-based nutrients from straw to grains (Wang *et al.* 2017a). Thus, enhancing the activities of N sources and sinks may conceivably prevent

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Abbreviations: APX – ascorbate peroxidase; 6-BA – 6-benzylaminopurine; CAT – catalase; CK – control; MDA – malondialdehyde; NHI – nitrogen harvest index; P_N – net photosynthetic rate; POD – peroxidase; SOD – superoxide dismutase; SPAD – reading of relative leaf chlorophyll content; Φ_{NO} – constitutive nonphotochemical energy dissipation; Φ_{NPQ} – regulated thermal dissipation; Φ_{PSII} – actual photochemical efficiency.

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premature tiller aging and extend the rice leaf functional stage and grain-filling time.

The generation and elimination of free radicals serve to maintain balance in the normal process of plant growth. The active mechanism of antioxidant enzymes proceeds through disproportionation reactions to eliminate excessive free radicals and to reestablish equilibrium (Alguacil *et al.* 2003). With plant aging, antioxidant activity gradually declines and the active oxygen metabolism balance becomes disordered, resulting in the accumulation of bio-toxic substances, such as MDA and H₂O₂ (Procházková *et al.* 2001). Thus, a viable strategy for increasing the yields of inferior tillers involves free radical elimination, which effectively delays senescence in late-emerging tillers.

For decades, plant growth regulators, such as 6-benzylaminopurine (6-BA), which functionally approximates endogenous cytokinins, have been used for their direct and indirect effects on the elimination of reactive oxygen species (ROS), which reduces membrane lipid peroxidation and other metabolic functional disorders (Leshem 1981, Burke 2013). Apart from delaying senescence, 6-BA has been shown to promote the transport of assimilates from vegetative to reproductive organs, thereby enhancing the organ's attractiveness to photosynthetic products in the provisioning of additional materials for spike formation (Zahir *et al.* 2001). The present study focused on inferior rice tillers and sought to: (1) evaluate the antioxidant effects of 6-BA on inferior rice tiller yields and (2) examine and elucidate the morphological and physiological responses of inferior tillers to 6-BA application under different N treatments.

Materials and methods

Site description: Field experiments were conducted at two adjacent fields, located in Wuxue County (30°11'N, 115°59'E), Hubei Province, in Central China, during the 2014, 2015, and 2016 rice growing seasons. The soil type is hydromorphic paddy soil, a silty clay loam derived from quaternary yellow sediments. Prior to the investigation, soil samples were collected from the upper 20-cm layer for chemical analyses. The experiments in 2015 and 2016 were conducted at the same field, whereas the experiments in 2014 were conducted at an adjacent field. In 2014 and 2015, the soil pH, organic matter, total N content, available P, and available K were: 5.58 and 5.62, 26.9 and 29.4 g kg⁻¹, 1.87 and 1.67 g kg⁻¹, 14.2 and 13.3 mg kg⁻¹, and 121.4 and 109.8 mg kg⁻¹, respectively.

Plant materials and experimental design: Experiment I (6-BA treatments): The Indica hybrid variety 'Liangyou-287' was grown in 2014, and pregerminated seeds were sown in a seedbed on 28 March. The seedlings were transplanted on 3 May, with a hill spacing of 0.167 × 0.200 m and a single seedling per hill. The Indica hybrid variety 'C-Liangyouhuazhan' was grown in 2015, and pregerminated seeds were sown in a seedbed on 25 May. The seedlings were transplanted on 23 June, with a hill spacing of 0.175 × 0.214 m and a single seedling per hill. The study was conducted using a randomized complete

block design with three replications. The treatments comprised of four N application rates: (1) N₀ (no N fertilizer application); (2) N_{82.5} [82.5 kg(N) ha⁻¹]; (3) N₁₆₅ [165 kg(N) ha⁻¹]; and (4) N_{247.5} [247.5 kg(N) ha⁻¹]. Nitrogen (urea) was applied as follows: 50% at the basal stage, 25% at the tillering stage (15 d after transplanting; DAT), and 25% at the panicle initiation stage (40 DAT). Phosphorus, in the form of calcium superphosphate [75 kg(P₂O₅) ha⁻¹], and zinc, in the form of zinc sulfate heptahydrate [5 kg(Zn) ha⁻¹], were applied at the basal stage. Potassium (potassium chloride) was applied at 75 kg(K₂O) ha⁻¹, 70% at the basal stage and 30% at the panicle-initiation stage. To prevent seepage and nutrient flow, each plot was separated with 0.2-m wide bunds, covered with a double-layered plastic film (at a 0.3-m soil depth). Flooding was maintained in the field during transplantation until 10 d before crop maturity.

For each primary plot, 60 plants with uniform growth were screened and divided into two subplots: one was treated with 1.33 × 10⁻⁴ mol L⁻¹ 6-BA (BA treatment, the recommended concentration for rice; Pan *et al.* 2013) and the other was treated with distilled water (CK). The 6-BA (*Aladdin Industrial Corporation*, Shanghai, China) was dissolved in 0.1 mol L⁻¹ HCl, diluted with distilled water (the HCl concentration was far too low to impart any negative physiological effect on the plants). A volume of 0.5 g of *Tween-20* (polyethylene glycol sorbitan monolaurate; *Aladdin Industrial Corporation*, Shanghai, China) was also added to the 1-L solutions to serve as a surfactant. At the booting stage, 6-BA was applied with a brush to both sides of the flag leaves on the inferior tillers between 17:00 and 18:00 h. This was repeated approximately 7 d later.

Experiment II (tiller removal treatments) was performed in order to study the yield potentials of the inferior tillers. In 2016, 40 plants with uniform growth were screened from each N-treatment group and divided into two subplots. In one subplot, half of the early-emerging tillers were manually removed (Remove), whereas tillers in the other subplot were not removed (CK). The manual removal was performed for the entire subplot at 30 DAT.

Tiller tagging and morphological observations: Inferior tillers have the characteristic short growth periods and low grain yields. To determine inferior tillers in this study, the tillers of plants in each subplot were labeled with small plastic tags according to their order of emergence; the latest emerging tiller with at least three fully expanded leaves was designated as an inferior tiller (when the main stem began elongating). The first tiller to emerge was designated as the superior tiller. Within each subplot, the growth characteristics of 12 inferior tillers were measured and the Chl content of the flag leaf segments was determined using a SPAD meter (*SPAD-502*, *Minolta*, Japan).

Photosynthesis and Chl fluorescence measurements: At the grain-filling stage, eight tagged inferior tillers from each subplot were selected in the morning (10:00–11:30 h). The flag leaves were used to quantify the net photosynthetic rate (P_N) using a portable photosynthesis apparatus,

equipped with a red and blue LED light source leaf chamber (*Li-6400*, *Li-Cor*, Lincoln, NE, USA). For each measurement, the middle section of a flag leaf was enclosed in the leaf chamber and the P_N was recorded following equilibration to a steady state.

At the grain-filling stage, four tagged tillers of different types (from each plot) were selected and the flag leaves were used to measure the fluorescence parameters with a portable photosynthesis apparatus coupled with a *6400-40* leaf chamber. First (between 2:00–3:00 h), the initial fluorescence yield (F_0) was measured at a dark-acclimated state, followed by a saturating pulse to measure the maximum fluorescence yield (F_m). Next (9:00–11:00 h), the minimum Chl fluorescence yield (F_0'), steady-state Chl fluorescence (F_s), and maximum fluorescence (F_m') were recorded in a light-acclimated state. Both F_m and F_m' were measured with a 0.8-s saturating light pulse [$\sim 8,000 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$]. Within the leaf chamber, the leaf temperature was maintained at 28°C, the PPFD was maintained at $1,200 \mu\text{mol} \text{m}^{-2} \text{s}^{-1}$, the CO_2 concentration was set at $400 \mu\text{mol} \text{mol}^{-1}$, and the relative humidity was 60–70%.

The allocation of photons, which were absorbed by the PSII antennae for photosynthetic electron transport and thermal dissipation, was assessed by defining the actual photochemical efficiency (Φ_{PSII}), regulated thermal dissipation (Φ_{NPQ} ; Genty *et al.* 1996), and constitutive non-photochemical energy dissipation (Φ_{NO} ; Genty *et al.* 1996). The Φ_{PSII} , Φ_{NPQ} , and Φ_{NO} values were calculated based on the above-mentioned parameters (Wang *et al.* 2017b).

Plant biochemical analyses: Following the photosynthesis and Chl fluorescence measurements, half of the inferior tiller samples were dissected into straw and panicle and separately oven-dried at 75°C to a constant mass. The plant materials were then combined, ground to pass through a 1-mm mesh screen, and digested *via* H_2SO_4 and H_2O_2 . The total N concentration of the digested samples was determined using an automated continuous flow analyzer (*Seal*, Norderstedt, Germany).

For later quantification of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and antioxidant enzymes [including superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6) and ascorbate peroxidase (APX; EC 1.11.1.1)], the leaves of the samples were immediately frozen in liquid nitrogen and stored at -80°C in an ultra-cold storage freezer.

MDA concentration was determined according to the methods described by Zhao *et al.* (2012); 0.5 g of fresh leaves was homogenized, containing 10% trichloroacetic acid and 0.5% 2-thiobarbituric acid, and then heated in boiling water for 20 min. After rapid cooling with ice, the mixture was centrifuged at 3,000 rpm for 10 min. The absorbance of the supernatant was measured at 450, 532, and 600 nm using a spectrometer (*UV-1800*, *Shimadzu*, Japan). The concentration of MDA was calculated as: $\text{MDA} [\mu\text{mol} \text{L}^{-1}] = 6.45 \times (\text{OD}_{532} - \text{OD}_{600}) - 0.56 \times \text{OD}_{450}$.

The extraction strategy for the H_2O_2 supernatant was the same as detailed for MDA. To measure H_2O_2 concentration, 1 mol L^{-1} KI and 100 mmol L^{-1} K_2SO_4 was added to

0.5 mL of the supernatant. The mixture was then incubated for 1 h in the darkness and the absorbance recorded at 390 nm (*UV-1800*, *Shimadzu*, Japan) (Chakrabarty and Datta 2008).

Approximately 0.2 g of leaves were homogenized with a pestle in a chilled mortar, containing 5 mL of a 0.05 mol L^{-1} phosphate buffer solution (PBS, pH = 7.8). The homogenate was separated by centrifugation (10,000 rpm for 20 min at 4°C) and the supernatant was used as the crude extract for assays. One unit of SOD activity was the amount of enzyme required to inhibit 50% of the initial reduction of nitroblue tetrazolium under light exposure (Zhou *et al.* 1997). The specific SOD activity was expressed as $\text{U g}^{-1}(\text{FM})$. One unit of POD activity was defined as an increase of 0.01 in absorbance (guaiacol oxidation products) per min at 470 nm (Muñoz-Muñoz *et al.* 2009). The specific POD activity was expressed as $\text{U g}^{-1}(\text{FM}) \text{min}^{-1}$. One unit of CAT activity was defined as the quantity of enzyme required to decompose 1 $\mu\text{M} \text{min}^{-1}$ of H_2O_2 (Aebi 1984). The specific CAT activity was expressed as $\text{U g}^{-1}(\text{FM}) \text{min}^{-1}$. One unit of APX activity was defined as the quantity of enzyme required to decompose 1 $\mu\text{M} \text{min}^{-1}$ of hydrogen peroxide (Nakano and Asada 1981). The specific APX activity was expressed as $\text{U g}^{-1}(\text{FM}) \text{min}^{-1}$.

The statistical analyses focused on the split-plots design (N rate and 6-BA addition) and their interactions with various parameters of crop agronomics and physiological traits. All data were tested for normality using the *Shapiro-Wilk's* method and homoscedasticity using a *Levene's* test. When appropriate, the data were transformed (logarithmic and square root transformation) to meet the assumption of homogeneity of variance and normality. However, some transformed data still did not fulfill the requirements of variance homogeneity and normality, so nonparametric analysis of variance (*ANOVA*) was used. All data were tested with *ANOVA*, using the general linear model procedure in *SPSS 20.0* (Chicago, IL, USA) and the treatment means were compared with *Duncan's* new multiple range method. The figures were plotted in *Origin 9.0* (*Microcal Software*, Northampton, MA, USA).

Results

Yield potential of inferior tillers: The average grain yield of the superior tillers was significantly higher than that of the inferior tillers; the inferior tillers accounted for 38.5–42.4% of the rice population (Fig. 1). After manual removal of the superior tillers, the inferior tiller yield increased by 3.4 (N_0), 6.3 ($\text{N}_{82.5}$), 12.1 (N_{165}), and 20.0% ($\text{N}_{247.5}$) compared with the control group, under different N applications. The only significant yield increase was in the $\text{N}_{247.5}$ treatment. Enhanced inferior tiller yield was primarily driven by increased grain numbers and grain filling percentages, following superior tillers removal (Table 1S, *supplement*).

Grain yield and its components: Two-way *ANOVA* revealed that the grain yields and spikelets per panicle

were significantly affected by 6-BA treatment (BA) in 2014 and 2015 (Table 2S, *supplement*). The spikelets per panicle were also affected by N application rates in 2015. N significantly influenced the grain mass in 2014 and BA affected grain mass in 2015. Grain filling was significantly influenced by N and the interaction of N and BA in 2015. The interaction of N and BA did not affect the grain yield, spikelets per panicle, or grain mass in either year.

The application of 6-BA enhanced the grain yield of the inferior tillers grown under different N contents; the range

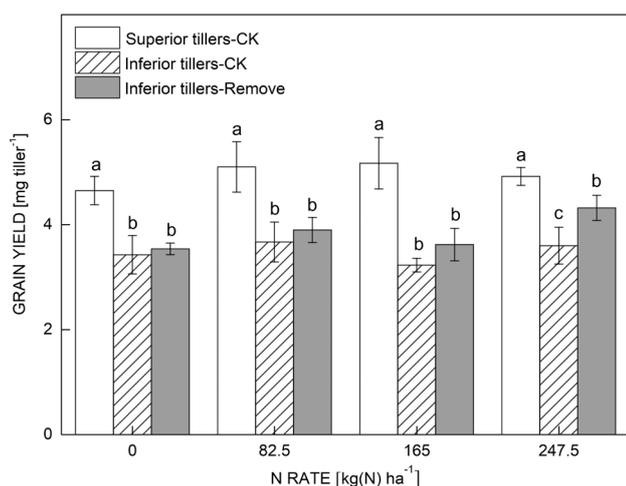


Fig. 1. Grain yields of different types of tillers grown under increasing N treatments. CK represents no tiller removal; Remove represents the manual removal of half of the early-emerging tillers of tillers group. Different letters denote significant differences according to the *Duncan's* new multiple range method ($p < 0.05$) between the different treatments at the same N level. Each bar represents the mean \pm SD of 12 rice plants.

of increase was 13.8–18.4% in 2014, and 0.4–17.2% in 2015 (Table 1). The application of 6-BA also increased the spikelets per panicle with higher contents of N fertilizer. The grain filling percentage increased by 6-BA addition under various N treatments but there was a dramatic drop in filling at the $N_{247.5}$ in 2015. In contrast to CK, the 6-BA-treated grain mass declined significantly at N_0 in 2014 and $N_{247.5}$ in 2015. Decreased grain filling or grain mass masked the effect of increased spikelet numbers, which resulted in an insignificant increase of inferior tiller grain yields.

N concentration and N harvest index: N fertilizer application enhanced the N concentration in the straw and grains at the physiological maturity stage (N concentration in the grain was higher than that in the straw; Fig. 2). With increased N fertilization, the effects of the 6-BA treatment on the plant N concentrations were stronger. A large quantity of N trapped within the straw caused a decrease in the N harvest index (Fig. 3). Under sufficient N supply (N_{165} and $N_{247.5}$), 6-BA application stimulated N concentrations in the straw and grain. However, there was no significant effect of 6-BA that promoted the transport of N assimilates, from the straw to the grain.

Photosynthesis-related parameters: Two-way *ANOVA* revealed that the leaf area, SPAD value, and net photosynthetic rate (P_N) were significantly affected by the 6-BA treatment in 2014 and 2015 (Table 2S). However, no significant $N \times BA$ effect was observed for the two years.

The flag leaf area, SPAD, and P_N increased with higher N supply and the application of 6-BA improved these parameters (Table 2). In 2014, the leaf area and SPAD values significantly increased by exogenous 6-BA under the N_{165} treatment. In 2015, they also significantly increased,

Table 1. Effects of 6-BA application on yield and yield components of inferior rice tillers at maturity grown under different N supply in 2014 and 2015. Values are means \pm SD of 12 tillers. Significant differences between two 6-BA treatments at the same N level are indicated by * ($p < 0.05$), ** ($p < 0.01$), and ns (no significance).

Treatments		Grain yield [g]	Spikelets [panicle ⁻¹]	Grain filling [%]	Grain mass [mg]	
2014	N_0	CK	1.95 \pm 0.27	89 \pm 9	83.9 \pm 3.2	26.0 \pm 1.6
		6-BA	2.22 \pm 0.29 ^{ns}	104 \pm 12*	87.4 \pm 1.2*	24.4 \pm 1.0*
	$N_{82.5}$	CK	1.95 \pm 0.23	89 \pm 9	84.8 \pm 2.7	25.8 \pm 1.2
		6-BA	2.62 \pm 0.15**	115 \pm 9**	88.1 \pm 1.2*	26.0 \pm 0.7 ^{ns}
	N_{165}	CK	2.08 \pm 0.34	94 \pm 26	84.9 \pm 1.8	26.2 \pm 1.8
		6-BA	2.54 \pm 0.20*	116 \pm 21*	88.3 \pm 2.7*	25.2 \pm 1.1 ^{ns}
	$N_{247.5}$	CK	2.07 \pm 0.45	97 \pm 17	86.4 \pm 2.9	24.4 \pm 1.3
		6-BA	2.45 \pm 0.37 ^{ns}	125 \pm 21*	84.2 \pm 2.8 ^{ns}	24.1 \pm 1.6 ^{ns}
2015	N_0	CK	2.60 \pm 0.17	126 \pm 6	87.4 \pm 1.5	23.5 \pm 0.6
		6-BA	2.61 \pm 0.28 ^{ns}	123 \pm 21 ^{ns}	89.7 \pm 3.4 ^{ns}	24.0 \pm 3.1 ^{ns}
	$N_{82.5}$	CK	2.57 \pm 0.33	125 \pm 19	88.5 \pm 2.8	24.2 \pm 2.7
		6-BA	2.73 \pm 0.28 ^{ns}	126 \pm 12 ^{ns}	93.7 \pm 3.6*	23.2 \pm 0.7 ^{ns}
	N_{165}	CK	2.61 \pm 0.45	124 \pm 13	93.8 \pm 1.4	22.9 \pm 1.8
		6-BA	3.06 \pm 0.27*	141 \pm 21 ^{ns}	96.9 \pm 1.6**	22.4 \pm 0.8 ^{ns}
	$N_{247.5}$	CK	2.77 \pm 0.41	127 \pm 10	95.6 \pm 0.7	23.7 \pm 0.9
		6-BA	2.97 \pm 0.23 ^{ns}	150 \pm 12*	91.1 \pm 2.9**	21.7 \pm 1.1*

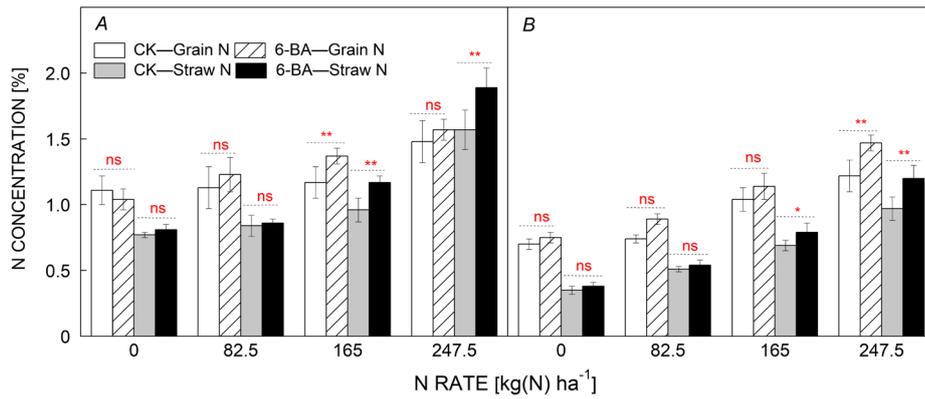


Fig. 2. Effects of 6-BA on the N concentration of inferior rice tillers grown under different N levels in 2014 (A) and 2015 (B). Significant differences between two 6-BA treatments at the same N concentration are indicated by * ($p < 0.05$), ** ($p < 0.01$), and ns (no significance). Each bar represents the mean \pm SD of four leaves from inferior tillers.

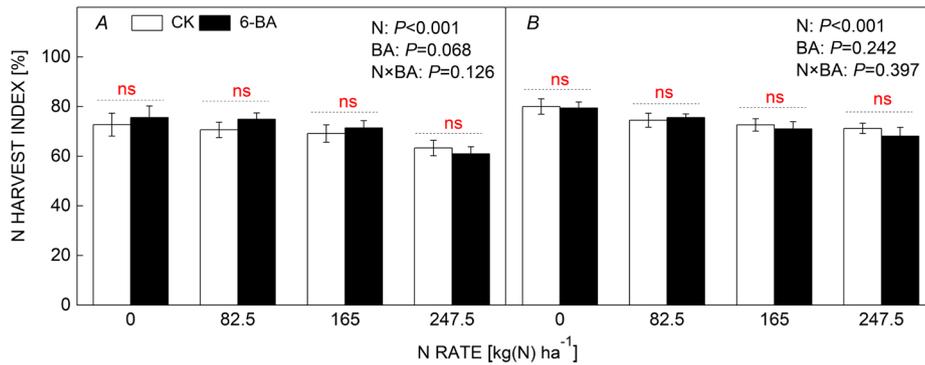


Fig. 3. Effects of 6-BA on the N harvest index of inferior rice tillers grown under different N treatments in 2014 (A) and 2015 (B). ns represents no significant differences between two 6-BA treatments at the same N treatments. Each bar represents the mean \pm SD of 12 inferior tillers.

Table 2. Effects of 6-BA application on leaf area, SPAD value, and net photosynthetic rate (P_N) of inferior rice tillers at maturity grown under different N levels. Values are means \pm SD of 12 tillers. Significant differences between two 6-BA treatments at the same N level are indicated by * ($p < 0.05$), ** ($p < 0.01$), and ns (no significance).

Treatments		Leaf area [cm ²]	SPAD value	P_N [$\mu\text{mol}(\text{CO}_2) \text{ m}^{-2} \text{ s}^{-1}$]	
2014	N ₀	CK	24.4 \pm 2.0	30.5 \pm 2.1	13.1 \pm 1.2
		6-BA	25.6 \pm 3.5 ^{ns}	33.7 \pm 1.9 ^{ns}	16.0 \pm 0.5 ^{**}
	N _{82.5}	CK	27.0 \pm 3.7	31.1 \pm 2.1	14.6 \pm 0.8
		6-BA	31.5 \pm 3.8 ^{ns}	32.4 \pm 1.1 ^{ns}	16.1 \pm 0.9 [*]
	N ₁₆₅	CK	29.3 \pm 2.8	32.8 \pm 2.2	14.7 \pm 1.9
		6-BA	37.7 \pm 2.8 ^{**}	35.7 \pm 1.0 [*]	18.1 \pm 0.4 [*]
	N _{247.5}	CK	38.9 \pm 1.8	37.3 \pm 1.5	17.5 \pm 2.0
		6-BA	39.1 \pm 2.8 ^{ns}	37.6 \pm 1.3 ^{ns}	18.5 \pm 2.1 ^{ns}
2015	N ₀	CK	26.6 \pm 1.9	30.1 \pm 1.3	11.7 \pm 0.2
		6-BA	30.4 \pm 2.9 ^{ns}	31.3 \pm 0.9 ^{ns}	13.4 \pm 0.7 [*]
	N _{82.5}	CK	25.7 \pm 2.8	33.2 \pm 0.5	13.9 \pm 1.2
		6-BA	29.5 \pm 1.1 [*]	35.6 \pm 1.2 [*]	15.1 \pm 1.3 ^{ns}
	N ₁₆₅	CK	37.0 \pm 2.3	36.9 \pm 1.6	16.1 \pm 0.9
		6-BA	38.6 \pm 1.4 ^{ns}	37.8 \pm 0.6 ^{ns}	17.6 \pm 1.0 ^{ns}
	N _{247.5}	CK	32.4 \pm 5.2	38.1 \pm 0.4	13.6 \pm 0.9
		6-BA	37.2 \pm 3.0 ^{ns}	39.7 \pm 1.0 ^{ns}	15.7 \pm 1.7 ^{ns}

but only under the N_{82.5} treatment. The application of 6-BA significantly increased the leaf P_N , by 15.3% in 2014 and 12.0% in 2015. It also increased the average leaf P_N values under different N treatments. In 2014, 6-BA application substantially improved the leaf P_N for all N treatments, except N_{247.5}. In 2015, the effect of 6-BA on leaf P_N was only apparent in the no-N treatment.

During the 2015 growing season, the leaf Chl fluorescence results indicated that the application of 6-BA significantly increased the light energy quantum yields in Φ_{PSII} under the same N supply, except for N_{247.5} (Fig. 4). Under low N (N₀ and N_{82.5}), 6-BA application significantly decreased Φ_{NPQ} . Nevertheless, a significant reduction in Φ_{NO} was observed in the N₁₆₅ treatment. The application

of 6-BA slightly increased Φ_{PSII} and decreased Φ_{NO} in the $N_{247.5}$ treatment, but the differences were not significant.

Lipid peroxidation and hydrogen peroxide content:

MDA and H_2O_2 are the key factors affecting plant resistance and accelerating aging; the exogenous application of 6-BA significantly reduced these harmful substances in the leaves (Fig. 5). Leaf resident MDA and H_2O_2 concentrations decreased with higher N supply. The scavenging effect of 6-BA on H_2O_2 was significantly stronger than that for MDA. There was also a significant interaction between the N content and the 6-BA treatment and in no-N treatments, the elimination of H_2O_2 and MDA was the highest. The highest reduction in H_2O_2 reached 63.7% (2014) and 75.7% (2015), whereas the highest reduction in MDA reached 30.0% (2014) and 16.9% (2015).

Antioxidant enzyme activities: The decrease of MDA and H_2O_2 in the 6-BA treatment was related to an increase in antioxidative activity in the plants. The variable trend of different antioxidant enzymes was not consistent following 6-BA addition (Fig. 6). The SOD activity significantly decreased following exogenous 6-BA addition under the

different N supply, except for the $N_{247.5}$ treatment in 2014. No obvious variations in SOD activity between the CK and 6-BA treatments were observed for the same N supply in 2015. Unlike SOD, the application of 6-BA increased the POD activity by 143.2% in 2014 and 44.2% in 2015.

The difference between CAT activity in the CK and 6-BA treatments decreased with the higher N supply in both years. In 2015, the APX activity increased with increasing N and the addition of 6-BA enhanced the APX activity under the same N treatment. However, these patterns were not observed in 2014.

Discussion

Effect of 6-BA on senescence inhibition in inferior rice tillers:

Due to different nodal positions, tillering proceeds in sequence. The inferior tillers occur later than the primary stems and superior tillers, which together with prematurity, may significantly curtail their growth (Mohapatra and Kariali 2008, Kariali *et al.* 2012). Endogenous cytokinins were surmised to be the primary hormone that inhibited plant aging, and its primary site of synthesis is the root tip, from which it is transported upward through the xylem

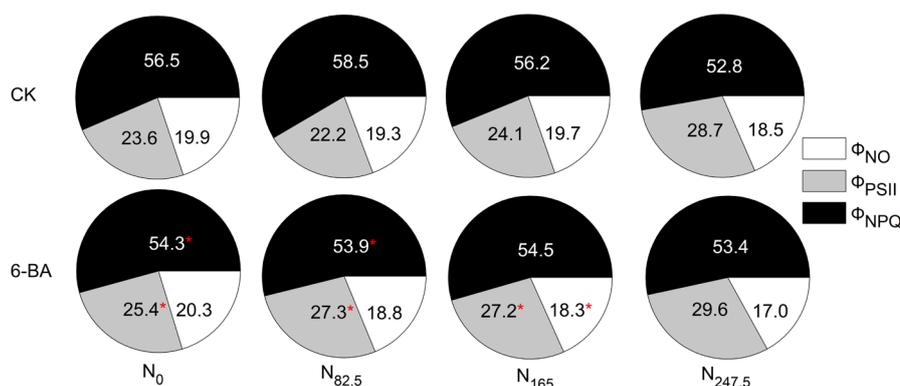


Fig. 4. Effects of 6-BA on the quantum yields in the flag leaves of inferior rice tillers grown under different N supply in 2015. Significant differences between two 6-BA treatments at the same N are indicated by * ($p < 0.05$).

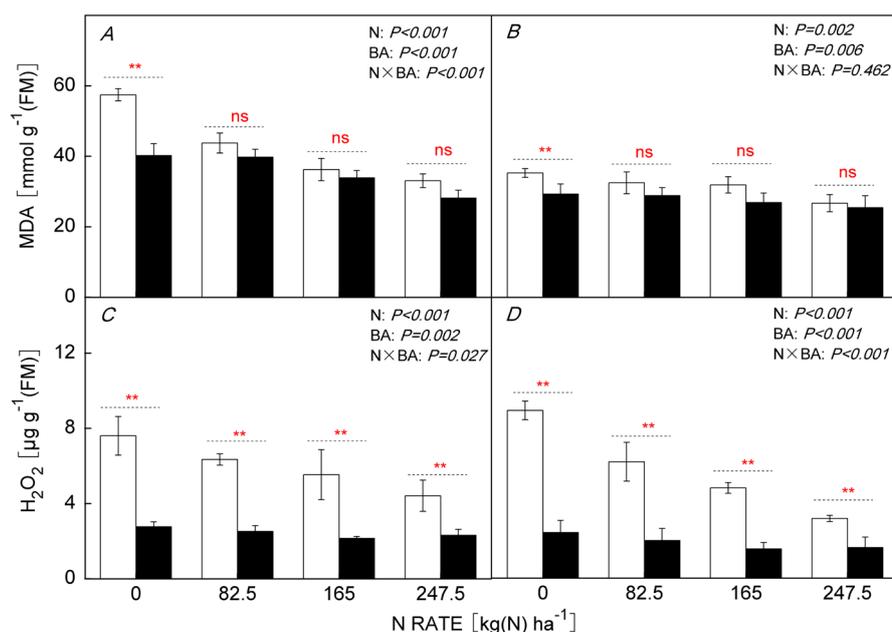


Fig. 5. Effects of 6-BA on the malondialdehyde (MDA) and H_2O_2 contents in inferior rice tillers grown under different N treatments in 2014 (A,C) and 2015 (B,D). Significant differences between two 6-BA treatments at the same N are indicated by ** ($p < 0.01$) and ns (no significance). Each bar represents the mean \pm SD of four leaves in inferior tillers.

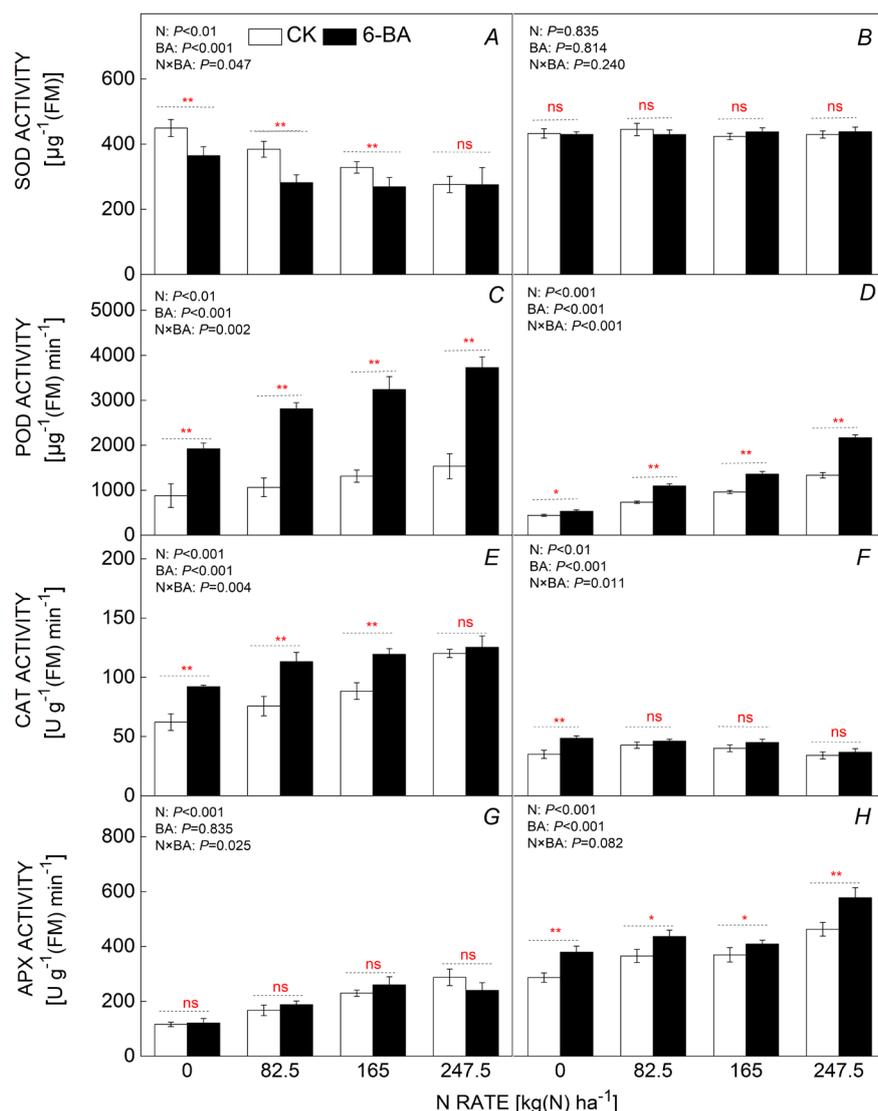


Fig. 6. Effects of 6-BA on the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) in inferior rice tillers grown under different N treatments in 2014 (A,C,E,G) and 2015 (B,D,F,H). Significant differences between two 6-BA treatments at the same N are indicated by * ($p < 0.05$), ** ($p < 0.01$), and ns (no significance). Each bar represents the mean \pm SD of four leaves in inferior tillers.

via transpiration (Li and Bangerth 2003).

However, root growth began senescing during the late reproductive period and the cytokinin supply became insufficient, which directly impacted the physiological photosynthetic function of the leaves. In our study, the SPAD values of the inferior tillers increased significantly with higher N loads. The SPAD value was further increased through 6-BA addition, but significant improvement occurred only with the introduction of suitable N supply (Table 2).

The health of the inferior tiller leaves was prolonged, which not only extended their photosynthetic functionality but also strengthened their source activity (Talla *et al.* 2016). The increased Chl content of the leaves indicated that the harmful intracellular substances (*i.e.*, MDA and its analogs), which typically destroy and degrade Chl, was reduced. MDA and H₂O₂ damage the stability of cell membrane structures and, in this study, were significantly reduced by 6-BA addition. The extent of the reduction of these two substances was greater under lower N supply (Fig. 5).

The antioxidant effect of 6-BA was extremely remarkable under the lower N fertilizer treatments. Also, H₂O₂ reduction was greater than that of MDA, indicating that 6-BA more strongly influenced H₂O₂ removal (Fig. 5). This might be due to MDA formation from H₂O₂-related inorganic free radicals (*e.g.*, oxyanion radicals and hydroxyl radicals), and other organic free radicals (*e.g.*, peroxy radicals, alkoxy radicals, polyunsaturated fatty acids, *etc.*; Leshem *et al.* 1986). The major antioxidant effect of 6-BA in plant tissue should be mediated through the induction of further enzymatic and nonenzymatic pathways (Synková *et al.* 2004). In the present study, 6-BA application enhanced the activity of POD, CAT, and APX enzymatic pathways, which led to a decrease in MDA and H₂O₂ (Fig. 6). CAT and APX are H₂O₂ specific scavengers in mesophyll cells and chloroplasts, respectively (Chen and Asada 1989). 6-BA might also directly remove intracellular H₂O₂; it appears that the deprotonation process occurred on the purine ring of the cytokinin when free cytokinin encountered superoxide. This process may be conducive for the catalyzation of the disproportionation reaction of

superoxide (Frimer *et al.* 1983). 6-BA is thought to reduce inorganic free radical populations through the inhibition of xanthine oxidase (Sheu *et al.* 1997), where MDA and H₂O₂ decrease is a secondary effect initiated by slower senescence or the suppression of senescence (Dhindsa *et al.* 1981). However, the antioxidant enzyme activity was not consistent following 6-BA application in different N treatments. Our results revealed variability in SOD activity that was not consistent across years. The difference in the CAT activity between the CK and 6-BA treatments was lesser at higher N contents in both years. The POD activity increased with higher loadings of N fertilizer (Fig. 6). The dominant duration of each antioxidant enzyme following the addition of 6-BA was inconsistent (Bailly *et al.* 2001, Pan *et al.* 2013), whereas overall, the oxidase enzyme activity was enhanced in contrast to no 6-BA addition.

In addition to enhancing antioxidant enzyme activity, which effectively inhibited free radicals, the more rational use of light by the inferior tiller leaves also reduced free radical production. For the present study, the application of 6-BA improved the capacity of the leaves to absorb and utilize light energy and reduced the ratio of regulated thermal dissipation. Thus, additional light energy was involved in photosynthetic reaction under the appropriate nitrogen supply (Fig. 4). A decrease in the proportion of Φ_{NPO} energy might have been a consequence of the reduced risk of chloroplasts damaged due to excessive excitations, as thermal dissipation is a protective mechanism for photosynthetic machinery (Correia *et al.* 2006, Mishra *et al.* 2008).

Effect of 6-BA on increasing the yield of inferior rice tillers: In theory, each axillary bud has the same cell functionality and should have similar yield potential (Oinam and Kothari 1995). Our findings revealed enhanced inferior tiller growth rates with increasing N application, following the removal of the superior tillers. However, there was a considerable yield gap between the superior and inferior tillers (Fig. 1).

The addition of exogenous 6-BA increased inferior tiller grain yield, but the increasing yield effect was not significant for the no or excessive N treatments (Table 1). The yield results suggest that the increase in grains per panicle was primarily due to increased inferior tiller yields (Table 1). 6-BA inhibited the expression of *OsCKX2* (cytokinin oxidase-critical gene) and enhanced the panicle cytokinin content, which led to an increase in the grain population (Yeh *et al.* 2015). The addition of 6-BA may also partially compensate for growth restrictions caused by insufficient N (Song *et al.* 2013). With excess N, the application of 6-BA significantly increased the grain population and decreased the seed-setting rate and grain mass of the inferior tillers. Hence, a production increase was not obvious (Table 1). At the grain-filling stage, obstacles to the transport of dry mass and N nutrients by source to sink organs might have initiated a reduction in grain mass and filling percentage. In the present study, 6-BA application enhanced the flag leaf area, SPAD value, and net photosynthetic rate of the inferior tillers (Table 2), where more light energy was allocated to

PSII for photochemical reactions (Fig. 4). These results demonstrate that the source of leaf supply capacity was significantly augmented under various N treatments. Nevertheless, it seems 6-BA did not promote the transport of N into the grains of the inferior tillers. The effect of 6-BA application on the N harvest index of the inferior tillers was not significant in this study (Fig. 3). The NHI results observed here are not consistent with those of Qiu *et al.* (2004) and Zahir *et al.* (2001).

6-BA did not significantly improve inferior tiller yields in the excessive-N treatments, which may be related to the lack of sunlight. Wang *et al.* (2017b) suggested that the photosynthetically active radiation intercepted by the inferior tillers was seriously low under high N supply, due to the shading produced by taller, early-emerging tillers. Insufficient light limited the physiological photosynthetic functions of leaves treated with excess N, resulting in no obvious yield increase following 6-BA application. Previous studies also reported weak plant responses to 6-BA when treated with excessive N (Luo *et al.* 2016). The application of no or excessive N impacted the nitrate to ammonium nitrogen ratio in plants, which significantly reduced the plant's responses to 6-BA (Garnica *et al.* 2010). Under the appropriate application of N, exogenous 6-BA exhibited the optimal promoting effect for inferior rice tillers.

Conclusions: Inferior rice tillers have the potential to increase yields without disrupting the performance of superior tillers. The foliar application of 6-BA improved the leaf area, SPAD, P_N , and light energy distribution of inferior tillers; the strongest effects were observed in the N_{82.5} and N₁₆₅ treatments. Leaf POD, CAT, and APX activity were also enhanced by 6-BA addition (in different N treatments), which significantly inhibited the accumulation of MDA and H₂O₂. The antioxidant effect was the strongest under lower N treatments. The addition of exogenous 6-BA significantly enhanced the grain yield of inferior tillers in the 82.5–165 kg(N) ha⁻¹ treatments, but the effects of 6-BA were negligible under no or excess N.

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