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Genotypic Variations in Terms of NH_3 Volatilization in Four Rice (*Oryza sativa* L.) Cultivars

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Abstract: NH_3 volatilization from the cultivated rice (*Oryza sativa* L.) canopy is one of the major contributing factors of the N loss in the rice production. The objectives of this study are to examine the genotypic variations in NH_3 volatilization from canopy among the cultivars and to determine the mechanisms responsible for NH_3 volatilization from rice. With these aims, two pot experiments were conducted using Yoshida culture solution and four rice genotypes (the Yangdao-6, the Wuyujing-3, the BG34-8 and the Zhenshan-97) in greenhouse at Huazhong Agricultural University, Wuhan City, China. The amount of ammonia volatilized from different rice cultivars was evaluated at booting, heading and milking stages. At booting stage, inhibitors of glutamine synthetase (methionine sulfoximine (MSO)) and photorespiration (isonicotinyl hydrazine (INH)) were sprayed on leaves at 0.1 mmol L^{-1} and 2 g L^{-1}, respectively. Genotypic variations in amount of NH_3 volatilized from canopy existed among the four rice cultivars at different growth stages and N treatments. According to results, spraying of the MSO at 0.1 mmol L^{-1} was significantly increased the NH_3 volatilization from the rice canopy, while spraying INH at 2 g L^{-1} decreased the NH_3 volatilization. Obtained results were showed that rate of ammonia volatilization was negatively correlated with the leaf GS activity, while the leaf GO activity related positively to the rice ammonia volatilization.

Key words: Genotypic variation, NH_3 volatilization, glutamine synthetase (GS), photorespiration, rice (*Oryza sativa* L.)

INTRODUCTION

Nitrogen is quantitatively the most essential macro nutrient for plants and a major limiting factor in the plant productivity (Tabuchi *et al*., 2007). It is mostly available in the form of ammonium to the rice (*Oryza sativa* L.) grown under anaerobic flooded conditions (Fan *et al*., 2007). Most of the world current nitrogen has been reported to be consumed in China (Peng *et al*., 2006). And this extensive use of N fertilizer has resulted in serious environmental problems in intensive agricultural areas of China, mainly because of atmospheric, soil and water enrichment with reactive N of agricultural origin (Ju *et al*., 2009). Excessive application of the N also results in lodging of the rice plants which directly affects the yield and indirectly decreases the fertilizer use efficiency of the crop (Lin *et al*., 2007). The NH_3 volatilization is one of the major factors contributing to the loss of anthropogenic input N-fertilizer from the soil (Saffigna and Freney, 2006; Zou *et al*., 2009) and shoots of cultivated plants (Stutte and Weiland, 1978; Farquhar *et al*., 1980; Abrol *et al*., 1986; Mattsson *et al*., 1993; Mattsson and Schjoerring, 1996). And from 10 to 60% of the N is lost from the system through this pathway in rice fields (Song *et al*., 2004). This loss of N through volatilization on one side results in wastage of the applied N (Hayashi *et al*., 2008) and on the other side can cause soil acidification, eutrophication and indirect enhancement of the greenhouse gas (N_2O) (Win *et al*., 2009). Xu *et al*. (2008), while comparing the ammonia volatilization in four different rice genotypes reported that maximum ammonia volatilization occurs at flowering than any other stage. On the other hand, this process (Ammonia volatilization) depends on the supply of nitrogen i.e., ammonia release increases with abundant fertilizer application (Hartwig and Bockman, 1994). Method of fertilizer application and fertilizer source along with soil pH, temperature, moisture or relative humidity and wind speed are some other important factors affecting the ammonia volatilization (Knight *et al*., 2007). Most of the N losses through ammonia volatilization occur within the 7-10 days after fertilizer application and losses from urea may be considerably high as compared to most of the ammonium fertilizers like $(NH_4)_2SO_4$ (Griggs *et al*., 2007).

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Glutamine Synthetase (GS) found in sub-cellular units (chloroplast and cytoplasm) of different tissues and organs is the major assimilatory enzyme for the ammonia produced from N fixation and nitrate or the ammonia nutrition (Miflin and Habash, 2002). Application of GS inhibitors can increase the process of ammonia volatilization and decrease the efficiency of N utilization (Yamaya and Oaks, 2004). Methionine sulphoximine is an effective inhibitor of GS and has been used to increase the NH_3 emissions from leaves (Mattsson and Schjoerring, 1996). A single foliar application of the MSO can inhibit the activity of GS for a period of 8-9 days (Pearson *et al.*, 1998). Photorespiration which takes place in the chloroplasts and peroxisomes, leads to the evolution of CO_2 and NH_3 in the mitochondria of C_3 plants (Husted *et al.*, 2002). Release of the ammonia as a result of photorespiration is reassimilated by the GS (Miflin and Habash, 2002). The inhibition of photorespiration by inhibitors can increase the efficiency of N in the C_3 plants like rice by reducing the NH_3 volatilization (Yamaya and Oaks, 2004). To our knowledge, very few literatures have been documented on the genotypic variations in the ammonia volatilization among the rice cultivars. An insight into the relative importance of these final yield contributing factors like selection of appropriate genotypes, improvement of crop management and increase in the usage efficiency of resources such as nitrogen (N) fertilizer is essential (Jing *et al.*, 2008).

The aim of this study are (1) to determine the genotypic variations in ammonia volatilization among rice (*Oryza sativa* L.) cultivars at various growth stages and N levels and (2) to examine the effect of GS activity and photorespiration in ammonia volatilization of various rice varieties at the booting stage under greenhouse conditions.

MATERIALS AND METHODS

Pot experiments were conducted in the greenhouse at the Huazhong Agricultural University Wuhan, China in 2008. The used culture solution was prepared according to Yoshida and Coronel (1976). The seed was sown in plastic trays having filts soil in the screen house. The soil was kept saturated after sowing to enhance seed germination and crop establishment. Fifteen days after sowing, the seedlings were pulled out from the trays; roots were washed thoroughly and then transplanted into pots. Plastic pots were filled with 10.0 L of nutrient solution in both experiments and a wooden cover with four holes was put inside each pot for supporting plant growth. One rice plant was transplanted in each hole of the wooden cover using sponge and thus each pot has four plants. In pot experiment 1, four rice varieties (the Yangdao-6, the Wuyujing-3, the BG34-8 and the Zhenshan-97) and four treatments (0, 20, 40 and 80 mg N L^{-1}) were used (Table 1) while in pot experiment 2, two rice varieties (theYangdao-6 and the Wuyujing-3) and one treatment (40 mg N L^{-1}) were used (Table 1). In both pot experiments, different stock solutions were added to the pots filled with 10 L of tap water (containing 1.3 mg N L^{-1}) and the pH o f the solution was adjusted as 5.0-5.5 using 1 mol L^{-1} NaOH and HCl (Yoshida and Coronel, 1976). Pots were placed in a greenhouse using a completely randomized design. Each treatment was replicated four times with four pots. The culture solutions were changed once a week and pH of the culture solution was adjusted every other day. Pesticides were sprayed 5-6 times to control loss by insects/pests and other agronomic practices were applied as recommended to the region growers.

In pot experiment 1, the Ammonia Volatilization Rate (AVR) from the rice plants was evaluated among rice varieties at booting, heading and milking stages using the closed chamber method (Wang *et al.*, 2001; Farquhar *et al.*, 1979). The container included a basic unit and a column organic glass covering whose diameter was 41 cm with a height of 120 cm. There was a hole on upper and lower sides in order to pass the suction pipe. The ammonia was collected at the booting, heading, milking and maturity stages. The input air was passed through the absorbent solution with 1 mol L^{-1} H_2SO_4 to remove the ammonia present in the air. The air sample in container was extracted by sucking pump and then absorbed into

Table 1: Rice varieties used in the experiments and the detail of N treatments

Nitrogen treatments	Treatment code	Variety code	Given name	Type	Seed source
1 week 40 mgN L^{-1}*+ 0 mg N L^{-1}+ 10 L tap water (1.3 mg N L^{-1})	N1	V1	Yangdao-6	Hybrid indica rice	Yangzhou Academy of Agricultural Sciences
1 week 40 mgN L^{-1}+ 20 mg N L^{-1}+ 10 L tap water (1.3 mg N L^{-1})	N2	V2	Wuyujing-3	Inbred japonica rice	Yangzhou Academy of Agricultural Sciences
1 week 40 mgN L^{-1}+ 40 mg N L^{-1}+ 10 L tap water (1.3 mg N L^{-1})	N3	V3	BG34-8	Inbred indica rice	International Rice Research Institute(IRRI)Rice Germplasm Resource Center
1 week 40 mgN L^{-1}+ 80 mg N L^{-1}+ 10 L tap water (1.3 mg N L^{-1})	N4	V4	Zhenshan-97	Inbred indica rice	Rice Research Institute of Fujian Agricultural Science College

*15-day-seedlings were cultured in a 40 mg N L^{-1} Yoshida solution in the first week following cultured solutions with various N treatments as indicated in Table 2 till one week before maturity. The N concentration of tap water was 1.3 mg N L^{-1}

the solution of 0.1 mol L^{-1} H_2SO_4 with an air moving velocity of 0.5 L min^{-1}. NH_4^+ concentration in the H_2SO_4 solution was then measured by Segmented Flow Analysis using Futura (France). At the heading stage, Photorespiration Rate (Pr), GS and Glycolate Oxidase (GO) activities of leaf were measured. Pr of flag leaf was measured with a portable photosynthesis system (LI-6200, LI-COR) between 10:00 to 12:00. Each measurement was repeated four times. GS and GO activities of leaf were measured by the methods of chromatometry (Zhang *et al.*, 1997) and HCl-Phenylhydrazine chromatometry (Eisenberg *et al.*, 1997; Gough *et al.*, 2001), respectively. Glycolate oxidase in fresh leaf was extracted in a buffer solution containing 100 mmol L^{-1} of Tris-HCl, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} DTT, 10 mmol L^{-1} $MgSO_4$, 100 mmol L^{-1} KCl, 0.5% Triton X-100 and 20% glycerol with a pH of 7.8. Glycolate oxidase activity was measured according to Lea *et al.* (1991) by incubation of extracts in an assay medium (containing 100 mmol L^{-1} of Tris-HCl, 50 mmol L^{-1} glutamate, 5 mmol L^{-1} hydroxylamine hydrochloride and 50 mmol L^{-1} ATP for 20 min at 30°C. GO activity was measured by HCl-Phenylhydrazine chromatometry. Extracted buffer solution was 100 mmol L^{-1} of H_3PO_4 with a pH of 8.0. Assay medium contained 1.0 mmol L^{-1} FMN, 50 mmol L^{-1} glycolate acid, 2 mmol L^{-1} HCl, 1.82 NaOH and 0.33% HCl-Phenylhydrazine. The activity was then measured by incubation of extracts in an assay medium containing 1.65% K $Fe(CN)_4$ for 20 min at 30°C.

In pot experiment 2, inhibitors of glutamine synthetase (methionine sulfoximine (MSO)) and photorespiration (isonicotinyl hydrazine (INH)) were sprayed on leaves at 0.1 mmol L^{-1} and 2 g L^{-1}, respectively, at the booting stage. After 12 h of MSO and INH spray, the amount of ammonia volatilized, the Pr, the GS and the GO activities were measured.

Data were statistically analyzed following analysis of variance by SAS and mean comparison was performed based on the Least Significant Difference (LSD) test at the 0.05 probability level (Yu and Xiao, 2007).

RESULTS

In pot experiment 1, genotypic variations existed in the AVR from canopy among the four rice varieties at different growth stages and N treatment (Fig. 1a-d). At the booting stage, the AVR from the Wuyujng-3 was significantly higher than the other three varieties. While at the heading and milking stages, the Zhenshan-97 volatilized more NH_3 at each N treatment, compared with the other three varieties. For varieties of the Yangdao-6 and Wuyujing-3, the AVR from canopy was higher at the booting stage than the heading and the milking stages, however, the AVR from the BG34-8 and Zhenshan-97 was significantly higher at the heading stage than the booting and the milking stages. Among the four rice varieties, the highest and lowest amounts of AVR from canopy at the three growth stages were observed in

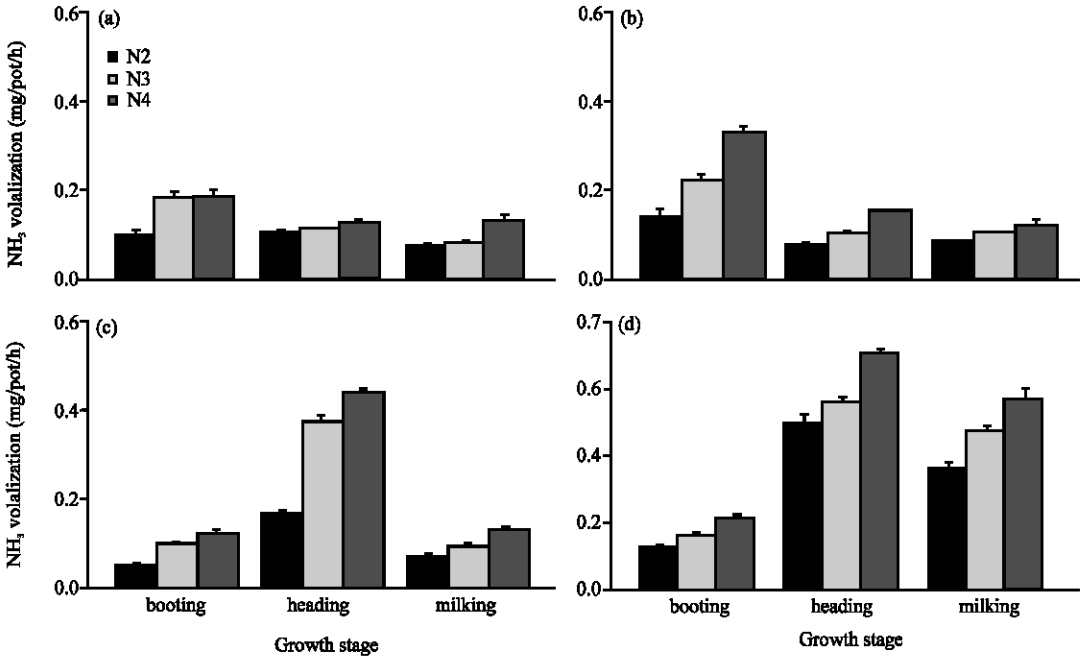

Fig. 1: NH_3 volatilization rate (AVR) from different rice canopies at various growth stages and under various N treatments; (a) Yangdao-6, (b) Wuyujing-3, (c) BG34-8 and (d) Zhenshan-97

Table 2: NH_3 volatilization rate (AVR) of rice genotypes at different growth stages and under various nitrogen treatments based on Duncan test

Source	Booting stage	Heading stage	Milking stage
	----------	(mg NH_3/pot/h)	----------
V1	0.10^b	0.08^c	0.06^b
V2	0.15^a	0.07^c	0.07^b
V3	0.06^c	0.21^b	0.06^b
V4	0.10^b	0.36^a	0.29^a
Means	0.10	0.18	0.12
N1	-	-	-
N2	0.09^c	0.18^c	0.12^c
N3	0.14^b	0.24^b	0.16^b
N4	0.18^a	0.30^a	0.20^a
Means	0.10	0.18	0.12
LSD 0.05	0.009	0.011	0.013

Different letters represent significance of F-values at 5% probability

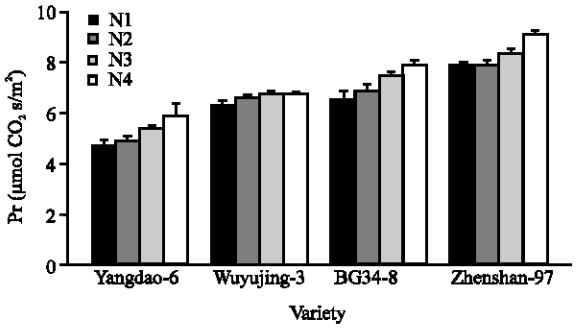

Fig. 2: Leaf photorespiration rate (Pr) of different rice genotypes and under various N treatments

Zhenshan-97 and the Yangdao-6, respectively. The AVR from the Zhenshan-97 canopy was 0.20, 0.48 and 0.41 mg pot^{-1} at the booting, heading and the milking stages, respectively. While, the AVR from the Yangdao-6 was 0.17, 0.16 and 0.14 mg pot^{-1} h^{-1} at the three growth stages, respectively.

The AVR from the rice canopy varied at different growth stages and it significantly increased as the N concentration in the culture solution was increased at different growth stages. Within different stages, the AVR from rice canopy increased from booting to heading stage and decreased from the heading to the milking stage for varieties the Yangdao-6, BG34-8 and the Zhenshan-97, while for the Wuyujing-3, the AVR decreased from the booting to the milking stage. At N3 and N4, the AVR decreased from the booting to the milking stage for the Yangdao-6 and the Wuyujing-3, while in the other two varieties, the trend of NH_3 volatilization was the same as found for N2 (Table 2).

At the heading stage, the leaf Pr increased as N concentration in the culture solution was increased for all the four rice varieties (Fig. 2). The leaf Pr of the

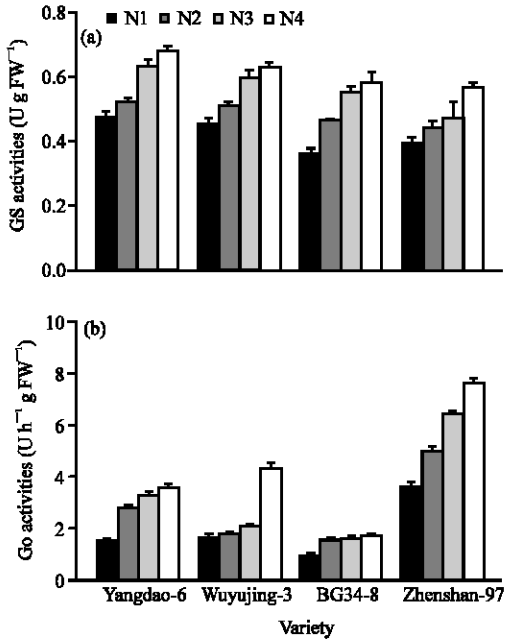

Fig. 3: (a) Leaf Glutamine synthetase (GS) and (b) Glycolate Oxidase (GO) activities of different rice varieties and under various N treatments

Wuyujing-3 and the Zhenshan-97 was higher than the Yangdao-6 and the BG34-8 for all N treatments.

Genotypic variations in the GS activity also existed among the four rice varieties at heading stage (Fig. 3a, b). The GS activity of BG34-8 and the Zhenshan-97 was lower than that of the other two varieties. For each variety, GS activity increased as N concentration in the culture solution increased. While, GO activity of the Zhenshan-97 was significantly higher than the other three varieties. In pot experiment 2, at the booting and milking stages, the AVR from both varieties were significantly increased after the spray of GS inhibitor (MSO) at 0.1 mmol L^{-1}(Fig. 4a-c). At booting, the relative percent of the AVR increases over the untreated control for the Wuyujing-3 and the Yangdao-6 were 234 and 158%, respectively. At the heading stage, the AVR from the Wuyujing-3 was significantly increased, while the AVR from the Yangdao-6 did not respond to the MSO application. Compared with the untreated/control, spray of photorespiration inhibitor (INH) at 1 g L^{-1} significantly decreased the AVR for both varieties and at the three growth stages. When MSO+INH were sprayed in a combination at the booting, heading and the milking stages, the AVR was in between the values obtained when sole the MSO and the INH were sprayed. The results showed that the Yangdao-6 was not as sensitive to the spray of the GS and the photorespiration inhibitors, as was the Wuyujing-3.

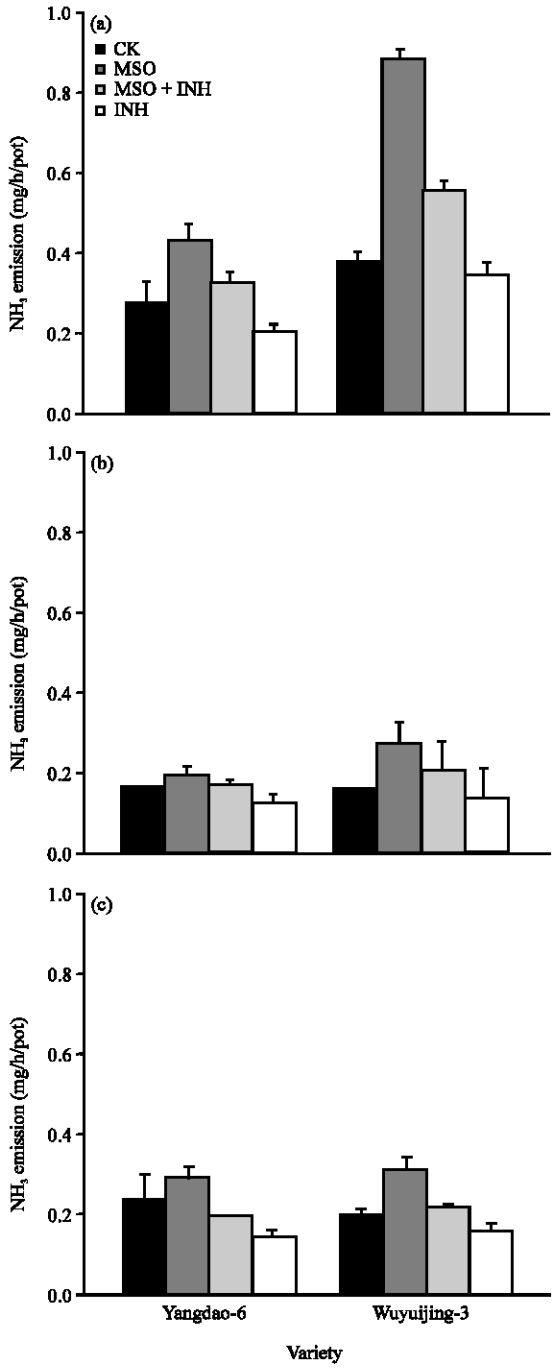

Fig. 4: NH_3 volatilization rate (AVR) of different rice varieties after spraying GS inhibitor (MSO) and photorespiration inhibitor (INH); (a) Booting, (b) Heading and (c) Milking

At the booting stage, the Pr of the Wuyujing-3 under each treatment was relatively higher than that of the Yangdao-6 (Fig. 5).Yangdao-6 significantly. However, spray of sole INH significantly decreased the

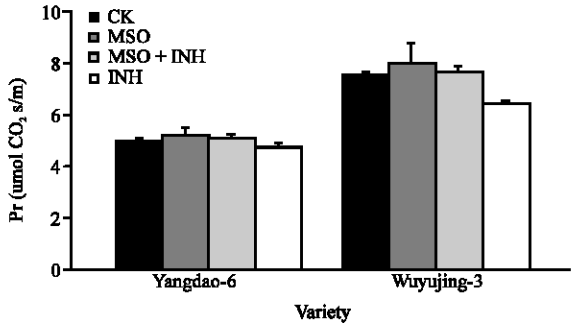

Fig. 5: Photorespiration rate (Pr) of different rice genotypes after the spray of GS inhibitor (MSO) and photorespiration inhibitor (INH)

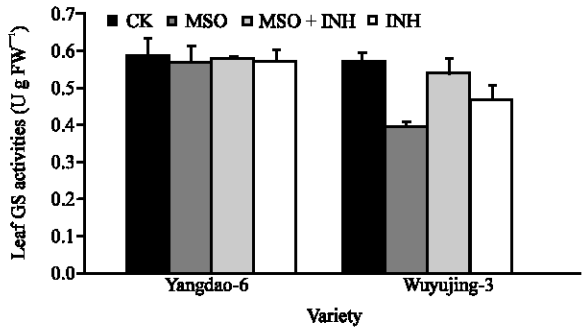

Fig. 6: Glutamine synthetase (GS) activity of different rice genotypes after spraying GS inhibitor (MSO) and photorespiration inhibitor (INH)

photorespiration rate in the Wuyujing-3, though the photorespiration rate did not respond to the spray of sole MSO or MSO+INH. The leaf GS activity of Wuyujing-3 decreased significantly when the MSO and INH were sprayed, while the decrease was not significant when the MSO+INH were sprayed at the booting stage compared with control (Fig. 6). Whereas for the Yangdao-6, the leaf GS activity was not sensitive to the sole or mixed sprays of inhibitors.

DISCUSSION

Genotypic variations in the NH_3 volatilization from rice canopy existed among rice varieties and at different growth stages (Fig. 1). Compared with the Yangdao-6 and the Wuyujing-3, more NH_3 was volatilized from canopy of the BG34-8 and the Zhenshan-97. The lowest AVR was recorded for the Yangdao-6, while the highest was observed for the Zhenshan-97. The possible reason for this variation in the AVR might be due to the differences of nitrogen use efficiency in these varieties. Generally the leaf GS activity of varieties of the BG34-8 and the

Zhenshan-97 at each N level was relatively lower than that of the Yangdao-6 and the Wuyujing-3 (Fig. 3). These results point towards the negative correlation of the AVR with the leaf GS activity. The importance of GS activity in controlling the NH_3 emission from plant leaves has also been demonstrated in mutant barley (*Hordeum vulgare* cv. Maris Mink), which possessed only 66 or 47% of the normal chloroplastic GS activity. Barley mutant plants had higher NH_3 emissions than wild-type plants. The 66% GS mutant showed higher NH_3 emissions than the 47% GS mutant, in agreement with the higher NH_4^+ and lower amino acid contents in the 66% mutant compared to mutants with less than the 4 7% GS activity (Mattsson *et al.*, 1997). Mattsson *et al.* (1997) also reported that more NH_3 was volatilized from mutant barley whose GS activity was 47% of the wild type, compared with the wild type. The AVR from rice canopy increased as N concentration in the culture solution was increased, which is consistent with the findings of some other researchers (Parton *et al.*, 1988; Schjoerring, 2000; Chen *et al.*, 2008). Husted *et al.* (2000) for example, reported that the amount of NH_3 volatilization of oilseed rape (*Brassica rapa* L.) was 3 times higher at the medium N level than at the low N level and 50 times higher at the high N level than at the medium N level. Similarly, increasing the N availability to plants increases the NH_4^+ concentration within the plants which in turn enhances the NH_3 emission as also reported by Schjoerring *et al.* (2000) for high NH_4^+ content of the roots. A linkage was observed by Mattsson *et al.* (1998) among measured NH_3 compensation points and which were calculated on the basis of apoplastic pH and NH_4^+ concentrations, indicating that the latter two parameters indeed are important in determining of the NH_3 volatilization from the plant leaves. These results show the influence of applied nitrogen on the AVR and importance of applying proper dose of nitrogen to get maximum nitrogen use efficiency.

The AVR from rice canopy differed at various growth stages, though the time courses of AVR differed based on various genotypes. For Yangdao-6 and Wuyujing-3, the AVR from rice canopy decreased from booting to heading and milking stages, while the AVR from the BG34-8 and Zhenshan-97 increased from booting to heading stages and decreased from heading to milking stage. The time course of NH_3 emission from cereal species seems to have two distinct peaks during the growth cycle (Parton *et al.*, 1988; Schjoerring, 1991) and the NH_3 emission increased from the booting to the maturing stage (Wu *et al.*, 2006). This may be due to the possible reduction of the activities of leaf GS and some N metabolism related enzymes after the booting or heading stage. In our experiment, the AVR values of different rice genotypes have one distinct peak, while the corresponding curve shows variation with developmental stages during the growth cycle. One possible explanation of this result could be the genotypic variation of the used varieties. Measuring the ammonia volatilization at all of the phenological stages may be helpful in the better understanding of the relationship between volatilization process and growth stages.

Spraying of the MSO at 0.1 mmol L^{-1} significantly increased the AVR from rice canopy, while it was decreased by the spray of INH at 2 g L^{-1}. Similar results have also been found by some other researchers in rapeseed and wheat (Olsen *et al.*, 1995; Mattsson and Schjoerring, 1996). The increase in NH_3 volatilization from rice canopy after spraying the 0.1 mmol L^{-1} MSO might be induced by the NH_4^+ accumulation in the rice leaf. The photorespiration is one of the main factors involved in ammonia volatilization (Lea *et al.*, 1992; Leegood *et al.*, 1995). Addition of the MSO and HPMS (photorespiration inhibitors) to the culture solution can increase NH_4^+ accumulation in the rice leaf and the NH_3 volatilization in the barley, while only HPMS addition to the culture solution results in a reduced the NH_3 volatilization (Platt and Anthon, 1981). This suggests the positive relationship of AVR with GS activities and its negative correlation with the Pr. One of the possible causes also reported by Wu *et al.* (2006) may be the prohibition of N metabolism, leading to the decomposition of protein and amino acid.

CONCLUSIONS

This study has clearly revealed that the differences in the AVR of rice genotypes and has also determined the influencing factors affecting the ammonia volatilization. The findings lead us to conclude that genotypic variation in the ammonia volatilization exists among rice genotypes and it changes (also shows one peak) as the crop passes through different growth stages. Nitrogen plays an important role in volatilization as increasing the amount of added nitrogen to the crop enhanced the process of volatilization. Our findings also show a distinct role of the photorespiration and the GS activities in the amount of ammonia volatilized. The photorespiration shows positive while GS activity shows negative relationship with the rate and amount of ammonia volatilized. The mechanisms underlying these relationships are not clear and need further study. A comprehensive study taking into account the measurement of ammonia volatilization at all the growth stages will lead us to a deeper insight into the process of volatilization.

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