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BRIEF COMMUNICATION

Metabolic regulation of ammonia emission in different senescence phenotypes of *Nicotiana tabacum*

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Abstract

In order to reveal the character of ammonia emission in senescent tobacco (*Nicotiana tabacum*), the content of NH_4^+, total nitrogen, and soluble protein, and the activities of nitrogen metabolism-related enzymes were measured in leaves of a quick-leaf-senescence phenotype ZY90 and a slow-leaf-senescence phenotype NC89. Compared with NC89, ZY90 had a higher NH_4^+ accumulation, a lower glutamine synthetase activity, and a significantly higher stomatal ammonia compensation point, and ammonia emission during 40 to 60 d after leaf emergence. During senescence, the quick-leaf-senescence phenotype was characterized by nitrogen re-transfer by ammonia emmission, whereas the slow-leaf-senescence phenotype by nitrogen re-assimilation. The ammonia emission was primarily regulated by glutamine synthetase activity, apoplastic pH, and NH_4^+ content.

Additional key words: nitrogen metabolism, stomatal ammonia compensation point, tobacco.

Ammonia emission is the main form of plant nitrogen re-transfer (Sommer *et al*. 2004, Herrmann *et al*. 2009). During senescence, leaf proteins are degraded into amino acids, amides, and ammonium. This degradation leads to the accumulation of NH_4^+ in plant leaves, which caused re-assimilation and re-transfer of nitrogen (Schjoerring *et al*. 2001). Ammonium in senescing leaves is re-assimilated by nitrogen metabolism-related enzymes, such as glutamine synthetase (GS, E.C. 6.3.1.2; Cruz *et al*. 2006), and it is also lost through ammonia emission (Husted and Schjoerring 1996, Massad *et al*. 2008).

The stomatal ammonia compensation point (χ_s) is considered as value representing the intensity and direction of ammonia exchange between a plant and atmosphere (Mattsson *et al*. 1998, Schjoerring *et al*. 2000, Mattsson and Schjoerring *et al*. 2003, Herrmann *et al*. 2009). However, few researches have focused on metabolic regulation of ammonia emission in the course of leaf ontogeny.

Seeds of two tobacco (*Nicotiana tabacum* L.) phenotypes (quick-leaf-senescence ZY90 and slow-leaf-senescence NC89) were supplied by the College of Tobacco Science, the Henan Agricultural University, Zhengzhou, China. The seeds were sowed in a seedbed, and then transplanted in pots filled with soil and grown in a greenhouse at a temperature of 25 ± 5 °C, a relative humidity of 70 ± 5 %, and a 16-h photoperiod with an irradiance > 400 μmol m^{-2} s^{-1}. Leaf age was counted from the day when the leaf was 1 cm long and 0.5 cm wide. The seedlings were transplanted into plastic drums when the fourth euphyllas were 7-d-old. Each drum contained 30 kg of soil, a plant per drum, and 20 individuals of each phenotype, repeated 6 times. The soil was fertilized with NH_4^+-N + NO_3-N in the ratio of 1.36:1.14 and finally contained [g (nutrient) kg^{-1}(soil)] 0.2 N, 0.4 P, and 0.5 K. At 40, 50, 60, and 70 d after leaf emergence (DAE), the eleventh leaf, which was counted from the bottom of a plant, was collected.

A vacuum infiltration technique according to Husted and Schjorring (1995) was used to extract the apoplastic

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Abbreviations : DAE - days after leaf emergence; GDH - glutamate dehydrogenase; GS - glutamine synthetase; $[H^+]_{apo}$ - apoplastic H^+content; $[NH_4^+]_{apo}$ - apoplastic NH_4^+content; pH_{apo}- apoplastic pH; χ_s - ammonia stomatal compensation point.

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solution. An *AMFIA* NH_4^+ analyzer (*Bran Luebbe*, Hamburg, Germany) was used to determine NH_4^+ concentration in the extracted solution. A calibration solution and a zero standard were made with deionized water which contained 1.0 and 10 μg(NH_4^+) kg^{-1}. A microelectrode inserted in a microcentrifuge tube was used to determine pH (*Inlab* 423, *Mettler*, Toledo, Spain). The extraction of the apoplastic solution and measurements of $[NH_4^+]_{apo}$ and pH_{apo} were done at a temperature of 25 °C.

Ten cubic centimetres of formic acid (10 mM) in a cooled mortar with fine sand was used to homogenize 2.00 g of the plant tissue. The homogenate was centrifuged at 25 000 *g* and 2 °C for 10 min and the supernatant was transferred on 0.45 μm polysulphone centrifugation filters (*Micro VectraSpin*, *Whatman*, Maidstone, UK) and spun at 5 000 *g* and 2 °C for 5 min.

An HPLC system (*Waters Corp*., Milford, MA, USA) with a pump, a column oven with a 3.3 m stainless steel reaction coil and a scanning fluorescence detector was used to determine ammonium. An alkylthio-isoindole fluorochrome was formed through reaction between NH_4^+ and *o*-phthalaldehyde (OPA) at the neutral pH with 2-mercaptoethanol as reducing agent. An excitation wavelength of 410 nm and an emission wavelength of 470 nm (Husted *et al*. 2000) were chosen to detect this fluorochrome. According to Horneck and Miller (1998), a model *1106* elemental analyzer (*Carlo Erba*, Milan, Italy) was used to determine total N content.

Samples (1.00 g) were ground in 5 cm^3 of a Tris-HCl (50 mM) buffer (pH 7.9, measured at 23 °C) and in 5 cm^3 of a buffer with pH 7.2 (measured at 23 °C) containing Tris-HCl (40 mM), Na_2EDTA (l mM), 5 % (v/v) glycerol, and bromophenol blue (0.01 mg cm^{-3}) for determining GS and glutamate dehydrogenase (GDH, EC1.4.1.2) activities, respectively. Debris was removed from the samples by centrifugation at 13 000 *g* for 10 min. Glutamine synthetase activity was measured using the spectrophotometric method according to O'Neal and Joy (1973) by measuring formed γ-glutamyl-hydroxamate, and GDH activity was detected according to Turano *et al*. (1996) based on the measurement of decrease or increase in absorbance of samples (respective of the direction of the reaction) at 340 nm using a spectrophotometer *Novaspec II* (*Pharmacia,* Uppsala, Sweden). A GS activity unit (U) was defined as the amount of GS which produced 1 μmol of γ-glutamyl-hydroxamate per min at 37 °C. A GDH activity unit (U) was defined as the reduction of NADH (l μmol) per min at 30 °C in the reaction mixture. Soluble protein content was determined in the fresh leaf extracts used for GS activity measurement using a commercially available kit (a Coomassie protein assay reagent; *Bio-Rad*, Munich, Germany) using bovine serum albumin as standard. An equation by Herrmann *et al.* (2009) was used to calculate the ammonia stomatal compensation point: χ_s [nmol(NH_3) mol^{-1}(air)] = $\Gamma \times K_H \times K_d$, where Γ is a dimensionless ratio between $[NH_4^+]_{apo}$ and $[H^+]_{apo}$, and K_H and K_d are thermodynamic constants.

Ammonia emission rate was determined by a self-design device (Fig. 1). In case of a good air-tightness,

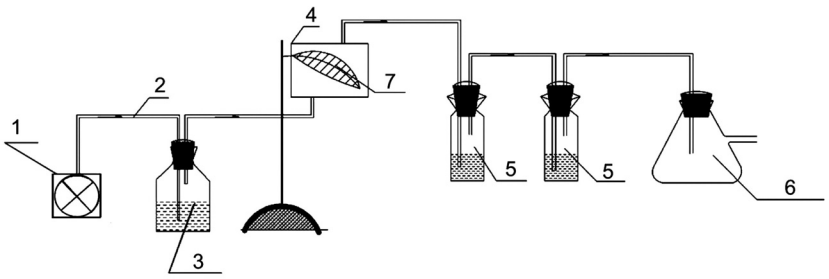

Fig. 1. A schematic ammonia collecting device diagram. A leaf sample (No. 7) was encased in a polyethylene plastic bag (100 cm long and 80 cm wide, 95 % light transmittance, No. 4), and its mouth was focused on the petiole of the leaf base, twinned with a rubber band firmly to ensure the bag had a good air-tightness. Air went into an aspirator bottle (No. 3) containing 0.5 M H_2SO_4 to remove NH_3 by using a micro air pump (No. 1). Then the air entered the sealed polyethylene plastic bag from the bottom and flowed out of the bag in the top and then through two absorption bottles (No. 5) containing 0.01 M H_2SO_4. It finally got in a buffering bottle (No. 6) opened to atmosphere. Individual parts were interconnected with a polyethylene pipe (No. 2).

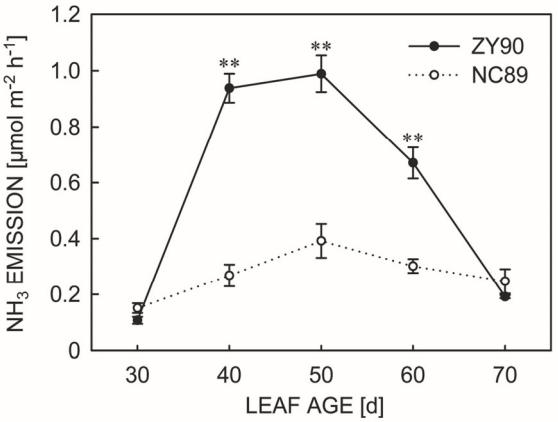

Fig. 2. Variations in NH_3 emission depending on tobacco leaf age. Means ± SE, $n = 6$.

ammonia emitting from a tobacco leaf was collected for 12 h (8:00 - 20:00). No temperature difference was found between the inner space and outside. The absorption liquid was replaced every 3 h, and all them were collected to determine NH_4^+ concentration with a continuous flow analyzer (*Bran Luebbe AA3*). Leaf surface area was measured with a portable leaf area meter (*LI-3000*, *LI-COR*, USA). The collected absorption liquid of a cycle without a sample leaf was used as blank.

Means and standard errors (SEs) were calculated based on six biological replicates. Data were subjected to the analysis of variance. Statistical analysis was performed using the *SAS v. 9.2* software (*SAS Institute*, Chicago, USA). Means were tested according to

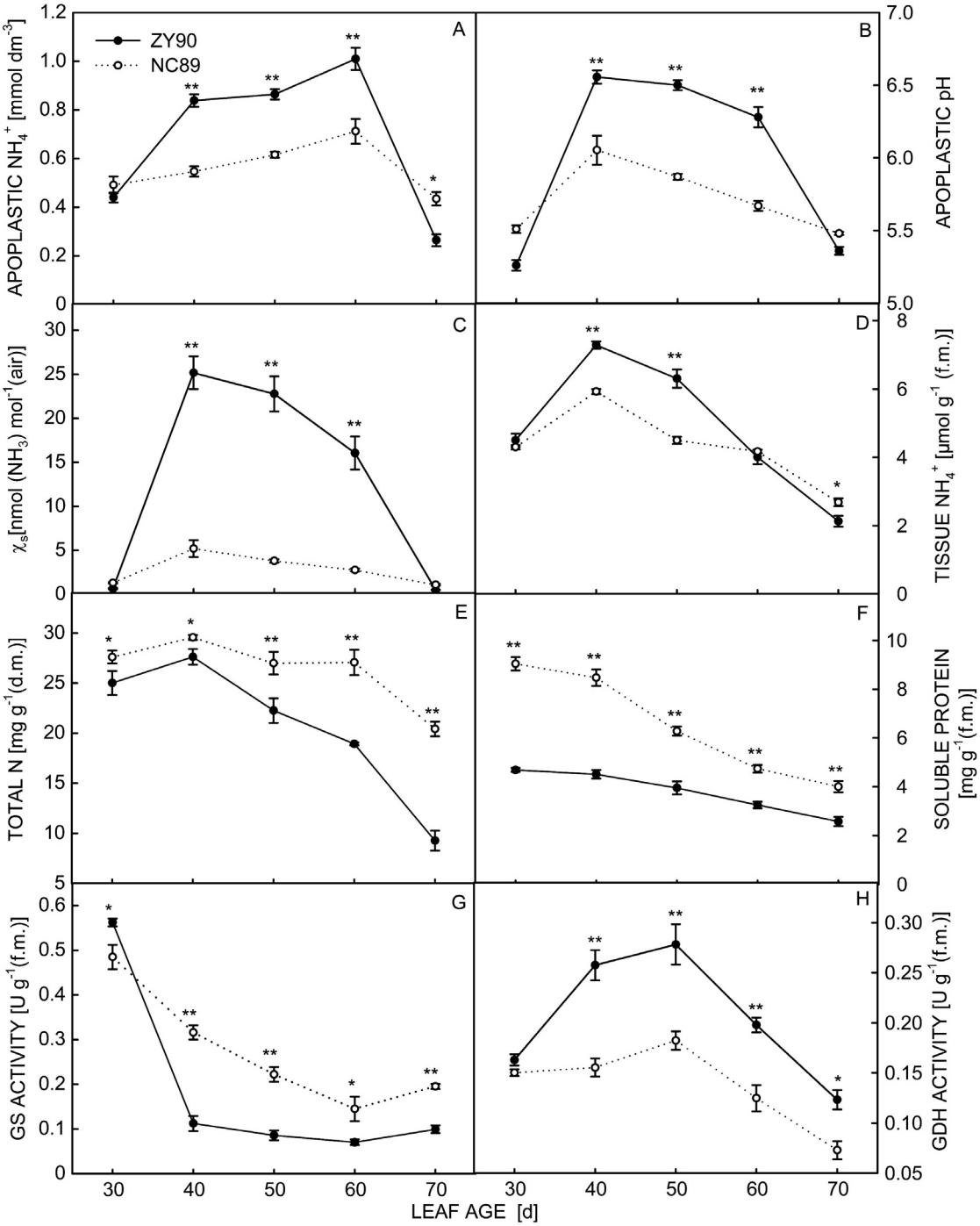

Fig. 3. Variations in $[NH_4^+]_{apo}$ (*A*), pH_{apo} (*B*), χ_s (*C*), leaf tissue NH_4^+content (*D*), total leaf N (*E*), soluble protein content (F), GS (*G*), and GDH (H) activities in tobacco leaves of different age. Phenopytes ZY90 and NC89 are compared. Mean ± SE, $n = 6$.

Table 1. Correlation coefficients between different parameters of two tobacco senescence phenotypes. Significant differences at * - $P \leq 0.05$, ** - $P \leq 0.01$.

Phenotype	Parameter	Soluble protein	Total N	Apoplastic NH_4^+	Apoplastic pH	Tissue NH_4^+	GS activity	GDH activity	χ_s
ZY90	NH_3 emission	0.209	0.446	0.829**	0.983**	0.784**	-0.634**	0.914**	0.979**
	χ_s	0.307	0.539*	0.849**	0.989**	0.823**	-0.575*	0.926**	-
	GDH activity	0.500	0.675**	0.771**	0.889**	0.897**	-0.346	-	-
	GS activity	0.570*	0.352	-0.46	-0.648**	-0.072	-	-	-
	tissue NH_4^+	0.738**	0.862**	0.619*	0.751**	-	-	-	-
NC89	NH_3 emission	-0.412	0.112	0.588*	0.594*	0.536*	-0.726**	0.194	0.630*
	χ_s	0.329	0.660**	0.412	0.992**	0.950**	-0.119	0.591*	-
	GDH activity	0.629*	0.813**	0.397	0.618*	0.764**	0.331	-	-
	GS activity	0.873**	0.391	-0.451	-0.087	0.010	-	-	-
	tissue $NH4^+$	0.448	0.758**	0.425	0.963**	-	-	-	-

Student's *t*-test at 0.05 and 0.01 probability levels.

The NH_3 emission from leaves of both phenotypes exhibited a similar tendency: it firstly raised and then decreased. When the leaf age was 30 DAE, the NH_3 emission was low. From 30 to 50 DAE, the NH_3 emission from ZY90 leaves increased rapidly from 0.11 ± 0.01 up to 0.99 ± 0.06 μmol m^{-2} h^{-1}, and from NC89 leaves, it increased slowly from 0.15 ± 0.01 to 0.32 ± 0.01 μmol m^{-2} h^{-1}. ZY90 had a significantly higher ($P \leq 0.01$) NH_3 emission rate than NC89 during the period of 40 to 60 DAE. In the leaf age from 50 DAE to 70 DAE, the NH_3 emission decreased sharply in ZY90 and slowly in NC89. At the end, the NH_3 emission from ZY90 leaves tended to be less than from NC89 leaves (Fig. 2).

The $[NH_4^+]_{apo}$ increased sharply in ZY90 and slowly in NC89 during the period from 30 to 60 DAE. The pH_{apo} increased during the period from 30 to 40 DAE in both phenotypes and declined earlier than $[NH_4^+]_{apo}$. The χ_s showed a similar tendency, it reached a maximum at 40 DAE. During the period from 40 to 60 DAE, $[NH_4^+]_{apo}$, pH_{apo}, and χ_s in ZY90 were significantly higher ($P \leq 0.01$) than in NC89 (Fig. 3*A*,*B*,*C*). The ammonia emission was correlated with χ_s, $NH_4^+{}_{apo}$, and pH_{apo} more tightly in ZY90 ($P \leq 0.01$) than in NC89 ($P \leq 0.05$).

In ZY90 and NC89 leaves, the content of NH_4^+, total N, and soluble protein continuously declined from 40 DAE (Fig. 3*D*,*E*,*F*). Significant differences ($P \leq 0.01$) in tissue NH_4^+ content were found between the two phenotypes during the period from 40 to 50 DAE, and NC89 had a significantly higher total leaf N and soluble protein content than ZY90. At 70 DAE, the total leaf N content decreased to 76.42 and 31.13 % of the maximum reached at 40 DAE in ZY90 and NC89, respectively. Leaf tissue NH_4^+ was correlated with the ammonia emission in ZY90 ($P \leq 0.01$) and in NC89 ($P \leq 0.05$) (Table 1).

Glutamine synthetase and GDH exhibited totally different variations with leaf age, and there were significant differences in the enzyme activities in the two phenotypes (Fig. 3*G*,*H*). The GS activity in ZY90 was significantly higher ($P \leq 0.05$) than in NC89 at 30 DAE but decreased to 34.81 % at 40 DAE, whereas the GS activity in ZY90 decreased only to 80.02 % at 40 DAE. The GDH activity firstly increased and reached a maximum of 0.28 ± 0.02 U g^{-1}(f.m.) in ZY90 and 0.18 ± 0.01 U g^{-1}(f.m.) in NC89 at 50 DAE and then decreased. The GS activity had a significant negative correlation ($P \leq 0.01$) with the ammonia emission for both phenotypes; there was also a positive correlation ($P \leq 0.01$) between the GDH activity and the ammonia emission in ZY90, but this correlation was not found in NC89 (Table 1).

It was reported that ammonia emission is derived from leaf tissue NH_4^+ which is a degradation product of soluble protein (Sommer *et al*. 2004). Its rate is influenced by leaf senescence severity (Agüera *et al*. 2010). There was actually a higher content of NH_4^+ in ZY90 leaves because of a larger degradation of soluble protein as compared to NC89 (Fig. 3*D*,*F*). An excessive accumulation of NH_4^+ in leaf tissue is toxic (Sparks 2009). Despite the content of total nitrogen and soluble protein decreased continuously, the leaf NH_4^+ did not exhibit a lasting increased tendency (Fig. 3*D*). Glutamine synthetase has been reported to be key enzyme of NH_4^+ assimilation and control of NH_4^+ content in leaves (Schjoerring *et al.* 2002). Expression of GDH is induced when N re-assimilation reaches higher levels, and functions in deamination during leaf senescence (Masclaux *et al*. 2002). In the tobacco, the concentration of $NH_4^+{}_{apo}$ increased until 60 DAE and then decreased (Fig. 3*A*), but the NH_4^+ content in leaf tissue declined from 40 DAE (Fig. 3*D*). This discrepancy is mainly because of NH_4^+ transfer from the leaf tissue to the apoplast (Schjoerring *et al.* 2000) and the transfer leads to an increase in χ_s (Duan *et al.* 2012).

Despite χ_s and the ammonia emission had a similar variation, χ_s reached a peak at 40 DAE, which was 10 DAE earlier than the ammonia emission (Figs. 2 and 3*C*). It indicates that the ammonia emission could be

influenced not only by χ_s but also by other factors such as leaf temperature, stomatal conductance, irradiance, *etc*. (Sutton *et al*. 1995, Husted and Schjoerring 1996).

Our results suggest that the slow leaf senescence in NC89, which had a lower GDH activity and higher GS activity, mainly rely on nitrogen re-assimilation to remove leaf NH_4^+ accumulation, however, the quick leaf senescence in ZY90, which had a higher GDH activity and lower GS activity, was characterized by a higher content of $[NH_4^+]_{apo}$ and χ_s, which led to a much larger ammonia emission (Fig. 2). This can explain the difference in ammonia emission from the two tobacco senescence phenotypes.

References

Agüera, E., Cabello, P., Haba, P.D.L.: Induction of leaf senescence by low nitrogen nutrition in sunflower (*Helianthus annuus*) plants. - Physiol. Plant. **138**: 256-267, 2010.

Cruz, C., Bio, A.F.M., Domínguez-Valdivia, M.D., Aparicio-Tejo, P.M., Lamsfus, C., Martins-Loução, M.A.: How does glutamine synthetase activity determine plant tolerance to ammonium. - Planta **223**: 1068-1080, 2006.

Duan, W.J., Yang, T.Z., Dai, Y., LI, D.L., Zhang, X.Q., Liu, H.B.,Wang, C.G.: Stomatal NH_3 compensation point and its metabolic regulation in senescence phenotypes of *Nicotiana tabacum*. - Biol. Plant. **56**: 771-774. 2012.

Herrmann, B., Mattsson, M., Jones, S.K., Cellier, P., Milford, C., Sutton, M.A., Schjoerring, J.K., Neftel, A.: Vertical structure and diurnal variability of ammonia exchange potential within an intensively managed grass canopy. - Biogeosciences **6**: 15-23, 2009.

Horneck, D.A., Miller, R.O.: Determination of total nitrogen in plant tissue. - In: Karla, Y.P. (ed.): Handbook of Reference Methods for Plant Analysis. Pp. 75-83. CRC Press, New York 1998.

Husted, S., Schjoerring, J.K.: Ammonia flux between oilseed rape plants and the atmosphere in response to changes in leaf temperature, light intensity and air humidity. - Plant Physiol. **112**: 67-74, 1996.

Husted, S., Hebbern, C.A., Mattsson, M., Schjoerring, J.K.: Determination of ammonium, low molecular weight amines and amides in plant tissue. - Physiol. Plant. **109**: 167-179, 2000.

Husted, S., Schjoerring, J.K.: Apoplastic pH and ammonium concentration in leaves of *Brassica napus* L. - Plant Physiol. **109**: 1453-1460, 1995.

Masclaux, D., C., Valadier, M.H., Carrayol, E., Reisdorf-Cren, M. Hirel, B.: Diurnal changes in the expression of glutamate dehydrogenase and nitrate reductase are involved in the C/N balance of tobacco source leaves. - Plant Cell Environ. **25**: 1451-1462, 2002.

Massad, R.S., Loubet, B., Tuzet, A., Cellier, P.: Relationship between ammonia stomatal compensation point and nitrogen metabolism in arable crops: current status of knowledge and potential modeling approaches. - Environ. Pollut. **154**: 390-403, 2008.

Mattsson, M., Schjoerring, J.K.: Senescence-induced changes in apoplastic and bulk tissue ammonia concentrations of ryegrass leaves. - New Phytol. **160**: 489-499, 2003.

Mattsson. M., Husted. S., Schjoerring. J.K.: Influence of nitrogen nutrition and metabolism on ammonia volatilization in plants. - Nutr. Cycl. Agroecosyst. **51**: 35-40, 1998.

O'Neal, D., Joy, K.D.: Glutamine synthetase of pea leaves. I. Purification, stabilisation and pH optima. - Arch. Biochem. Biophys. **159**: 113-122, 1973.

Schjoerring, J.K., Husted, S., Mäck, G., Mattsson, M.: The regulation of ammonium translocation in plants. - J. exp. Bot. **53**: 883-890, 2002.

Schjoerring, J.K., Husted, S.M.G., Nielsen, K., Finnemann, J. Matt, M.: Physiological regulation of plant-atmosphere ammonia exchange. - Plant Soil. **221**: 95-101, 2000.

Schjoerring, J.K., Mattsson, M.: Quantification of ammonia exchange between agricultural cropland and the atmosphere: measurements over two complete growth cycles of oilseed rape, wheat, barley, and pea. - Plant Soil. **228**: 105-115, 2001.

Sommer, S.G, Schjoerring, J.K, Denmead, O.T.: Ammonia emission from mineral fertilizers and fertilized crops. - Adv. Agron. **82**: 557-622, 2004.

Sparks, J.P.: Ecological ramifications of the direct foliar uptake of nitrogen. - Oecologia **159**: 1-13, 2009.

Sutton, M.A., Schjoerring, J.K. Wyers, G.P.: Plant-atmosphere exchange of ammonia. - Phil. Trans. roy. Soc. A **351**: 261-278, 1995.

Turano, F.J., Dashner, R., Upadhayaya, A., Caldwell, C.R.: Purification of mitochondrial glutamate dehydrogenase from dark-grown soybean seedlings. - Plant Physiol. **112**: 1357-1364, 1996.