DOI: 10.1007/s10535-012-0141-z BIOLOGIA PLANTARUM 56 (4): 771-774, 2012

BRIEF COMMUNICATION

Stomatal NH_3 compensation point and its metabolic regulation in senescence phenotypes of *Nicotiana tabacum*

W.J. DUAN[1,2], T.Z. YANG[1]*, Y. DAI[2], D.L. LI[2], X.Q. ZHANG[1], H.B. LIU[1], N. LI[2] and C.G. WANG[2]

Henan Agricultural University, Zhengzhou, 450002, P.R.China[1]
Research and Development Center, China Tobacco Chuanyu Industrial Corporation, Chengdu, 610066, P.R. China[2]

Abstract

We compared stomatal ammonia compensation point (χ_s) and its metabolic regulation in tobacco (*Nicotiana tabacum*) leaves of a quick-leaf-senescence phenotype ZY90 and a slow-leaf-senescence phenotype NC89. Compared with NC89, ZY90 had significantly higher χ_s values between 40 and 60 d after leaf sprouting in spite of its lower nitrogen content. During the same time, a steeper decline in glutamine synthetase activity was detected in ZY90 leaves, simultaneously with a steep increase in χ_s. These results suggested that the quick leaf senescence phenotype exhibited high NH_3 emission potential due to efficient nitrogen recycling and remobilization, and glutamine synthetase played a key role in regulating χ_s in ZY90.

Additional key words: glutamine synthetase, nitrogen content, quick- and slow-leaf-senescence phenotypes, tobacco.

Leaf senescence represents the final stage of leaf development and is characterized by the transition from nutrient assimilation to nutrient remobilization (Masclaux *et al.* 2000). Many different proteolytic activities associated with senescence at the whole leaf level ensure that during senescence proteinaceous components of leaf cells are degraded into amino acids, amides and ammonium. Ammonium is re-assimilated into amino acids for export from senescing leaves, whereas may also be lost through leaching or through volatilization of ammonia (Schjoerring and Mattsson 2001).

Stomatal ammonia compensation point (χ_s) of plants is a major parameter controlling the strength and the direction of NH_3 exchange between vegetation and the atmosphere (Schjoerring *et al.* 1998). It is defined as the atmospheric NH_3 concentration for which there is no exchange between the leaf and the atmosphere in dry conditions. The χ_s is influenced by environmental conditions, plant growth characteristics (Sutton *et al.* 1994), N content in the plant (Sommer *et al.* 2004), activity of glutamine synthetase (GS, EC 6.3.1.2) (Mattsson *et al.* 1997), *etc*. Tobacco plants with different genetic background have different senescence rates. However, very little information is available concerning the differences in χ_s and its metabolic regulation in different senescence phenotypes.

Two tobacco (*Nicotiana tabacum* L.) phenotypes (quick-leaf-senescence ZY90 and slow-leaf-senescence NC89) were used for the experiments. The seeds, provided kindly by College of Tobacco Science, Henan Agricultural University, Zhengzhou, China, were sowed in a seedbed. When the seedlings averaged about 12 cm in height, they were transplanted in pots filled with soil and grown in a greenhouse at temperature of 25 ± 5 °C, relative humidity of 70 ± 5 % and 16-h photoperiod with irradiance > 400 μmol m^{-2} s^{-1}. Soil contained [g(nutrient) kg^{-1}(soil)] 0.2 N, 0.4 P and 0.5 K after being fertilized with NH_4^+-N: NO_3^--N to 1.36: 1.14. The thirteenth leaf, numbered from the bottom of plant, was collected at 20, 30, 40, 50, 60, 70 d after leaf sprouting (DAS). Leaf age was counted from the first day when it was 1 cm long and 0.5 cm wide.

Received 2 March 2011, *accepted* 5 December 2011.
Abbreviations: DAS - days after leaf sprouting; 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.
Acknowledgements: This work was supported by grants from China Tobacco Chuanyu Industrial Corporation (Grant No. 2010-347-20).
* Author for correspondence: fax: (+86) 371 63558121, e-mail: yangtiezhao@126.com

Apoplastic solution was extracted with a vacuum infiltration technique according to Husted and Schjorring (1995). Concentration of NH_4^+ in the extracted solution was determined with *AMFIA* NH_4^+ analyzer (*Bran Luebbe,* Hamburg, Germany), using calibration solution (NH_4Cl in deionized water) of 0.1 and 1.0 μg(NH_4^+) kg^{-1} or 1.0 and 10 μg(NH_4^+) kg^{-1} depending on the concentration of the samples. Deionized water was used for the zero standard. The pH were determined directly by a microelectrode (*Inlab 423*, *Mettler*, Toledo, Spain) inserted in the microcentrifuge tube.

For extraction of leaf tissue, the plant tissue was homogenized in 10 mm formic acid in a cooled mortar with fine sand. The homogenate was centrifuged at 25 000 *g* (2 °C) for 10 min and the supernatant was transferred to 0.45 μm polysulphone centrifugation filters (*Micro VectraSpin*, *Whatman*, Maidstone, UK) and spun at 5 000 *g* (2 °C) for 5 min.

Ammonium was determined by fluorimetry on an HPLC system (*Waters Corp*., Milford, MA, USA) equipped with a pump, a column oven with a 3.3 m stainless steel reaction coil, an autosampler cooled to 2 °C and a scanning fluorescence detector. The reaction between NH_4^+ and *o*-phthalaldehyde (OPA) to form an alkylthioisoindole fluorochrome was performed at neutral pH with 2-mercaptoethanol as reducing agent. This fluorochrome was detected at an excitation wavelength of 410 nm and an emission wavelength of 470 nm (Husted *et al.* 2000). Total N content was determined by a model *1106* elemental analyser (*Carlo Erba*, Milan, Italy) according to Horneck and Miller (1998).

Glutamine synthetase (GS) and glutamate dehydrogenase (GDH, EC 1.4.1.2) activities were measured according to the method of O'Neal and Joy (1973) and Turano *et al.* (1996), respectively. Soluble protein content was determined in crude leaf extracts used for GS activity using a commercially available kit (Coomassie protein assay reagent; *Bio-Rad*, Munich, Germany) using bovine serum albumin (BSA) as a standard.

Ammonia stomatal compensation point, χ_s [nmol(NH_3) mol^{-1}(air)], was calculated following Husted and Schjoerring (1996) and Herrmann *et al.* (2009) taking into account that $K_d << [H^+]_{apo}$ within the range of apoplastic pH values: $\chi_s = \Gamma \times K_H \times K_d$, where Γ is the dimensionless ratio between $[NH_4^+]_{apo}$ and $[H^+]_{apo}$, and K_H and K_d are thermodynamic constants. Since the physiological ionic strength in the apoplast normally ranges between 14 and 28 mM (Cosgrove and Cleland 1983, Speer and Kaiser 1991) K_d was adjusted to an ionic strength of approximately 20 mM by the extended Debye-Hückel equation (Atkins 1990), giving $K_d = 10^{-9.32}$. Γ values represent a measure of the NH_3 exchange potential independent of temperature.

Both $[NH_4^+]_{apo}$ and pH_{apo} exhibited significant differences between ZY90 and NC89 despite of similar variations for the two senescence phenotypes (Fig. 1*A*,*B*). $[NH_4^+]_{apo}$ increased from 0.30 ± 0.01 up to 0.89 ± 0.01 mM in ZY90 and from 0.39 ± 0.01 up to 0.75 ± 0.01 mM in NC89 during the period from 20 to 60 DAS, respectively. ZY90 had significantly higher ($P \leq 0.05$) $[NH_4^+]_{apo}$ than NC89 during the period from 40 to 60 DAS. pH_{apo} increased from 5.03 ± 0.09 to 6.56 ± 0.05 in ZY90 and from 5.36 ± 0.08 to 6.11 ± 0.05 in NC89 during the period from 20 to 40 DAS, respectively, and declined earlier than $[NH_4^+]_{apo}$ for both phenotypes.

Measured $[NH_4^+]_{apo}$ and pH_{apo} were used to assess Γ, the ratio of $[NH_4^+]_{apo}$ and pH_{apo}. The calculated values of χ_s are shown in a separate axis (Fig. 2). χ_s also showed a significant difference between ZY90 and NC89 as it increased and reached up to 23.1 nmol(NH_3) mol^{-1}(air) for ZY90 and up to 6.9 nmol(NH_3) mol^{-1}(air) for NC89 at 40 DAS, respectively.

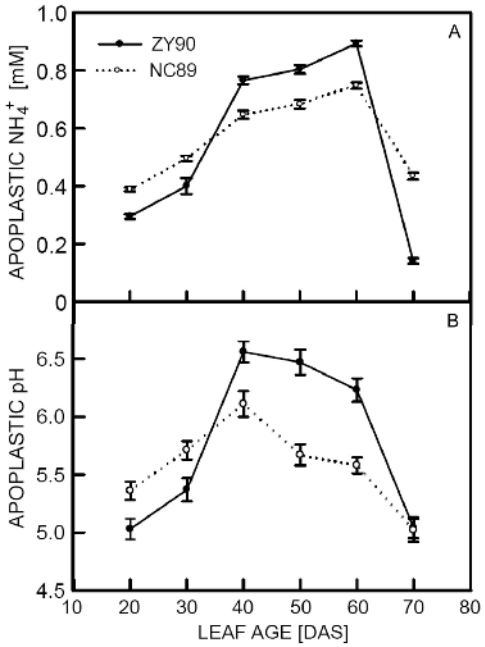

Fig. 1. Variations in $[NH_4^+]_{apo}$ (*A*) and pH_{apo} (*B*) in tobacco leaves with leaf age. Each value is the mean of six independent replicates ± SE.

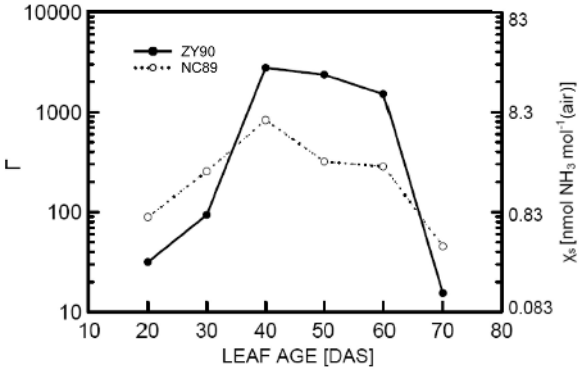

Fig. 2. Variations in Γ ($[NH_4^+]_{apo}$ to $[H^+]_{apo}$) and χ_s (ammonia stomatal compensation point at 25 °C) in tobacco leaves with leaf age.

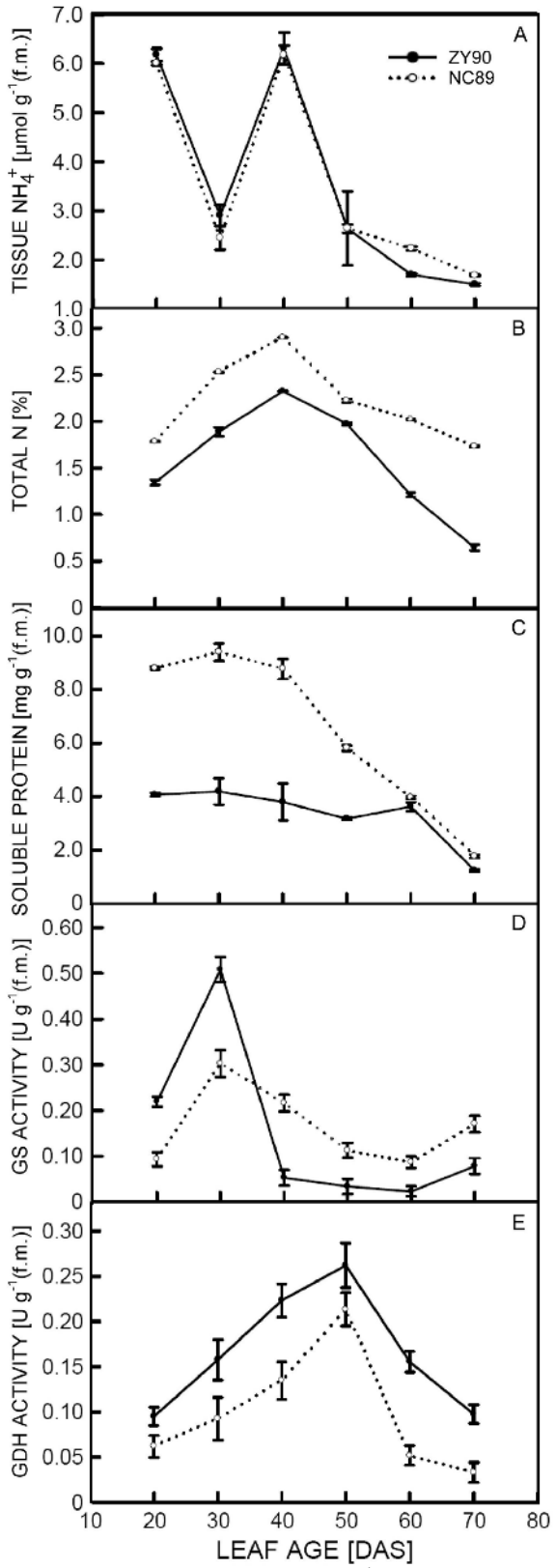

Fig. 3. Variations in leaf tissue NH_4^+ content (*A*), total leaf N (*B*) and soluble protein content (*C*), GS (*D*) and GDH (*E*) activities in tobacco leaves with leaf age. Means ± SE, $n = 6$.

ZY90 and NC89 also displayed a similar variation in leaf tissue content of NH_4^+, total N and soluble proteins (Fig. 3). No difference in tissue NH_4^+ content was found between the two phenotypes during the period from 20 to 50 DAS. NC89 had significantly higher ($P \leq 0.05$) total leaf N content throughout the experimental period and soluble protein content before 50 DAS compared with ZY90. Total leaf N content decreased 72.15 and 40.40 % of the maximum reached at 40 DAS for ZY90 and for NC89 at the last stage of senescence, respectively.

According to correlation analyses (Table 1), throughout the experimental period, χ_s positively correlated ($P \leq 0.01$) with total N rather than leaf tissue NH_4^+ and soluble protein content in ZY90, while it positively correlated with leaf tissue NH_4^+ content ($P \leq 0.05$) and total N ($P \leq 0.01$) in NC89. Tissue content of NH_4^+, N and soluble protein also showed mutual positive correlations ($P \leq 0.01$) for each phenotype, but all of them did not correlate with $[NH_4^+]_{apo}$.

GS and GDH exhibited a similar variation with leaf age for ZY90 and NC89, but there was a significant difference in enzyme activity (Fig. 3*D*,*E*). GS activity reached up to 0.51 ± 0.03 U g^{-1}(f.m.) for ZY90 and up to 0.30 ± 0.03 U g^{-1}(f.m.) for NC89 at 30 DAS, respectively. Thereafter the enzyme activity decreased to 10.14 % of the highest value for ZY90, which corresponds to 71.29 % for NC89. GDH activity reached up to 0.26 ± 0.02 U g^{-1}(f.m.) for ZY90 and up to 0.21 ± 0.02 U g^{-1}(f.m.) for NC89 at 50 DAS before a decrease.

GS activity negatively correlated ($P \leq 0.01$) with χ_s for ZY90 but did not correlate with χ_s for NC89 (Table 1). For both phenotypes GS activity showed no correlation with content of $[NH_4^+]_{apo}$, leaf tissue NH_4^+, total foliar N and soluble proteins.

It was reported that increased plant N content may influence ammonia volatilization and high N content seems to result in higher NH_3 emission, particularly during senescence (Parton *et al.* 1988). There was actually a positive correlation between χ_s and total nitrogen content for each phenotype in this study. However, ZY90 had larger χ_s despite of its lower foliar N content between 40 and 60 DAS and finally more relative reduction of total leaf N as compared to NC89 (Figs. 2, 3*B*). This discrepancy confirmed that the rate of senescence and the remobilization of leaf N are related to the N content of the plant (Masclaux *et al.* 2000), because senescence is important for the recycling of nitrogen and other nutrients, and according to a recent report, the N remobilization rate correlates with leaf senescence severity (Agüera and Cabello 2010).

The NH_3 compensation point was primarily regulated by GS activity in the senescing leaves, which declined considerably steeper in ZY90 compared with NC89 (Fig. 3*D*). GS has been reported to be a key enzyme involved in the assimilation of ammonia and very important for controlling the NH_4^+ content in plant tissues

(Schjoerring *et al.* 2002) and even the flux of NH_3 between vegetation and the atmosphere (Husted and Schjoerring 1995). Our results suggest that the decline in GS activity resulted in an increase in leaf tissue NH_4^+ content and χ_s value for each phenotype (Fig. 2 and 3*A*). The function of GDH in higher plants is still controversial. GDH may be expected to function in the deaminating direction in tobacco tissues (Masclaux-Daubresse *et al.* 2006, Skopelitis *et al.* 2007). This proposition can explain the difference in χ_s values of the two tobacco senescence phenotypes.

Table 1. Correlation between parameters of the two tobacco senescence phenotypes (* - $P \leq 0.05$, ** - $P \leq 0.01$).

Phenotype	Parameter	GS activity	Apoplastic NH_4^+	Leaf tissue NH_4^+	Total N	Soluble protein
ZY90	apoplastic NH_4^+	-0.46	-	-0.16	0.39	0.46
	χ_s	-0.60**	0.87**	0.20	0.48*	0.40
	leaf tissue NH_4^+	0.08	-0.16	-	0.77**	0.82**
	GS activity	-	-0.46	0.08	0.21	0.22
NC89	apoplastic NH_4^+	-0.22	-	-0.04	-0.02	-0.15
	χ_s	0.29	0.57*	0.50*	0.65**	0.41
	leaf tissue NH_4^+	-0.06	-0.04	-	0.83**	0.92**
	GS activity	-	-0.22	-0.06	0.33	0.42

References

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

Atkins, P.W. (ed.): Physical Chemistry (4th Ed.). - Oxford University Press, Oxford 1990.

Cosgrove, D.J., Cleland, R.E.: Solutes in the free space of growing tissues. - Plant Physiol. **72**: 326-331, 1983.

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., 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.

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.

Masclaux, C., Valadier, M.H., Brugière, N., Morot-Gaudry, J.F., Hirel, B.: Characterization of the sink/source transition in tobacco (*Nicotiana tabacum* L.) shoots in relation to nitrogen management and leaf senescence. - Planta **211**: 510-518, 2000.

Masclaux-Daubresse, D., Reisdorf-Cren, M., Pageau, K., Lelandais, M., Grandjean, O., Kronenberger, J., Valadier, M., Feraud, M., Jouglet, T., Suzuki A.: Glutamine synthetase-glutamate synthase pathway and glutamate dehydrogenase play distinct roles in the sink-source nitrogen cycle in tobacco. - Plant Physiol. **140**: 444-456, 2006.

Mattsson, M., Häusler, R.E., Leegood, R.C., Lea, P., Schjoerring, J.K.: Leaf-atmosphere ammonia exchange in barley mutants with reduced activities of glutamine synthetase. - Plant Physiol. **114**: 1307-1312, 1997.

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

Parton, W.J., Morgan, J.A., Altenhofen, J.M., Harper, L.A.: Ammonia volatilization from spring wheat plants. - Agron. J. **80**: 419-425, 1988.

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., 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.

Schjoerring, J.K., Mattsson, M., Husted, S.: Physiological parameters controlling plant-atmosphere ammonia exchange. - Atmos. Environ. **32**: 491-498, 1998.

Skopelitis, D.S., Paranychianakis, N.V., Kouvarakis, A., Spyros, A., Stephanou, E.G., Roubelakis-Angelakis, K.A.: The isoenzyme 7 of tobacco NAD(H)-dependent glutamate dehydrogenase exhibits high deaminating and low aminating activities *in vivo*. - Plant Physiol. **145**: 1726-1734, 2007.

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

Speer, M., Kaiser, W.M.: Ion relations of symplastic and apoplastic space in leaves from *Spinacia oleracea* L. and *Pisum sativum* L. under salinity. - Plant Physiol. **97**: 990-997, 1991.

Sutton, M.A., Asman, W.A.H., Schjoerring, J.K.: Dry deposition of reduced N. - Tellus **46B**: 255-273, 1994.

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.