INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
12–639/MFA/2012/14–6–1009–1013
http://www.fspublishers.org

Full Length Article

High Nitrogen Rate Inhibits Proteolysis and Decreases the Export of Leaf Pre-stored Proteins to Grains in Wheat (*Triticum aestivum*)

LINGAN KONG[1], FAHONG WANG‡, RONGTING ZHANG†, BO FENG, JISHENG SI, SHENGDONG LI AND BIN ZHANG
Crop Research Institute, Shandong, Academy of Agricultural Sciences, 202 Gongyebei Road, Jinan 250100, China
†Jinan Academy of Agricultural Sciences, Jinan 250316, China
‡Shandong Provincial Key Laboratory of Crop Genetic Improvement, Ecology and Physiology, Jinan 250100, China
[1]Corresponding author's e-mail: kongling-an@163.com

ABSTRACT

Pre-anthesis-synthesized proteins stored in the vegetative organs of cereal crops are re-mobilized and transferred to the grains. In this study, winter wheat (*Triticum aestivum* L.) was grown at levels of 225 kg N ha^{-1} (normal) and 330 kg N ha^{-1} (high) to investigate the effects of excessive N rates on the N re-mobilization from vegetative organs to the developing grains and on the grain protein yield. At a high N dose, the activities of the major flag leaf proteases were greatly decreased ~8-24 days after anthesis. From the beginning of the grain filling to the maturity, the post-anthesis flag leaf protein contents reduced by 80.50% and 70.40% at normal and high N rates, respectively ($P < 0.05$). The wheat flag leaves grown under high N conditions had a higher residual N at maturity than those grown under normal N conditions. Thus, up to maturity, the flag leaf N translocation efficiency was 67.4% in wheat exposed to a high N rate, significantly lower than the 73.8% of the plants exposed to a normal N rate ($P < 0.05$). Consequently, the grain protein content was not significantly increased with high N fertilization. In conclusion, the application of excessive N inhibits the proteolysis and decreases the export of flag leaf-stored protein to the developing grains and therefore, may not improve the protein yield in wheat.

Key Words: Chlorophyll fluorescence; Leaf senescence; Nitrogen re-mobilization; Proteases; Protein yield

INTRODUCTION

Proteins stored in the vegetative parts of wheat (*Triticum aestivum* L.) undergo hydrolysis and the resulting amino acids are largely translocated to the grains; these processes are accompanied by gradual plant senescence (Yang *et al*., 2004; Parrott *et al*., 2010). Approximately 60%-95% of the wheat grain N is derived from the re-mobilization of the N stored in the vegetative organs before anthesis, and, consequently, a smaller proportion is taken up after anthesis (Kichey *et al*., 2007; Palta *et al*., 2007; Chen *et al*., 2011). The process of protein re-mobilization is highly organized and well regulated and occurs via the hydrolysis of the leaf proteins by proteolytic enzymes, with the resulting compounds being transported to the developing grains (Andersson & Johansson, 2006). However, the mechanisms by which the proteolysis and amino acid export are regulated remain unclear (Barneix, 2007).

A variety of factors, including temperature, illumination, nutrient availability, hormonal balance, and source-sink relationships for nutrients and assimilates, modifies leaf senescence and assimilate re-mobilization (Izumi *et al*., 2010; Chen *et al*., 2011). It has been shown that N re-mobilization commences later when maize (*Zea mays* L.) plants are subjected to a high N regime compared to low N fertilization (Uhart & Andrade, 1995). In wheat, numerous studies have reported that a high dose of N fertilizer favors post-anthesis N uptake and often delays senescence and reduces pre-anthesis N re-mobilization and, hence, leads to slow grain filling and a low harvest index (Yang *et al*., 2002; Yang & Zhang, 2006; Barneix, 2007). Nevertheless, detailed information on the differences in N re-mobilization due to various N input levels has not been well documented for wheat.

Because proteolytic enzymes are critical for many plant physiological and developmental possesses involved in protein mobilization, such as in seed germination and grain filling (Martínez *et al*., 2008; Jinka *et al*., 2009; Parrott *et al*., 2010), we investigated the effects of an excessive N rate on the activities of the major leaf proteases that are involved in leaf proteolysis, aiming to analyze the importance of N application regimes for post-anthesis N re-mobilization to the developing grains and to improve our understanding of N-use efficiency during grain filling in wheat.

To cite this paper: Kong, L., F. Wang, R. Zhang, B. Feng, J. SI, S. Li and B. Zhang, 2012. High nitrogen rate inhibits proteolysis and decreases the export of leaf pre-stored proteins to grains in wheat (*Triticum aestivum*). *Int. J. Agric. Biol*., 14: 1009–1013

MATERIALS AND METHODS

Experimental site description and plant material: Winter wheat (*Triticum aestivum* L.) of cultivar Jimai 22, which is currently used in local production, was sown on October 7, 2010, at an experimental station (36°42' N, 117°4' E; elevation 48 m) at the Shandong Academy of Agricultural Sciences, China. The conditions in this region are semi-arid, with approximately 165 mm of long-term average total precipitation during the wheat-growing season and high temperature stress often occurring during the late grain-filling stages. The temperature information during the grain-filling stage of the experimental year was collected using the Zig Bee System and is given in Fig. 1.

The soil type of the experimental site was classified as sandy loam (pH 6.6). The top 40 cm of soil contains 0.98% organic matter, 75.6 mg kg^{-1} of water-hydrolyzable N, 38.0 mg kg^{-1} of rapidly available phosphorous, and 87.3 mg kg^{-1} of rapidly available potassium. Prior to tillage, 120 kg P_2O_5 ha^{-1} and 112.5 kg K_2O ha^{-1} were applied to all of the plots, with maize (the previous crop) residue retention. The topsoil (16 cm) was tilled twice using a rotary tiller. The plots were 4 m wide and 50 m long. Wheat was sown at 135 kg ha^{-1}, with 20-cm spacing. For the N treatment, a total of 225 or 330 kg N ha^{-1} (as urea) was applied at planting and Zadoks growth stages 31 and 41 (75 or 110 kg N ha^{-1} each stage). The topdressing of N fertilizer was surface supplied at 20 cm spacing and immediately incorporated with irrigation. These treatments were arranged in a randomized complete block design with four replications. The application of 330 kg N ha^{-1} is considered an excessive amount because, at this rate in field practice, the wheat grain yield cannot be increased relative to the normal rate of 225 kg N ha^{-1}.

Protease extract and activity assays: Approximately 0.5 g (FW) of flag leaves was ground to a fine powder in liquid nitrogen using mortar and pestle; the powder homogenized by additional grinding using a mortar and pestle in 5 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 1% (w/v) insoluble polyvinylpolypyrrolidone, 2 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.05% (v/v) Triton-X 100. These mixtures were centrifuged at 12,000 × g for 20 min at 4°C, and the supernatants were used for the protease activity assays. The samples were collected at 0, 8, 16, 24 and 32 days after anthesis (DAA).

The aminopeptidase activities were assayed using 2 mM L-leu-*p*-nitroanilide (as the substrate) in 100 mM PBS buffer (pH 7.0) containing 1% (v/v) DMSO, as described by Yang *et al*. (2004). The carboxypeptidase activity assay was performed using 2 mM *N*-carbobenzoxy-L-phe-L-ala in 100 mM Na-acetate buffer (pH 5.0), as described by Yang *et al*. (2004). The endopeptidase activity was measured as described by Jinka *et al*. (2009).

Nitrogen analyses and protein content: The flag leaves or grains were collected at 0, 8, 16, 24 and 32 DAA and oven dried at 60°C to a constant weight. The total N content was measured using an automatic N analyzer (BUCHI AutoKjeldahl Unit K-370, BUCHI Laboratory Equipment, Flawil, Switzerland), and a conversion factor of 6.25 was used to determine the protein content.

Grain yield and protein yield: For the grain weight, the wheat spikes were harvested at random (1-m^2) in each plot that was treated with 225 or 330 kg N ha^{-1} at 0, 8, 16, 24 and 32 DAA. The harvested spikes were then air dried and manually threshed. The grains were weighed to calculate the yield per square meter. The grain protein yield was calculated by multiplying the grain protein content by the grain yield and was expressed as g m^{-2}.

Chlorophyll fluorescence measurement and imaging: Flag leaves were selected at random at 0, 16 and 32 DAA for the measurements of the maximum PSII quantum yield (F_v/F_m). These leaves were darkened for 20-30 min and then used to measure the chlorophyll fluorescence induction kinetics at the red chlorophyll fluorescence band (ca. 690 nm) using a kinetic imaging fluorometer (FluorCam, Photon System Instruments Ltd., Brno, Czech Republic) (Nedbal *et al*., 2000; Jin *et al*., 2011). The duration for the F_0 measurement was 5.04 s. After measuring the minimum fluorescence in the dark-adapted state (F_0), the samples were illuminated with a saturating pulse (1,500 μE m^2 s^{-1}) to determine the maximal fluorescence in the dark-adapted state (F_m). The chlorophyll fluorescence emission transients were captured using a CCD camera at a resolution of 512 × 512 pixels. Numerical analyses of the classical physiological parameters were performed on the maximum PSII quantum yield $F_v/F_m = (F_m - F_0)/F_m$ using more than ten replicates.

Statistical analysis: The data were subjected to an ANOVA using the DPS statistical software (v.7.55, Refine Information Tech. Co., Ltd., Hangzhou, Zhejiang, China). The mean values are presented with their standard errors.

RESULTS

Flag leaf protease activity: The activities of aminopeptidases (Fig. 2a), carboxypeptidases (Fig. 2b) and endopeptidases (Fig. 2c) showed similar trends of temporal changes: rising as grain filling progressed, reaching a maximum approximately 16 DAA, and gradually decreasing thereafter. From 8 to 24 DAA, the flag leaf protease activities were significantly lower in the wheat supplied with 330 kg N ha^{-1} than the plants supplied with 225 kg N ha^{-1}. These data suggest that excessive N application reduces the activities of flag leaf proteases.

Leaf protein content: The wheat supplied with 330 kg N ha^{-1} developed higher leaf protein content than the plants exposed to 225 kg N ha^{-1} during the grain-filling period, but a significant difference was observed only at 16 DAA and thereafter. From the beginning of grain filling to the maturity, the flag leaf protein content decreased by 80.50% in the wheat supplied with 225 kg N ha^{-1} but decreased by 70.40% in the plants exposed to 330 kg N ha^{-1} ($P < 0.05$). These data suggest that the transport of protein from the

leaves to grains is reduced in wheat treated with excessive N compared to that fertilized with a normal N level (Fig. 3).

Total leaf N content: At 0 DAA, the treatment with 330 kg N ha^{-1} resulted in only a slightly higher leaf N content than the treatment with 225 kg N ha^{-1}, however the difference in the leaf residual N was significant up to 32 DAA ($P < 0.05$) (Table I). As a result, the translocation percentage for the wheat exposed to excessive N was significantly lower than that exposed to the normal N treatment ($P < 0.05$). The variation in the translocation efficiency was coincident with the difference in the leaf protein content between the N treatments. These data indicate that the flag leaf N transfer rate in the wheat under excessive N conditions was significantly lower than that under normal N conditions.

Protein yield: No significant difference in the protein yield was observed between the wheat plants exposed to 225 or 330 kg N ha^{-1}, though the normal amount of N slightly enhanced the protein yield prior to 24 DAA compared with the excessive N dose (Table II).

Maximum PSII quantum yield: At the end of anthesis (0 DAA), the flag leaf F_v/F_m (maximum PSII quantum yield) values of the wheat plants exposed to 225 or 330 kg N ha^{-1} were 0.81±0.04 and 0.83±0.05, respectively. These values decreased to 0.75±0.04 and 0.77±0.04, respectively, at 16 DAA and to 0.55±0.03 and 0.58±0.03, respectively at 32 DAA. No significant differences in the values of the flag leaf F_v/F_m were observed between the two N treatments at any of the three stages (Fig. 4).

Table I: Effects of different N rates on the flag leaf N content and translocation efficiency

N rate (kg ha^{-1})	Total N content (% DW)		N translocation efficiency (%)
	0 DAA	32 DAA	
225	2.9±0.2a	0.8±0.5b	73.8±3.5a
330	3.1±0.2a	1.0±0.6a	67.4±3.2b

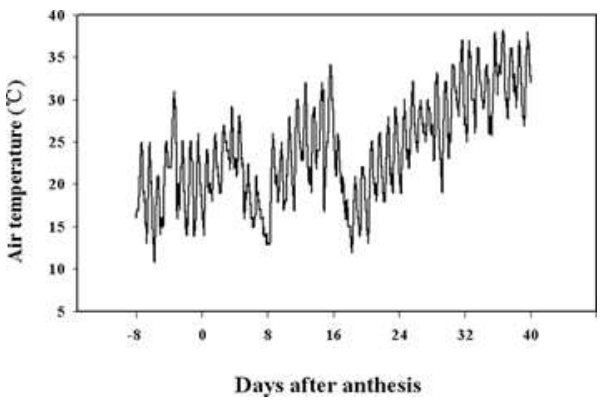
DAA = Days after anthesis
The means followed by different letters within a column are significantly different using a Tukey test ($P < 0.05$)
N translocation efficiency (%) = (N translocation/N content at anthesis) × 100

Table II: Protein yield (g m^{-1}) at two levels of N fertilization during grain filling

N rate (kg ha^{-1})	Days after anthesis				
	0	8	16	24	32
225	0	9.9±0.6a	48.6±3.9b	88.7±5.3c	113.3±6.7d
330	0	9.4±0.6a	46.8±3.2b	87.1±5.8c	115.8±5.8d

The means followed by same letters within a column are not significantly different using a Tukey test ($P < 0.05$)
The data were averaged from four replicates

Fig. 1: The hourly air temperature of the experimental station around the grain-filling stage of wheat



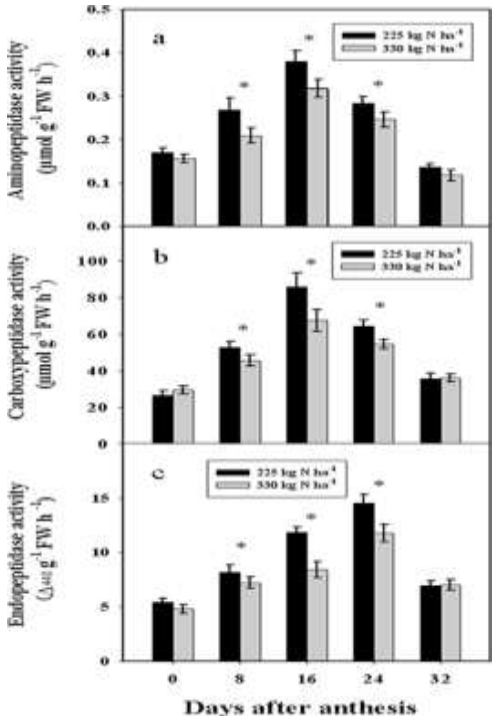
DISCUSSION

Most plant N in vegetative organs is stored as protein, a major potential component of crop grains (Ishida *et al*., 2008; Izumi *et al*., 2010). Cereal grain proteins originate from two principle N sources: post-anthesis and pre-anthesis absorbed N. It is well established that a larger fraction (60%-95%) of grain N is derived from vegetative N re-mobilization, a process regulated by the actions of proteases, such as aminopeptidases, carboxypeptidases, and endopeptidases (Yang *et al*., 2004; Martínez *et al*., 2008).

In this study, we found that excessive N (330 kg ha^{-1}) significantly restrained the activities of aminopeptidases, carboxypeptidases, and endopeptidases at the middle stages of grain filling. However, the mechanism of the actions of high N on the protease activity has remained unclear to date. In barley (*Hordeum vulgare* L.), Parrott *et al*. (2010) observed that the expression levels of protease genes and activities of proteolytic enzymes were strongly induced by lower N/C ratios, namely, relatively lower N conditions. Therefore, it is reasonable to postulate that a higher N content in wheat plants (Table I) exposed to a high amount of N fertilizer results in a higher N/C ratio compared to plants exposed to a normal N level, leading to a lower induction of proteolytic enzyme activity and subsequent lower N translocation efficiency.

Consequently, we observed substantial reductions in the N and protein contents of wheat plant parts after anthesis for both of the N treatments; however, a higher amount of proteins or total N remained in the leaves of the wheat plants after excessive N application compared with the normal N rate. Consequently, the translocation efficiency was lower in the wheat supplied with excessive N than the normal N rate. These data strongly indicate that plant N re-mobilization to the developing grains may be depressed by high N doses. In high-protein barley, earlier leaf proteolysis, as catalyzed by the action of proteases, enhanced the amino acid availability and promoted both N re-translocation to the developing grains and grain protein accumulation (Jukanti & Fischer, 2008). Therefore, we speculate that the appropriate activity of plant proteases regulated by the plant N regime may be critical for the degradation of stored protein and organic N re-mobilization to the developing grains.

Fig. 2: Effects of a high amount of N on the activities of flag leaf aminopeptidases (a), carboxypeptidases (b) and endopeptidases (c). The means and standard deviations of four replicates are shown for each data point. The error bars are one standard error of the mean. *significant at 0.05 probability levels



In many plants, it has been shown that a high N supply increases the cytokinins concentration and, therefore, delays proteolytic activity, protein degradation and plant senescence (Yang *et al*., 2002; Kiba *et al*., 2011); thus, the re-mobilization of released N to the seeds ultimately decreases the N harvest index (Barneix, 2007; Gregersen *et al*., 2008). The effects of a high N status were closely associated with increases in the active forms of cytokinins (Garnica *et al*., 2010) or with the promotion of the root-shoot translocation of active cytokinins (Sakakibara, 2006). However, whether cytokinins are directly involved in the excessive N-regulated repression of leaf proteases remains to be elucidated.

The leaf N is mainly located in the chloroplasts as ribulose-1-5 bis-phosphate carboxylase-oxygenase (EC: 4.1.1.39) (Rubisco) (Ishida *et al*., 2008; Martínez *et al*., 2008; Izumi *et al*., 2010), an essential enzyme in plant photosynthesis and accounting for more than 50% of leaf cell protein (Lawlor, 2002; Barneix, 2007; Jukanti & Fischer, 2008). It is reasonable to suggest that excessive N may promote leaf photosynthetic capacity, which, in combination with a slower plant senescence and longer duration of grain filling, may favor the crop yield. However, we did not observe significant increases in grain yields (0.786 & 0.794 kg m^{-2} for applications of 225 & 330 kg N ha^{-1}, respectively) or leaf photosynthesis (Fig. 4) in the

Fig. 3: Comparison of the flag leaf protein content in wheat plants exposed to 225 and 330 kg N ha^{-1} during grain filling. The means and standard deviations of four replicates are shown for each data point. The bar is one standard deviation of the mean. *and **significant at 0.05 and 0.01 probability levels, respectively

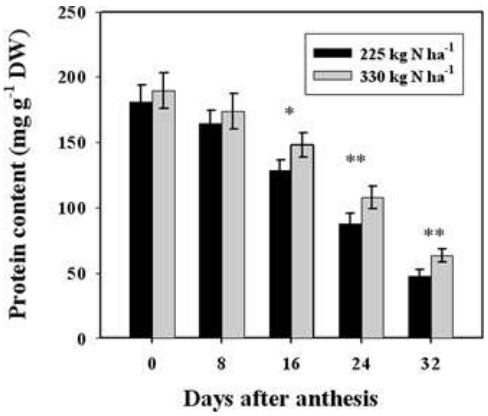
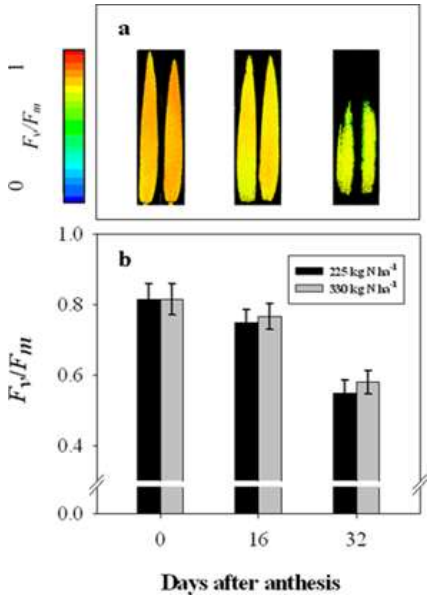


Fig. 4: Comparison of the flag leaf F_v/F_m values between wheat plants exposed to 225 and 330 kg N ha^{-1} at different stages. (a) Representative pseudocolor images for F_v/F_m at 0, 16, and 32 DAA, vertically corresponding to that shown in (b); (b) the chlorophyll fluorescence parameters F_v/F_m in flag leaves. Each data point in (b) represents the average and the standard deviation of at least 10 individually selected and analyzed leaves



wheat supplied with 330 kg N ha^{-1} compared to 225 kg N ha^{-1}. One explanation may be that the Rubisco levels are more abundant than the requirements for photosynthesis (Lawlor, 2002); hence, a small degree of protein degradation

at the early stages of grain filling cannot significantly affect leaf photosynthesis. Secondly, a delayed onset of senescence often reduces the grain-filling rate, and, thus, more assimilates may be left in the straw (Gregersen *et al*., 2008; Chen *et al*., 2011). Thirdly, at the late stages of grain filling, the air temperature is too high for wheat photosynthesis, therefore eliminating the photosynthetic differences between the N levels. Moreover, the temperature greater than 35°C frequently occurring at the late stages of grain filling in this region (Fig. 1) often causes a sudden plant death and thus, grain filling ceases, even for the plants that remain green.

In summary, the data presented here suggest that an excessive N rate during the grain-filling stage inhibits the activities of flag leaf proteases, decreases N export from the flag leaves to the grains, and therefore, reduces the applied N economy.

Acknowledgement: Funding for this research was provided by grants from the Shandong and National Earmarked Fund for Modern Agro-industry Technology Research System (CARS-3-1-21) and from Special Fund for Agro-scientific Research on Public Causes, Minister of Agriculture, China (201303109-7, 201203079, 200903010).

REFERENCES

Andersson, A. and E. Johansson, 2006. Nitrogen partitioning in entire plants of different spring wheat cultivars. *J. Agron. Crop Sci.,* 192: 121–131

Barneix, A.J., 2007. Physiology and biochemistry of source-regulated protein accumulation in the wheat grain. *J. Plant Physiol.,* 164: 581–590

Chen, C.C., G.Q. Han, H.Q. He and M. Westcott, 2011. Yield, protein, and remobilization of water soluble carbohydrate and nitrogen of three spring wheat cultivars as influenced by nitrogen input. *Agron. J.,* 103: 786–795

Garnica, M., F. Houdusse, A.M. Zamarreño and J.M. Garcia-Mina, 2010. The signal effect of nitrate supply enhances active forms of cytokinins and indole acetic content and reduces abscisic acid in wheat plants grown with ammonium. *J. Plant Physiol.,* 167: 1264–1272

Gregersen, P.L., P.B. Holm and K. Krupinska, 2008. Leaf senescence and nutrient remobilisation in barley and wheat. *Plant Biol.,* 10: 37–49

Ishida, H., K. Yoshimoto, M. Izumi, D. Reisen, Y. Yano, A. Makino, Y. Ohsumi, M.R. Hanson and T. Mae, 2008. Mobilization of Rubisco and stroma-localized fluorescent proteins of chloroplasts to the vacuole by an ATG gene-dependent autophagic process. *Plant Physiol.,* 148: 142–155

Izumi, M., S. Wada, A. Makino and H. Ishida, 2010. The autophagic degradation of chloroplasts via rubisco-containing bodies is specifically linked to leaf carbon status but not nitrogen status in Arabidopsis. *Plant Physiol.,* 154: 1196–1209

Jin, Z., T. Tian, M.S. Naeem, G. Jilani, F. Zhang and W. Zhou, 2011. Chlorophyll fluorescence responses to application of new herbicide ZJ0273 in winter oilseed rape species. *Int. J. Agric. Biol.*, 13: 43–50

Jinka, R., V. Ramakrishna, S.K. Rao and R.P. Rao, 2009. Purification and characterization of cysteine protease from germinating cotyledons of horse gram. *BMC Biochem.,* 10: 28

Jukanti, A.K. and A.M. Fischer, 2008. A high-grain protein content locus on barley (*Hordeum vulgare*) chromosome 6 is associated with increased flag leaf proteolysis and nitrogen remobilization. *Physiol. Plant.,* 132: 426–439

Kiba, T., T. Kudo, M. Kojima and H. Sakakibara, 2011. Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. *J. Exp. Bot.,* 62: 1399–1409

Kichey, T., B. Hirel, E. Heumez, F. Dubois and J. Le Gouis, 2007. In winter wheat (*Triticum aestivum* L.), post-anthesis nitrogen uptake and remobilisation to the grain correlates with agronomic traits and nitrogen physiological markers. *Field Crops Res.,* 102: 22–32

Lawlor, D.W., 2002. Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. *J. Exp. Bot.,* 370: 773–787

Martínez, D.E., M.L. Costa, F.M. Gomez, M.S. Otegui and J.J. Guiamet, 2008. 'Senescence-associated vacuoles' are involved in the degradation of chloroplast proteins in tobacco leaves. *Plant J.,* 56: 196–206

Nedbal, L., J. Soukupová, D. Kaftan, J. Whitmarsh and M. Trtílek, 2000. Kinetic imaging of chlorophyll fluorescence using modulated light. *Photosynth. Res.,* 66: 3–12

Palta, J.A., I.R.P. Fillery and G.J. Rebetzke, 2007. Restricted-tillering wheat does not lead to greater investment in roots and early nitrogen uptake. *Field Crops Res.,* 104: 52–59

Parrott, D.L., J.M. Martin and A.M. Fischer, 2010. Analysis of barley (*Hordeum vulgare*) leaf senescence and protease gene expression: a family C1A cysteine protease is specifically induced under conditions characterized by high carbohydrate, but low to moderate nitrogen levels. *New Phytol.,* 187: 313–331

Sakakibara, H., 2006. Cytokinins: activity, biosynthesis, and translocation. *Annu. Rev. Plant Biol.,* 57: 431–449

Uhart, S.A. and F.H. Andrade, 1995. Nitrogen and carbon accumulation and remobilization during grain filling in maize under different source⁄sink ratios. *Crop Sci.,* 35: 183–190

Yang, J.C. and J.H. Zhang, 2006. Grain filling of cereals under soil drying. *New Phytol.,* 169: 223–236

Yang, J.C., J.H. Zhang, Z.Q. Wang, Q.S. Zhu and L.J. Liu, 2002. Abscisic acid and cytokinins in the root exudates and leaves and their relationship to senescence and remobilization of carbon reserves in rice subjected to water stress during grain filling. *Planta,* 215: 645–652

Yang, L.T., S. Mickelson, D. See, T.K. Blake and A.M. Fischer, 2004. Genetic analysis of the function of major leaf proteases in barley (*Hordeum vulgare* L.) nitrogen remobilization. *J. Exp. Bot.,* 55: 2607–2616

(Received 08 August 2012; Accepted 18 September 2012)