Plant Production Science

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/tpps20

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To cite this article: Hideki Araki, Hirotaka Ueno, Keisuke Mizuta, Tadashi Takahashi, Masaru Shibata & Jun'ichi Mano (06 Feb 2025): Timing of nitrogen fertilization and shading affect the transition of nitrogen metabolism in senescing leaves of ripening wheat (*Triticum aestivum* L.), Plant Production Science, DOI: 10.1080/1343943X.2025.2461776

To link to this article: https://doi.org/10.1080/1343943X.2025.2461776

Published online: 06 Feb 2025.

PLANT PRODUCTION SCIENCE
https://doi.org/10.1080/1343943X.2025.2461776

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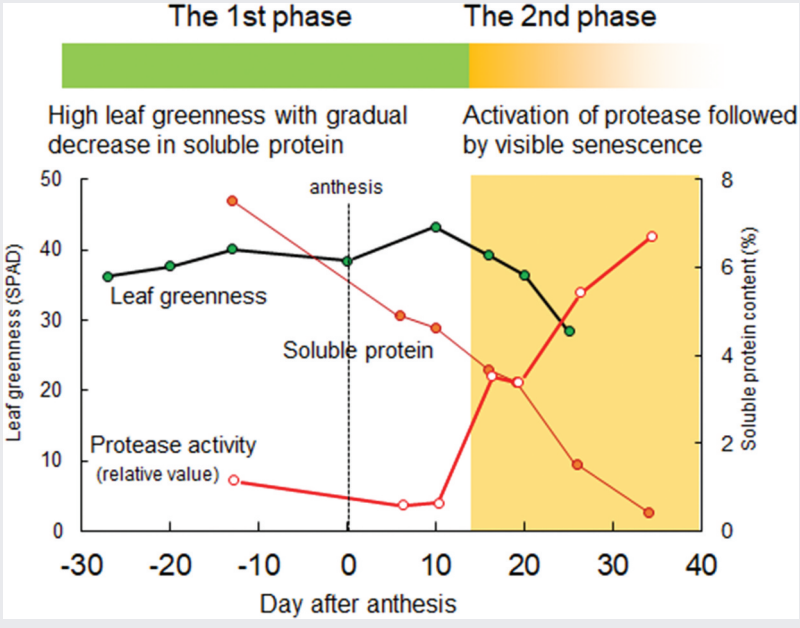
Timing of nitrogen fertilization and shading affect the transition of nitrogen metabolism in senescing leaves of ripening wheat (*Triticum aestivum* L.)

Hideki Araki[a], Hirotaka Ueno[a], Keisuke Mizuta[b], Tadashi Takahashi[a], Masaru Shibata[c] and Jun'ichi Mano[d]

[a]Graduate School of Sciences and Technology for Innovation, Yamaguchi University, Yamaguchi, Japan; [b]Faculty of Agriculture, Kagawa University, Miki, Japan; [c]Faculty of Education, Yamaguchi University, Yamaguchi, Japan; [d]Science Research Center, Yamaguchi University, Yamaguchi, Japan

ABSTRACT

The grain yield and protein content of wheat is influenced by the transition of nitrogen metabolism in senescing leaves from pre-anthesis to maturity. To characterize the biochemical processes of the transition, we here analyzed the interactions among greenness, soluble protein, amino acid and protease activity in the flag and second leaves of ripening wheat plants. Nitrogen regimes were adjusted by intensive nitrogen applications in the early (IN (E)) or late stages (IN (L)) along with or without top-dressing at anthesis (±TD). The progress of leaf nitrogen metabolism towards terminal senescence appeared to be split into two phases: the first phase (from pre-anthesis to about 15 days after anthesis (DAA), when leaf greenness did not change appreciably) was characterized by a decrease in soluble protein, and the second phase (after 15 DAA) was characterized by drastic increases in the protease activity followed by a rapid decrease in leaf greenness. The decrease in soluble proteins during the first phase was not accompanied by a rise in protease activity. The rise in protease activity during the second phase was most apparent in the IN (E)-TD plants that were likely under the most severe nitrogen starvation among the treatments. The IN (L) and TD were effective to defer the leaf senescence processes. The shading treatment delayed the rise in protease activity and decrease in leaf greenness. This study suggests that the terminal leaf senescence proceeds in two phases, and the transition between these was accelerated by low nitrogen and slowed by mild shading.



ARTICLE HISTORY
Received 28 August 2024
Revised 9 January 2025
Accepted 27 January 2025

KEYWORDS
Wheat; Leaf senescence; Nitrogen starvation; Protease; Amino acid; Soluble protein

CONTACT Hideki Araki ahide@yamaguchi-u.ac.jp

1. Introduction

Leaf senescence in wheat is physiologically important if the filling grains are to gain access to the minerals stored in the leaves and culms. On the other hand, too early leaf senescence can lead to yield reduction due to insufficient carbon assimilation for grain filling. Early leaf senescence can be triggered by abiotic stresses, including those caused by nitrogen deficit (Distelfeld et al., 2014), water deficit (Islam et al., 2021; Yang et al., 2001), waterlogging (Araki et al., 2012; Belford, 1981; Hossain et al., 2011) or high temperature (Chen et al., 2018). In western Japan, abnormally early leaf senescence (known locally as *kareure*) can occur in both wheat and barley and across several regions (Araki et al., 2018; Hossain et al., 2009). To obtain more consistent wheat yields year by year, early leaf senescence can be suppressed by genetic modifications (e.g. the 'stay green' modification as suggested by Kipp et al., 2014) and/or by modifications to the agronomic management of the crop.

The onset and rate of leaf senescence are often influenced by leaf nitrogen status (Distelfeld et al., 2014). It is thought that leaf senescence can involve the programmed cell death of mesophyll cells (Blankenship, 2015). This is usually assessed by measuring changes in leaf greenness; this being correlated with the quantity of photosynthetic pigments; and this with the level of nitrogen in the mesophyll, as most such pigments are bound with proteins to form 'pigment-protein complexes' (Blankenship, 2015). As leaf senescence progresses, these pigment protein complexes are decomposed into simpler molecules by proteases and by the chlorophyll degradation processes (Martínez et al., 2008; Vassileva et al., 2012). Most of the decomposed nitrogen molecules are then remobilized to the grains (Distelfeld et al., 2014). Degraded stromal proteins are also a source of nitrogen for remobilization of the grains (Crafts-Brandner et al., 1998; Mae et al., 1984). However, relatively few studies have investigated the degradation processes in the mature leaves of ripening wheat. The leaf degradation processes operating during grain ripening would be more complex than those in leaves, while the plant is substantially vegetative or in isolated (detached) leaves in the laboratory due to the rapid development of the spike and possibly to a declining rate of nitrogen uptake by the roots.

The timings and rates of nitrogen fertilizer applications influence the nitrogen status of leaves in wheat (Dick et al., 2016). In conventional managements of wheat cropping in western Japan, the amounts of nitrogen applied before sowing and at tillering account for more than 60% of the total nitrogen, with the aim of obtaining stronger vegetative growth and reducing the risk of lodging. However, recent field experiments in which most of the nitrogen was applied after panicle initiation or stem elongation have shown increased yield and quality (higher grain protein), and this is attributed to increased aboveground nitrogen accumulation (Mizuta et al., 2020, 2023). The delay in nitrogen application to the later growth stages probably improves leaf nitrogen status and defers the onset of senescence metabolism.

Light intensity may also affect the rate of senescence. Leaves senesce when they are exposed to lower levels of light or to longer dark periods (Ishida et al., 2008). However, it has often been observed that the leaves and spikes of lodged field-grown wheat and barley plants senesce more slowly than those of standing plants. This indicates that shading may also slow protein degradation. Nevertheless, the effects of light intensity on leaf nitrogen metabolism have not been fully elucidated in the field at crop level.

The objective of this study is to examine changes in leaf greenness, in association with levels of soluble protein and amino acid and protease activity in the flag and second (from top) leaves of wheat plants pre- and post-anthesis. In this study, the effects of nitrogen fertilizer application timings and mild post-anthesis shading treatments were examined. The positive effects of more intense fertilizer applications after panicle initiation on yield and aboveground nitrogen accumulation (Mizuta et al., 2020) are also discussed from the perspective of nitrogen metabolism and delayed leaf senescence.

2. Materials and methods

2.1. Plant materials

Samples were taken from wheat plants (*Triticum aestivum* L. cv. 'Setokirara') grown in the experimental fields of the Faculty of Agriculture, Yamaguchi University (N34° 8′5″, E131°28′5″) in 2017/18, 2018/19 and 2019/20. As the wheat was cultivated on upland fields temporarily converted from rice paddy fields under a double-cropping system, shallow furrows were formed at 1.5 m intervals to improve drainage. The soil excavated from the furrows was redistributed so as to slightly-elevate the central area forming a low ridge about 1.2 m wide. Seeds were sown at a rate of 6 g m^{-2} on the ridges in four rows about 0.3 m apart on 14 November 2017, 13 November 2018 and 12 November 2019. In each growing season, P_2O_5 and K_2O were applied at sowing at 10 g m^{-2}. A mineral fertilizer, containing 35–38% lime, 2–4% MgO, 13–18% FeO, 2–4% MnO, 1.2–2% P_2O_5,

0.1–0.5% K_2O and a trace of MoO_3 was applied at 100 g m^{-2}.

As the main treatment (the main plots), two different fertilizer timings were employed in 2017/18 and 2018/19. These will be referred to as Early-stage Intensive Nitrogen application (IN (E)) and Late-stage Intensive Nitrogen application (IN (L)). Two subplots were also established with top-dressing at anthesis (+TD) or without top-dressing at anthesis (–TD). These treatments were arranged in a split-plot design with three replications in 2017/18 and four replications in 2018/19. In treatment IN (E) in 2017/18, nitrogen was applied at a rate of 6 g m^{-2} just before sowing, at 3 g m^{-2} at GS13 (GS indicates the Zadoks growth stage (Zadoks et al., 1974)) and at 4 g m^{-2} at GS30. In 2018/19, nitrogen was applied at 5 g m^{-2} at sowing, at 3 g m^{-2} at GS13 and at 3 g m^{-2} at GS30. In treatment IN (L) in 2017/18, nitrogen was applied at a rate of 3 g m^{-2} at GS13, at 6 g m^{-2} at GS30 and at 4 g m^{-2} at GS37. In 2018/19, nitrogen was applied at 2 g m^{-2} at GS13, at 6 g m^{-2} at GS30 and at 3 g m^{-2} at GS37. For TD, 6 g m^{-2} of nitrogen was applied at anthesis. All nitrogen was applied as urea.

In 2019/20 shading was imposed from day 1 to day 28 after anthesis (DAA). The shading treatment was applied to four plots, selected at random. For this, a non-woven fabric (that intercepts 22% of normal sunlight) was suspended over an area of approximately 1.0 × 1.5 m. The nitrogen was applied at a rate of 4 g m^{-2} at sowing, at 2 g m^{-2} at GS14 and at 3 g m^{-2} at G31.

2.2. Measurements

In 2018/19 and 2019/20, leaf greenness was measured with a chlorophyll meter for 10 flag and 10 second (from top) leaves randomly selected

Protease activity, soluble protein content and amino acid content were determined for the topmost fully developed leaf, before the flag leaf was fully expanded or for the flag and the second leaf after the flag leaf was fully expanded. The samples were collected from the flag leaves in 2017/18, and from both the flags and the second leaves in 2018/19 and 2019/20. In 2017/18, nine leaves were collected per plot and placed in 5 ml polyethylene (PE) tubes. The tubes containing the leaves were quickly frozen in liquid nitrogen. In 2018/19 and 2019/20, 15 leaves per plot were collected. The leaves were placed in PE bags and quickly frozen in liquid nitrogen. The frozen PE tubes and bags were stored at –90°C pending assay.

Protease activity was assessed by fluorescence intensity after the leaf extract reacted with FITC-casein. To make the leaf extract, 1.2 g of frozen leaf fragments were weighed and ground in a mortar cooled by liquid nitrogen. The ground leaves were mixed with 4.8 g of phosphate buffer (20 mM, pH7.5), containing 1% (w/v) polyvinylpyrrolidone and 0.1% (v/v) β-mercaptoethanol (Thoenen et al., 2007). The mixture was filtered through Miracloth (Calbiochem). The filtrate was centrifuged at 15,000 rpm for 10 min, and the supernatant was used as the leaf extract. The leaf extracts were mixed with 100 µl of FITC-casein and 100 µl of phosphate buffer containing 0.1% (v/v) β-mercaptoethanol and incubated at 37°C for 60 min. The incubated samples were reacted with 5% trichloroacetic acid and incubated at 37°C for 30 min. After the second incubation, the samples were centrifuged at 13,000 rpm for 5 min, and the supernatant was reacted with 1 M sodium bicarbonate for 5 min. Reacted samples were measured for fluorescence intensity (excitation wavelength 485 nm/fluorescence wavelength 535 nm). The protease activity is shown fluorescence intensity on a dry weight basis.

Leaf soluble protein content was determined by the Bradford method (Bradford, 1976) and used the same leaf extract samples as were used to assay protease activity.

Amino acid content was measured by fluorescence detection using high performance liquid chromatography (HPLC). The HPLC was configured as reversed-phase chromatography with a system controller (CBM-20A lite, Shimadzu Corporation), two pumping units (LC-20AT, Shimadzu Corporation), a column oven (CTO-10A, Shimadzu Corporation), a fluorescence spectroscopic detector (RF-10AXL, Shimadzu Corporation) and column (Develosil C30-UG-5, Nomura Chemical Co., Ltd.). The eluents were solution A, a mixture of tetrahydrofuran, methanol and 10 mm phosphate buffer in the ratio 1:8:91, v:v:v and solution B, a mixture of methanol and 10 mm phosphate buffer in the ratio 8:2, v:v. Amino acids were separated by changing the ratio of solution A to solution B during the measurement. To make the amino acid extract, approximately 50 mg of the frozen leaves were crushed with 800 µl of 10 mM phosphate buffer and 50 µl of 1.5 mM glycylglycine in a bead crusher (Shake man 6, Bio Medical Science Co., Ltd.). The crushed samples were centrifuged at 12,000 rpm for 10 min, and the supernatant was collected. The sediment was mixed with 400 µl of 10 mm phosphate buffer and centrifuged at 12,000 rpm for 10 min. The supernatants of the first and second centrifugal separations were combined to form the amino acid extract. The amino acid extract was mixed with 3-mercaptopropionic acid diluted to 0.1% (v:v) using 0.1 M borate buffer and o-phthalaldehyde diluted to 0.2% (w:w) using 0.1 M borate buffer at a ratio of 1:6:3 v:v:v and reacted for 1 min. The reacted samples were injected into the HPLC for measurement. The detected peaks were adjusted to 100% collection

using glycylglycine which showed a peak as the internal standard.

2.3. Weather data

The meteorological data in Yamaguchi City were downloaded from a website provided by the Japan Meteorological Agency. Mean temperatures were calculated from the daily means averaged over three 10-day periods (the first and second 10-day periods of each calendar month and residual days of the month). Cumulative precipitation was calculated by summing the daily values over the relevant periods. Averaged data was calculated from the records in 1991–2020.

2.4. Statistical analyses

In 2017/18 and 2018/19, the data were analyzed by t-test before anthesis and by analysis of variance (ANOVA) after anthesis. In 2019/20, the data were also analyzed by t-test. Since we found small fluctuation within the same treatments in the measurements on protease activity, soluble protein content and amino acid content, we randomly selected three samples out of four plots and the data were subjected to two-way ANOVA. The differences among treatments were analyzed by Tukey's test.

3. Results

Figure 1 indicates the weather during the first, second and third 10-day periods in the months when the wheat was growing in 2017/18, 2018/19 and 2019/20. In 2017/18, the daily mean temperatures from November to February were 0.5–3.6°C lower than the averages calculated for 1991–2020. The mean temperatures after March were at similar levels to the average data. The rainfall was generally low throughout the growth period except for during the first 10-day period of January, the first and second 10-day periods of March and the first 10-day period of May. In 2018/19, the daily mean temperatures were similar to the averaged data. The rainfall from December to the first 10 days of March was 316 mm and was 22% higher than the average (260 mm). After the second 10-day period of March, rainfall was similar to the average data or less than half of the average data except during the third 10-day period of April. In 2019/20, the daily mean temperature was generally higher than average till the end of March. Overall, the rainfall was lower than average throughout the growing season.

Figure 2 indicates leaf greenness of the flag and second leaves during the pre- and post-anthesis periods in 2018/19. Flag leaf greenness (Figure 2a) gradually increased from 27 days before anthesis to 13 days after anthesis (DAA) regardless of nitrogen application. IN (E)-TD shows an apparent drop in leaf greenness after 16 DAA. In contrast, in IN (E)+TD, leaf greenness remained high till 20 DAA, then began to drop to 39.0 at 25 DAA. In IN (L), leaf greenness was about 3–8 points higher than in IN (E) throughout the measurement period regardless of TD application; for example at 0 DAA, leaf greenness was 44.8 in IN (L) and 38.4 in IN (E). The change in leaf greenness after 16 DAA was similar between +TD and -TD indicating that, unlike IN (E)-TD, IN (L) retained a high level of leaf greenness for longer, even without TD.

Meanwhile, in the second leaf (Figure 2b), leaf greenness in IN (E) decreased slightly from 40.2 27

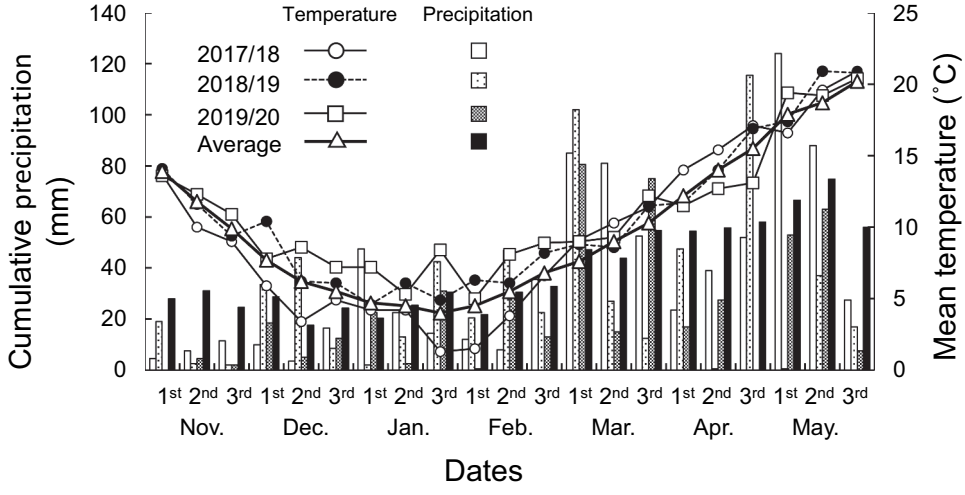


Figure 1. Precipitation and mean temperatures in 2017/18, 2018/19 and 2019/20. The data was calculated for the first and second 10-day periods in each calendar month and for the residual days of the month. The average data was calculated from records in 1991–2020.

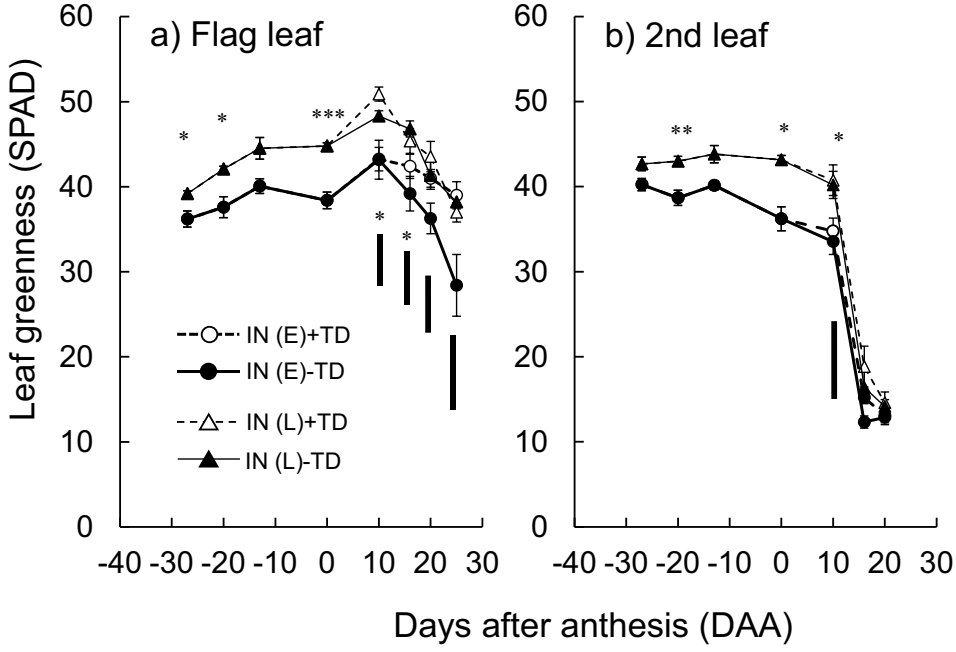


Figure 2. Change in leaf greenness of wheat plants intensively supplied with nitrogen in the early (IN (E)) and late stages (IN (L)) of growth with or without a nitrogen top-dressing at anthesis (TD) in 2018/19. *, ** and *** indicate significant differences between in (E) and in (L) at $p < 0.05$, 0.01 and 0.001, respectively. The bars on symbols indicate standard error. The vertical bars below symbols indicate honestly significant differences (HSD) at $p < 0.05$ by Tukey's test.

days before anthesis to 33.5 by 10 DAA. This was followed by a more rapid drop to 12.3 by 16 DAA. The TD application did not affect this significantly. In IN (L), although the values were about 3–6 points higher than in IN (E), the pattern of a rapid drop was the same as in IN (E).

Figure 3 shows the protease activity of the flag and second leaves pre- and post-anthesis in 2017/18 and 2018/19. In IN (E) of 2017/18 (Figure 3a), protease activity was low until 11 DAA followed by rapid increase in 23 DAA. The extent of increase was greater in IN (E)-TD than in IN (E)+TD, being 4287 mg^{-1} DW in IN (E)-TD and

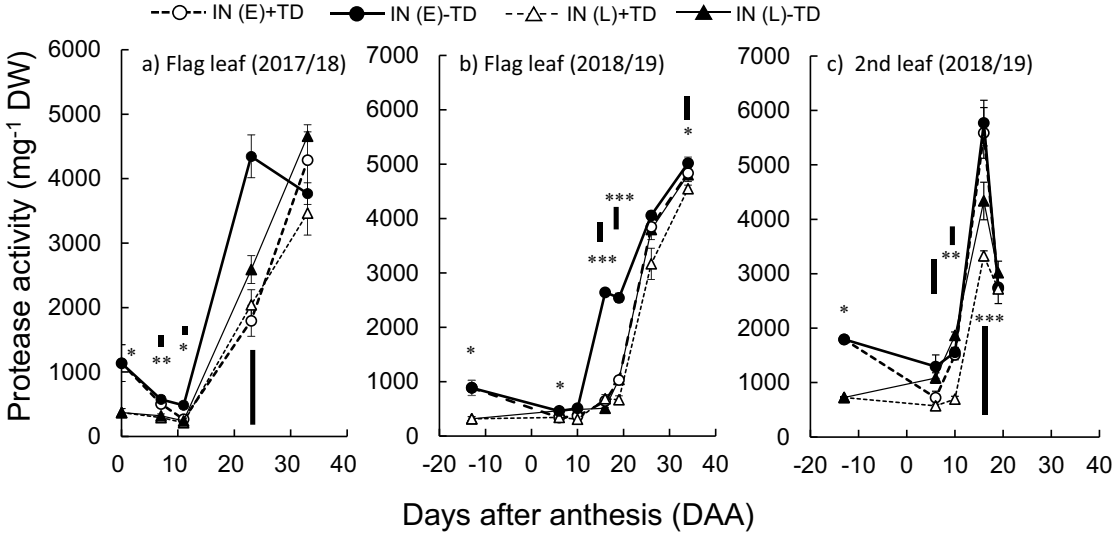


Figure 3. Change in protease activity of wheat plants intensively supplied with nitrogen in early (IN (E)) and late stages (IN (L)) of growth with or without a top-dressing of nitrogen at anthesis (TD) in 2017/18 and 2018/19. The protease activity is shown as fluorescence intensity per unit dry weight. *, ** and *** indicate significant differences between in (E) and in (L) at $p < 0.05$, 0.01 and 0.001, respectively. The bars on symbols indicate standard error. The vertical bars above or below symbols indicate honestly significant differences (HSD) at $p < 0.05$ by Tukey's test.

1796 mg^{-1} DW in IN (E)+TD. In IN (L), the protease activity was low until 11 DAA but increased on 23 DAA although the increase in IN (L)-TD was milder than that in IN (E)-TD. In 2018/19 (Figure 3b), changes in both IN (E) and IN (L) were about the same as in 2017/18. The short interval measurements in 2018/19 showed that protease activity except in IN (E)-TD increased slightly between 10 and 19 DAA and then increased strongly. The protease activity in IN (E)-TD increased at 16 DAA, earlier than in other treatments. From 6 DAA to 19 DAA, protease activity of IN (L)-TD was consistently lower than in IN (E)+TD even though IN (L)-TD was not applied with nitrogen at anthesis.

In the second leaf (Figure 3c), the protease activity 13 days before anthesis was significantly higher in IN (E) than in IN (L). Protease activity of IN (E)+TD and IN (L)+TD was lower than that of IN (E)-TD and IN (L)-TD at 6 DAA, indicating the effect of TD to restrict protease activity. However, the protease activity at 10 DAA in IN (E)+TD began to increase, while the activity in IN (L)+TD was still low. The protease activity rose exponentially at 16 DAA in all treatments. On 19 DAA, protease activity decreased in all treatments, probably due to partial tissue death (patch-wise senescence) where senesced areas were dry and protease activity was low.

Figure 4 indicates amino acid content of the flag and second leaves both pre- and post-anthesis in 2017/18 and 2018/19. In 2017/18 (Figure 4a), the amino acid content of flag leaf in IN (E)-TD ranged from 0.94% to 1.26% by 23 DAA, indicating the amino acid content was essentially unchanged before the rise in protease activity (Figure 3a). In IN (L), the amino acid contents post-anthesis were significantly higher than in IN (E). The TD application increased amino acid contents both in IN (E) and in IN (L). In 2018/19 (Figure 4b), the amino acid contents of IN (E)-TD were also constant until 16 DAA, as found in 2017/18. The amino acid content in IN (L)-TD and IN (L)+TD were significantly higher than in IN (E)-TD and IN (E)+TD, respectively, on 6 DAA and 10 DAA. For the second leaf, the amino acid content in IN (L) was already higher than in IN (E) at 13 days before anthesis (Figure 4c). The amino acid content in IN (E)-TD and IN (L)-TD was higher than that in IN (E)+TD and IN (L)+TD at 6 DAA and 10 DAA, and the content temporally increased at 10 DAA. In both flag and second leaves, the amino acid content did not drop before changes in leaf greenness became apparent (Figure 2) and the change in protease activity (Figure 3).

Figure 5 indicates the soluble protein content of the flag and second leaves pre- and post-anthesis. On 0 DAA in 2017/18 (Figure 5a), the soluble protein content in IN (E)-TD was 5.2%, almost 4.6 points lower than in IN (L)-TD. The soluble protein content reduced gradually to less than 1.0% on 34 DAA. The TD application raised the soluble protein content on 11 DAA in both IN (E)+TD and IN (L)+TD. In 2018/19 (Figure 5b), though the soluble protein content of IN (E) was identical to that in IN (L) at 13 days before anthesis, the content on 6 DAA in IN (E)-TD fell significantly to less than in IN (L)-TD. The content of IN (E)-TD and IN (L)-TD gradually fell to less than 0.5% on 34 DAA. The TD application kept the soluble protein content high until 19 DAA. The soluble protein content of IN (L)+TD was kept high compared with IN (E)+TD. The soluble protein content of the second leaf in IN (E) was already lower than in IN (L) at 13 days before anthesis (Figure 5c), unlike that in the flag leaves (Figure 5b). The reduction after anthesis and the effects of TD were similar to those in the flag leaves.

Figure 6 shows the effects of post-anthesis shading on leaf greenness, protease activity and soluble protein

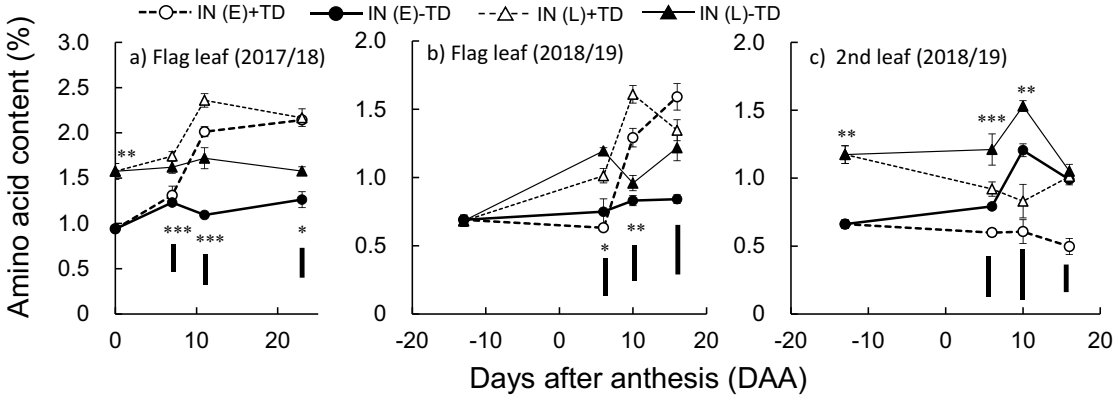


Figure 4. Change in amino acid content of wheat plants that were intensively supplied with nitrogen in the early (IN (E)) and late stages (IN (L)) of growth with or without a nitrogen top-dressing at anthesis (TD) in 2017/18 and 2018/19. The amino acid contents are the sum of each of the amino acids measured by HPLC and are shown on a leaf dry weight basis. *, ** and ***indicate significant differences between in (E) and in (L) at $p < 0.05$, 0.01 and 0.001, respectively. The bars on symbols indicate standard error. The vertical bars below symbols indicate honestly significant differences (HSD) at $p < 0.05$ by Tukey's test.

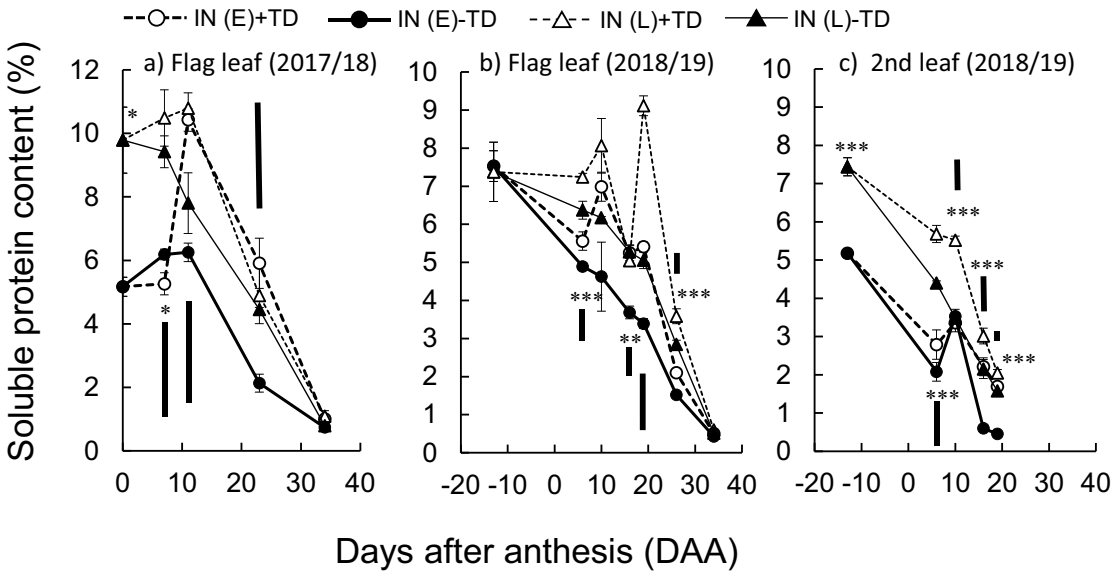


Figure 5. Change in soluble protein content of wheat plants that were intensively supplied with nitrogen in the early (IN (E)) and late stages (IN (L)) of growth with or without a nitrogen top-dressing at anthesis (TD) in 2017/18 and 2018/19. The soluble protein content is shown on a leaf dry weight basis. *, ** and *** indicate significant differences between in (E) and in (L) at $p < 0.05$, 0.01 and 0.001, respectively. The bars on symbols indicate standard error. The vertical bars above or below symbols indicate honestly significant differences (HSD) at $p < 0.05$ by Tukey's test.

content. The leaf greenness of unshaded (control) plants gradually decreased post-anthesis. In contrast, the leaf greenness of shaded plants was almost constant from pre-anthesis to 28 DAA. The protease activity was low up to 22 DAA but increased sharply in unshaded plants on 28 DAA although the increase was less in the shaded plants. The soluble protein content gradually decreased regardless of the light conditions until 22 DAA. However, the soluble protein content of shaded plants was significantly higher than in unshaded plants on 28 DAA.

4. Discussion

Our study examined the effects of modified fertilizer timings (IN (L)), which applied nitrogen intensively at the initiation of stem elongation and at flag leaf emergence, on nitrogen metabolism in the flag and second leaves both pre- and post-anthesis. The effects of nitrogen top-dressing at anthesis were also examined. It is confirmed that IN (L) helped maintain leaf greenness from 27 days before anthesis until approximately 20 DAA, compared with conventional fertilizer

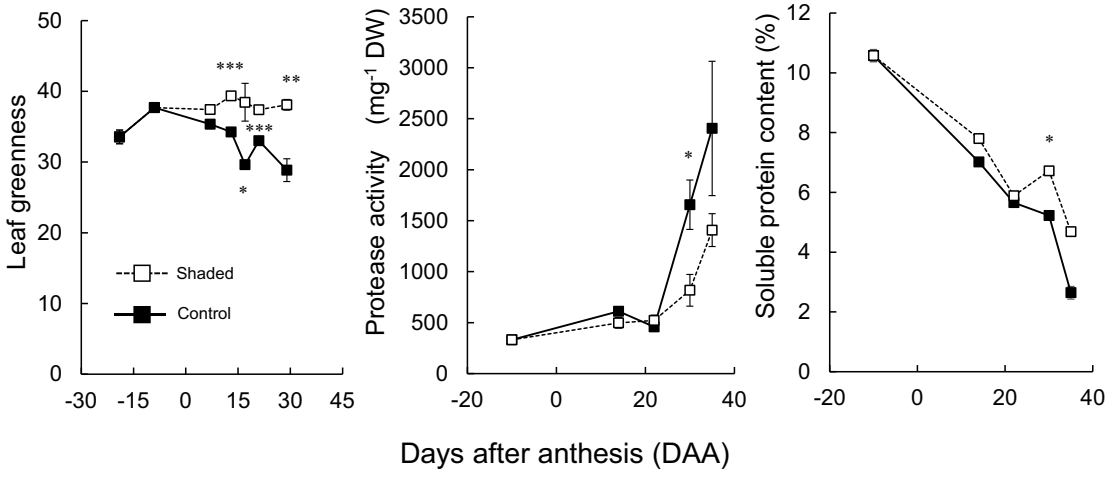


Figure 6. Change in leaf greenness, protease activity and soluble protein content of flag leaves of wheat plants in 2019/20 slightly shaded from anthesis. The bars indicate standard error. *, ** and *** indicate significant differences between control and shaded at $p < 0.05$, 0.01 and 0.001, respectively.

managements (IN (E)) (Figure 2). The high level of leaf greenness indicates that IN (L) promoted chlorophyll synthesis and helps explain the high CGR (crop growth rate) and yields found in previous reports (Mizuta et al., 2020, 2023). In IN (E)+TD, the nitrogen top-dressing at anthesis (TD), which are known to raise grain protein content (Takayama et al., 2004), slowed the reduction in leaf greenness after 25 DAA (Figure 2), indicating that the early reduction in IN (E)-TD was induced by leaf nitrogen starvation. This result indicated that the IN (L) delayed the process of leaf senescence as found in plants applied with TD.

The nitrogen metabolism of leaves towards terminal senescence appears to be split into two phases: the first phase, from pre-anthesis to approximately 15 DAA, when leaf greenness does not change appreciably (Figure 2a), was characterized with a decrease in soluble protein (Figure 5a, b) and the second phase after 15 DAA was characterized by a sharp increase in protease activity (Figure 3) followed by a rapid decrease in leaf greenness (Figure 2).

In the second phase, post-anthesis protease activity likely relates to visible senescence characterized by decreases in leaf greenness since the sharp rise in protease activity occurred just prior to the decrease in leaf greenness (Figure 2). Leaf greenness data (measured by chlorophyll meter) correlate well with leaf chlorophyll content (Ling et al., 2011). Most chlorophyll is bound with proteins to form a light harvest complex (LHC) and plays the role of 'light antenna' (Blankenship, 2015). The rise in protease activity may be the first process degrading the proteins bound with the pigment molecules.

Interestingly, the timing of top-dressing significantly affected the extent of the increase in protease activity. On 23 DAA in 2017/18 (Figure 3a) and 16 DAA in 2018/19 (Figure 3b) when the apparent rise in protease activity was first observed, the protease activity in IN (E)-TD was two to five times higher than in IN (L)-TD, indicating that severer nitrogen starvation induced a stronger and earlier rise in protease activity. In contrast, in treatments IN (E)+TD, IN (L)-TD and IN (L)+TD of 2018/19, protease activity stayed low on 16 DAA, indicating that IN (L) and +TD had significant effects on delaying the onset of metabolic process toward visible senescence. Since young grains begin to store carbohydrates and proteins from 5 DAA (Simpson et al., 1983) and the carbohydrate accumulation becomes most active after cell division is completed (around 10 DAA at early milk-ripe stage), grain nitrogen demand quickly grows after 10 DAA. The rise of protease activity seems to be correlated with grain growth and can be delayed by high leaf nitrogen status.

A question still remains: what is the trigger that raises protease activity? Leaf cells must recognize nitrogen status in the leaf tissue. Amino acid content is unlikely to be involved in a signaling process to raise protease activity. The amino acid content of the flag and second leaves in waterlogged wheat showed significant declines 10 days after waterlogging (Stieger & Feller, 1994), implying that the decrease in leaf amino acids is indicative of the extent of nitrogen starvation. However, the amino acid contents of the flag leaf in IN (E)-TD were not reduced during pre- and post-anthesis in either 2017/18 or 2018/19 (Figure 4). In particular, pre-anthesis in 2018/19, the amino acid contents of flag leaves in IN (E)-TD and IN (L)-TD were identical, even though the amount of fertilizer applied after stem elongation and leaf greenness (Figure 2) were quite different. Thus, it is suggested that amino acid content is not a good indicator of the extent of nitrogen starvation within the lamina. The change in amino acid content may be less related to the expression of proteolytic enzyme genes.

In the former phase mentioned above, the soluble protein contents decreased from pre-anthesis (Figure 5) without a decrease in leaf greenness (Figure 2). This indicates that the reduction in soluble protein was less involved in the visible characteristics of leaves. It is known that the soluble proteins of leaf lamina mainly consist of enzymes, and a major proportion of the soluble enzyme components are the ones involved in carbon assimilation and the synthesis of carbohydrates (Blankenship, 2015). The content of Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) accounts for 12–30% of the total proteins in a leaf (Evans, 1989; Ishida et al., 2008), may be important to explain the changes in the soluble protein content in this study. The important point found in this study was that the soluble protein decreased without a corresponding rise in protease activity (Figure 3). This indicates that Rubisco and other enzymes might not be decomposed by catalysis of proteases. Rubisco loss in wheat unrelated to proteases, is probably due to the formation of Rubisco-containing bodies and autophagy-like degradation, as discussed by Gregersen et al. (2008). Although it is possible that the gradual decline in soluble protein content may be due to degradation of pigment-protein complexes, the catabolic pathway has also not yet been elucidated (Martínez et al., 2008). Further studies on those pathways are necessary.

It is confirmed that light conditions may be one of the factors affecting leaf senescence rate in field-grown wheat as the shade placed above the canopy retained leaf greenness up to 28 DAA when unshaded plants showed earlier decreases in leaf greenness

(Figure 6). Interestingly, the soluble protein contents decreased in both shaded and unshaded plants due, probably, to remobilization of leaf nitrogen to the developing grains. On 28 DAA when the protease activity in unshaded plants was significantly higher than in shaded plants, the soluble protein contents in the unshaded plants was significantly lower than in the shaded ones. This indicates that nitrogen remobilization from leaves to grains was more active in the unshaded plants. It is known that high intensities and specific wavelengths of light can accelerate the senescence rate of wheat leaves through increases in reactive oxygen species (Causin et al., 2006). Moreover, nitrogen remobilization also affects the senescence rate in wheat (Srivalli & Khanna-Chopra, 2004). Thus, understanding of the mechanism involved in abnormally early senescence or 'stay-green' will require further elucidations of the interactive effects of nitrogen, light and grain development on the leaf senescence processes in wheat.

Acknowledgments

We thank Mr. Mitsuji Nagasuna and Mr. Akira Takata for their technical assistance.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Ministry of Agriculture, Forestry and Fisheries, Japan, under Grant [Development of Diagnostic Methods and Countermeasure Techniques for Overcoming High-Yield Inhibitory Factors]; JSPS KAKENHI [JPMXS0440400020].

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