The Occurrence of Delayed Stem Senescence in Relation to trans-Zeatin Riboside Level in the Xylem Exudate in Soybeans Grown under Excess-Wet and Drought Soil Conditions

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Abstract: Delayed stem senescence (DSS) after pod maturation in soybean (Glycine max) lowers the quality of products in the mechanized harvest. The effects of drought and excess wet soil conditions on the occurrence of DSS were studied with special reference to remobilization of vegetative nitrogen and supply of cytokinin via xylem. Excess wet soil treatment was applied throughout the reproductive period to field-grown soybean in 2003 (Exp.1) and short-term drought stress treatment was applied during the reproductive period to pot-grown soybean plants in 2004 (Exp.2). The degree of DSS at pod maturing was evaluated by the DSS score from “1” for severe DSS to “5” for completely synchronous senescence of pods and vegetative parts. The DSS score in Exp.1 varied from 2.2 to 2.5 and that in Exp.2 from 2.8 to 3.7. Excess wet soil treatment in Exp.1 promoted the occurrence of DSS, while drought stress treatments during the periods of flowering to pod elongation, later seed-filling and seed maturing decreased it. The soybean plants that showed distinct DSS had lower ratios of seed number to vegetative dry weight at seed maturity and a lower rate of nitrogen remobilization from vegetative organs to seeds during the latter half of the reproductive period. The trans-zeaxtin riboside (tZR) level in xylem exudate tended to be higher in soybeans with severer DSS than in those normally matured in both experiments showing increased tZR concentration and/or higher exudation rate. These results suggest that DSS can be caused by a wet soil water condition, which lowers pod/seed number and increases vegetative organs mediated by the increased supply of cytokinin through xylem during seed-filling.

Key words: Cytokinin, Delayed stem senescence, Drought stress, Sink and source, Soil water content, Soybean.

Delayed stem senescence (DSS) in soybean (Glycine max L.) is a phenomenon of retarded leaf and stem yellowing, where plants maintain a high stem water content and remain chlorophyll in stem at seed maturity. Since seeds are soiled during harvesting with a combine, DSS is a serious problem for production by mechanized harvesting, especially in Japan, where the appearance of seed is important in product quality.

Drought stress and high temperature during the reproductive periods are likely to cause DSS (Hashimoto and Oohama, 2002; Inoue, 2003; Takeda et al., 2003). However, the reproducibility of experimental results is not certain and the environmental factors causing DSS are not clear. To avoid DSS, we need to figure out the mechanism of DSS occurrence in relation to environmental factors, especially soil water condition.

There have been several reports that DSS is associated with the decrease in seed weight per stem weight at seed maturity resulting from accelerated flower abortion (Inoue, 2002; Shimada et al., 2005). DSS is readily induced by the flower or pod removal, because extra nutrients are left in the stem or leaves (Leopold et al., 1959; Wittenbach, 1982; Phillips et al., 1983; Inoue, 2003; Shimada et al., 2005). However, Crafts-Brandner and Egli (1987) found that sink removal do not necessarily disturb leaf and stem senescence, and Desclaux et al. (2000) reported that drought stress during flowering decreased pod number temporarily, but did not decrease pod production. DSS without decreasing seed yield was observed in a study with varied air-temperature by Mochizuki et al. (2005). The inconsistency of the experimental results shows that environmental factors causing DSS and the relationship between defective pods and the occurrence of DSS have not yet been fully established.

Cytokinins are known as senescence-inhibiting hormones. For example, Ookawa et al. (2004) suggested that cytokinin maintains high leaf nitrogen content and suppresses the reduction of Rubisco level in the leaves during senescence in rice. In soybean, the relationship between cytokinins in xylem exudate...
and plant senescence has been demonstrated by several investigators using explants or whole plants (Neumann et al., 1983; Garrison et al., 1984; Nooodén et al., 1990; Nooodén and Letham, 1993). Heindl et al. (1982) showed that the concentration of cytokinins in xylem exudate is high during the early flowering, and then drastically declines at late flowering and/or pod-set stages. It is supposed that cytokinins may relate to the occurrence of DSS, even in the field condition, but it has never been examined how such a cytokinin dynamics is related to the occurrence of DSS.

The objectives of this study were to examine the effect of drought stress and excess wet soil conditions on DSS, and to identify how vegetative and reproductive growth and cytokinin level in xylem exudate are related to the occurrence of DSS.

**Materials and Methods**

1. **Plant culture**
   
   (1) **Experiment 1** Soybean cultivar Tachinagaha was grown in the field under a rain shelter roofed with polyethylene film at the Graduate School of Agriculture, Kyoto University, Kyoto, Japan (35°2′ N, 135°47′E, 65 m altitude) in 2003. The soil was alluvial loam and fertilized with N : P2O5 : K2O = 4 : 10 : 10 (g m−2) before planting. Seeds were sown on 27 June, the plant density was 13.5 plant m−2 (10 × 74 cm) and irrigation tubes were laid on every two furrows. Soil water content was measured continuously with time domain reflectometry (TDR) every two or three days during developmental period. In the control, the soil water content was maintained at 20.7% (Fig. 1A), while in the excess wet treatment the soil water content was maintained at 25.3% on the average from the beginning of flowering (R1) to the beginning of seed maturing (R7) (from 5 August to 30 September) (Fehr et al., 1971). Soil water content was maintained uniformly at around 20% before the treatment. There were 4 replications for each treatment and the area of each replication was 6.5 m².
   
   (2) **Experiment 2** Soybean cultivar Tachinagaha was grown in pots (24 cm in diameter) containing 9 L soil in 2004 under the same rain-shelter as in Exp.1. The soil was fertilized with N : P2O5 : K2O = 1 : 2 : 2 (g pot−1) before transplanting. Seeds were first sown on 29 June in paper pots (4.7 cm × 5.0 cm). Two plants were
transplanted to each pot when the first trifoliolate was unrolled (14 days after sowing: 14 DAS) and thinned to one plant per pot one week after transplanting.

Daily values of the fraction of transpirable soil water (FTSW) in each treatment was determined by dividing daily loss of pot weight by the transpirable soil water at field capacity, which had been determined by preliminary water-withdrawal test.

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\text{Daily FTSW} = \frac{\text{daily pot weight} - \text{final pot weight}}{\text{initial pot weight} - \text{final pot weight}}
\]

Initial pot weight = pot weight when soil water in the pot was at field capacity. Final pot weight = pot weight when transpiration stopped. The initial and final pot weight was calculated in advance (initial pot weight – final pot weight = 3.2 kg).

All pots were under the same soil water condition until the beginning of flowering (R1), and thereafter subjected to the following four treatments. Drought stress was applied during flowering and pod elongation (FPE), early seed filling (SFI), later seed filling (SFII), and seed maturing (SM) in Exp.2 on the occurrence of delayed stem senescence (DSS) indicated by DSS score, water and N content of the vegetative plant parts and the date of seed maturity.

2. Sampling plant materials and xylem exudate

Dry weight and nitrogen concentration in the leaf, petiole, stem, pod (pod wall) and seed were measured in both experiments. The plant samples were collected at 36 DAS for R1 stage, 66 DAS for 15 days after the beginning of seed filling (R5), 94 DAS for R7 and 114 or 115 DAS for seed maturity (R8) in control and excess wet, respectively in Exp.1 and at 35 DAS for R1, 50 DAS for R5, 69 DAS for 20 d after R5, 90 DAS for R7 and 105, 108 or 113 (SM, SFII, Control/FPE/SFI, respectively) DAS for R8 in Exp.2 (Fehr et al., 1971). The concentration of Kjeldahl N of each organ was measured.

Each plant was cut at about 8 cm above the soil level at 4−5 a.m. The xylem exudate was collected with absorbent cotton from the decapitated stem for 3 hr around predawn. Xylem exudate was sampled at 36 DAS, 52 DAS, 66 DAS and 94 DAS in Exp.1 and at 35 DAS, 50 DAS, 69 DAS, and 96 DAS in Exp.2. The exudation rate (ml h⁻¹) was estimated from the increase in cotton weight. The xylem exudate absorbed by cotton were extracted by centrifuging (3000 rpm, 30 min), and stored in −38°C freezer until analysis after adding 1 ml methanol.

3. Purification of cytokinins with a Sep-pak C₁₈ cartridge

The methanol was evaporated in form the exudates sample at 40°C and then 5% methanol was added to the exudate. The exudate was eluted through a C₁₈ Sep-pack cartridge, which had been washed with 5% methanol. Cytokinins were trapped in the cartridge. The cartridge was eluted with about 10 ml 55% methanol and the extract was evaporated to dryness and dissolved in exactly 200 μl 5% methanol. The concentration of t-ZR which is one of main endogenous cytokinins retarding plant senescence (Letham and Palni, 1983) in the purified extracts were

Table 1. Effects of excess wet soil water treatment from the beginning of flowering (R1) to the beginning of seed maturing (R7) in Exp.1 and the drought stress during flowering and pod elongation (FPE), early seed filling (SFI), later seed filling (SFII) and seed maturing (SM) in Exp.2 on the occurrence of delayed stem senescence (DSS) indicated by DSS score, water and N content of the vegetative plant parts and the date of seed maturity.

| Treatment          | Date of seed maturity DAS | DSS score | Stem water content mg g⁻¹ | Stem + petiole N content mg g⁻¹ |
|--------------------|---------------------------|-----------|---------------------------|-------------------------------|
| Ex. 1 Control      | 114                       | 2.5 a*    | 584 a                     | 11.4 b                        |
| Excess wet         | 115                       | 2.2 b     | 662 a                     | 13.4 a                        |
| Ex. 2 Control      | 113                       | 2.9 b     | 662 ab                    | 7.2 a                         |
| FPE                | 113                       | 3.3 ab    | 653 ab                    | 5.5 a                         |
| SFI                | 113                       | 2.8 b     | 672 a                     | 10.5 a                        |
| SFII               | 108                       | 3.4 ab    | 543 b                     | 6.2 a                         |
| SM                 | 105                       | 3.7 a     | 614 ab                    | 5.1 a                         |

*Values followed by different letter (s) in a column within each experiment are significantly different at P = 0.05 with the Tukey test.
analyzed with ELISA kit (Phytodetek Kit; transzeatin riboside).

4. **Assessment of unsynchronized senescence**

At seed maturity, the degree of DSS was recorded by the DSS score which was proposed by Furuya and Umezaki (1993) as ‘degree of delayed stem maturation’. The five DSS scores are defined as follows. DSS1, the stem is green and yellow-green leaves or green leaves are remained at more than one-third of the nodes of the plant at seed maturity; DSS2, stem color is green or yellow-green and yellow-green or green leaves are remained at less than one-third of the nodes of the plant at seed maturity; DSS3, the stem is light green and contains some moisture and chlorophyll, and several leaves contain some moisture or several petioles without leaves remain at seed maturity, then changing to DSS4 a few days later; DSS4, the stem is yellow and retains some moisture, and occasionally a few leaves are yellow to yellow-green; DSS5, the stem is brown and dry, and all leaves have abscised. Soybeans with less than a 3 DSS score (DSS3) are regarded as DSS in general. The score in each treatment was determined with averages of 48 plants in Exp.1 and 7–18 plants in Exp.2. In Exp.2, more pots were used for control and earlier treated plots, e.g. FPE or SFI, than SFI or SM to spread risks of damage by disease and pest. The water content of stem and N content of stem and petioles were used as auxiliary factors for assessment of unsynchronized senescence.

**Results**

Both the excess wet treatment and control showed DSS symptoms in Exp.1 whose DSS scores were 2.2 and 2.5, respectively (Table 1). In Exp.2, the plants in control and drought-stressed during SFI (referred to as SFI plants hereafter), showed DSS2.9 and 2.8, respectively, and were diagnosed as DSS. The water content of stem and N content of stem+petiole negatively correlated with the DSS score showing that the DSS score indicates the extent of water and N contents retained in the stem. The plants drought-stressed during SFII and SM (SFII plants and SM plants) were matured normally. They showed DSS scores of 3.4 and 3.7, respectively, and their water content of stem and N content of stem+petiole were lower than those in the control and SFI plants in Exp.2. FPE plants matured normally, and had a DSS score of 3.3. The stem water content and N content of stem+petiole of FPE plants were lower than those of control and SFI plants.

Neither the yield nor yield components differed between the plants in the control and excess wet treatment groups in Exp.1. However the seed number per vegetative dry weight, which means the seed number per total dry weight of stem, leaves and petioles at seed maturity, was significantly lower in

| Treatment   | Seed dry weight | Pod number per plant | Ratio of ripened pod number | Seed number per pod | Seed number per plant |
|-------------|----------------|----------------------|----------------------------|-------------------|-----------------------|
| Control     | 15.1 a         | 34.5                 | 0.88 a                     | 1.47 a            | 50.7 a                |
| Excess wet  | 13.5 a         | 31.0                 | 0.87 a                     | 1.34 a            | 42.5 a                |
| Ex. 1 Control | 15.1 a         | 34.5                 | 0.88 a                     | 1.47 a            | 50.7 a                |
| Excess wet  | 13.5 a         | 31.0                 | 0.87 a                     | 1.34 a            | 42.5 a                |
| Ex. 2 Control | 25.5 ab | 61.2                 | 0.84 ab                    | 1.55 a            | 90.8 b                |
| FPE         | 25.8 ab        | 60.0                 | 0.95 ab                    | 1.61 a            | 98.8 ab               |
| SFI         | 21.8 b         | 60.0                 | 0.95 ab                    | 1.70 a            | 91.4 b                |
| SFII        | 21.9 b         | 60.0                 | 0.95 ab                    | 1.65 a            | 98.8 ab               |
| SM          | 28.1 a         | 58.8                 | 0.94 a                     | 1.94 a            | 114.2 a               |

*Values followed by different letter (s) in a column within each experiment are significantly different at P = 0.05 with the Tukey test.*
the excess wet treatment than that in the control in Exp.1 (Table 2). In Exp.2, the effect of short-term drought stress did not largely affect seed yield and yield components, with the exception of SFII plants and control groups, which showed a moderately low seed yield and low ratio of ripened pod, respectively. In contrast, the ripened-pod number per vegetative dry weight and the seed number per vegetative dry weight were evidently lower in the control and SFI than in the other treatments. In Exp.1, the lower seed number per vegetative dry weight in the excess wet condition was associated with a lower value of DSS score compared with the control. The seed number per vegetative dry weight was significantly correlated with the DSS score in Exp.2 ($r=0.90$) (Fig. 2). Besides, plants in Exp.1 which tended to show lower DSS score compared to that in Exp.2 had a smaller seed number per vegetative dry weight than in Exp.2.

Fig. 3 shows the change in stem dry weight during the growth of the plants in control and each treatment group in both experiments. In Exp.1 the stem dry weight in the excess wet treatment tended to be heavier than that in the control at 15 days after R5 although there was no significant difference at any stage (Fig. 3A). In Exp.2, the control and SFI had a heavier stem dry weight than FPE during the first half of the seed filling. The stem dry weight of SFII and SM plants decreased after the drought stress treatments (Fig. 3B).

The relative rate of daily nitrogen remobilization (RNR) from vegetative organs during the period from the middle of seed filling to seed maturity was calculated as follows:

$$\text{RNR} = \frac{N_{\text{veg. (T1)}} - N_{\text{veg. (T2)}}}{T2-T1} \cdot \frac{1}{N_{\text{veg. (T1)}}}$$

Where, $N_{\text{veg.}}$ is the amount of N in vegetative organs (stem, leaves and petioles), and T1 and T2 are 15 days (Exp.1) or 20 days (Exp.2) after R5 and seed maturity, respectively.

RNR is significantly correlated with the DSS score in Exp.2 ($r=0.99$). The lower value of DSS in the excess wet treatment in Exp.1 compared with the control was again associated with a lower RNR (Fig. 4). Plants in Exp.1 showed lower RNR than any plants in Exp.2, being associated with lower DSS scores.

The $t$-ZR concentration in xylem exudate was highest at R1 in both experiments (Table 3), as has been identified previously (Noodén et al., 1990). In Exp.1, there was a significant difference in the $t$-ZR concentration between the control and excess wet treatment only at R7. In Exp.2, the $t$-ZR concentration in FPE plants was higher than that in the control at R5, but it was equal or lower than that in the other treatments at later stages. The $t$-ZR concentration in SFI and SFII plants in Exp.2 did not differ from that in the control at any stage. The rate of xylem exudation
Sato et al. — Occurrence of Delayed Stem Senescence and Cytokinin in Soybean xylem exudate.

In Exp.1, the volume of xylem exudates during later seed filling, from R5 to R7, was significantly higher in the excess wet treatment than in the control during later seed filling, from R5 to R7. The plants in the control in Exp.2 maintained a higher rate of xylem exudation than in the other two treatments from R5 to R5 + 20d. The t-ZR flux (t-ZR concentration × the volume of xylem exudate, pmole h\(^{-1}\) plant\(^{-1}\)) in the excess wet treatment in Exp.1 was kept higher than in the control from R1 to R7, especially from R5 + 15d to R7 (Table 3). The t-ZR flux in the control in Exp.2 was higher than that in the other treatments from R5 to R5 +20, but there were no significant differences among any treatments at R7.

### Discussion

The symptom of DSS in excess wet treatment was severer than that in the control in Exp.1 (Table 1). In Exp.2, the plants in the control that received adequate water throughout the growth period exhibited DSS symptoms, while FPE, SFII and SM plants matured normally. However, the SFI plants subjected to water deficit during early seed filling exhibited distinct DSS like the control plants. The results of the two experiments indicate that a wet soil condition promotes the occurrence of DSS, and short-term water deficit ameliorates DSS depending on the developmental stage of the water deficit. This is not consistent with the results reported by Hashimoto and Oohama (2002) and Takeda et al. (2003), which suggested that DSS occurrence was promoted by water deficit during the flowering and/or pod elongation stages. One reason may be the difference in the degree of water stress between their experiments and this study. In our study, the drought treatments did not reduce the pod number significantly, but reduced yield and/or pod number as mentioned above. The drought stress treatment in our study was not strong enough to reduce yield and/or pod number. The occurrence of DSS in the control in Exp.2 suggests that the occurrence of DSS is influenced by the developmental stage of the water deficit. The results of the two experiments indicate that a wet soil condition promotes the occurrence of DSS, and short-term water deficit ameliorates DSS depending on the developmental stage of the water deficit.

### Table 3

| Treatment | t-ZR concentration (nM) (A) | The volume of xylem exudate (mL h\(^{-1}\) plant\(^{-1}\)) (B) | t-ZR flux (pmol h\(^{-1}\) plant\(^{-1}\)) (A×B) |
|-----------|-----------------------------|-------------------------------------------------|---------------------------------|
| Ex. 1     |                             |                                                 |                                 |
| Control   | 10.2 a*                     | 6.9 a 7.0 a 3.5 a                                | 0.60 a 0.65 a 0.16 a            |
| Excess wet| 10.9 a                      | 7.6 a 7.5 a                                    | 0.66 a 0.77 a 0.49 a            |
| Ex. 2     |                             |                                                 |                                 |
| Control   | 9.79                        | 2.05 b 1.13 a                                  | 0.08 0.12 b 0.24 b              |
| FPE       | 8.01 a                      | 0.81 b 2.3 a                                   | 0.12 b 0.38 b 0.20 a            |
| SFI       | 1.29 a                      | 2.2 a                                          | 0.24 b 0.20 a 0.13 a            |
| SFII      |                             |                                                 |                                 |

*Values followed by different letter (s) in a column within each experiment are significantly different at P = 0.05 with the Tukey test.
enough to reduce reproductive organs but was enough to accelerate plant senescence. Then, how soil water condition affects DSS occurrence would depend on how and to what extent it affects plant characteristics during reproductive stage. In Exp.1, the plants in control also showed DSS symptoms, which were less distinct than in the excess wet treatment but severer than in any treatment in Exp.2 (Table 1). The reason for this is not clear but the difference in soil environment between field (Exp.1) and pot (Exp.2) conditions may be involved. The increased soil water content around R7 (Fig. 1) might have affected the senescence of the plant in the control.

There was no relation between seed dry weight per plant and the DSS score in either experiment (Table 2). There was no definite relationship between any seed yield component and the occurrence of DSS. In contrast, the seed number per vegetative dry weight was correlated with the DSS score (Fig. 2). In the soybeans which showed DSS, the stem growth was relatively sustained until the middle of seed filling in both experiments (Fig. 3). Since the seed yield was not decreased by DSS, the increase in stem growth of DSS plants might contribute to the decrease of seed number per vegetative dry weight. The decrease in seed number per vegetative dry weight in soybeans with DSS might be caused both by the change in seed development and also by the change in vegetative growth (Table 2). In this study, the excessive growth of vegetative organs during the early reproductive period was promoted by DSS. Furthermore, RNR during the latter half of the reproductive period was related with the DSS score (Fig. 4). In SFII and SM in Exp.2, stem growth was sustained until the middle of seed filling, RNR was higher than in the other treatments, and the maturation was normal. Drought stress promotes the remobilization of leaf nitrogen and leaf senescence in soybean (De Souza et al., 1997). The effect of DSS could be alleviated by drought stress in the latter half of the reproductive period, even if vegetative organs grew excessively during the early half of reproductive period. These results suggest that the ratio of seed number to vegetative dry weight and the rate of nitrogen remobilization from vegetative organs are related to the occurrence of DSS.

The \( t-ZR \) flux during early seed filling tended to be higher in the plants with DSS than in the normally matured plants, and it tended to be associated with the change of stem dry weight in both experiments (Table 3, Fig. 3). The pod dry weight per stem dry weight at the middle of seed filling period tended to be negatively correlated with the level of \( t-ZR \) flux during early seed filling as shown in Fig. 5, in which \( t-ZR \) flux is presented with the average of two measurement times at R5 and R5 + 15d in Exp.1, and at R5 and R5 + 20d in Exp.2, respectively. There are several reports which suggest synthesis of cytokinins is promoted by the deficit of sink organs in terms of number of seeds or pods (Noodén et al., 1990; Noodén and Letham, 1993). Cytokinins also affected nitrogen and biomass partitioning in wheat seedlings (Simpson et al., 1982), a perennial herb \textit{Urtica dioica} L. (Wargner and Beck, 1993; Beck, 1996) and rice (Ookawa et al., 2004). They increased the nitrogen levels and dry weight in older leaves by promoting the accumulation of nitrogenous compounds in the leaves and also by maintaining high photosynthesis of the leaves (Jordi et al., 2000). From this point of view, the excessive growth of stem during early seed filling in the plants with DSS is considered to be the result of a temporary decrease in pod number mediated by the high \( t-ZR \) flux in xylem exudate.

Soil water condition can directly affect hormonal balance in plants. For example, Shashidhar et al. (1996) demonstrated that cytokinin flux in xylem was decreased under drought stress associated with increased ABA flux in sunflower. Moreover, it is well known that ABA is induced under a drought condition and competes with cytokinins. The \( t-ZR \) flux in xylem was generally low in Exp.2 compared with that in Exp.1 and this is supposed to be resulted from the large daily fluctuation of FTSW in the pot test. The plants in all plots might be exposed to a slightly drying condition in Exp.2.

This study suggests that DSS is caused by an excess water soil condition during the reproductive period when it decreases the number of seeds per vegetative dry weight, and the drought soil condition tends to control the occurrence of DSS. This is accompanied by the excessive growth of vegetative organs, which may be induced by the high level of cytokinin flux in xylem during seed filling.
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