Effects of Waterlogging on Nitrogen Fixation and Photosynthesis in Supernodulating Soybean Cultivar Kanto 100

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Abstract: The supernodulating soybean (Glycine max (L.) Merr.) cultivar Kanto 100 was previously characterized by superior nitrogen (N) fixation and photosynthesis, and resulting in high yields. However, this cultivar seems to be susceptible to waterlogging during the vegetative growth stage, which frequently occurs in major soybean producing areas in East Asia. The objective of this study was to compare the effects of waterlogging on nodulation, N fixation and photosynthesis in Kanto 100 with those in its normally-nodulating ancestral cultivar Enrei. Kanto 100 and Enrei were grown in pots, and subjected to waterlogging for 10 days at three vegetative growth stages in 2003 and 2004. Waterlogging significantly reduced the number of nodules of both cultivars, but the magnitude of the reduction was more pronounced in Kanto 100. The acetylene reduction activity (ARA) of nodules and apparent photosynthetic rate (AP) of leaves were generally depressed immediately after the start of waterlogging, but both functions recovered substantially at the pod-filling stage in both cultivars. No marked cultivar difference was found in the magnitude of the reduction of ARA per plant and AP measured immediately after waterlogging and at the pod-filling stage in both years, but growth impairment was more pronounced in Kanto 100 in 2003. These results suggest that the supernodulating cultivar Kanto 100 is more susceptible to waterlogging than its normally-nodulating ancestral cultivar.

Key words: Glycine max, Nitrogen fixation, Photosynthesis, Soybean, Supernodulation, Waterlogging.

Soybean is physiologically characterized as requiring more nitrogen (N) for grain production than other major crops (Sinclair and de Wit, 1975). Soybean seeds contain a large amount of N during the seed filling stage, and most of the N accumulated in the vegetative tissues is translocated to seeds. Thus, a rapid decline of leaf N content occurs during seed development, resulting in a decline of leaf photosynthesis capabilities (Sinclair and de Wit, 1975). This self-destructive N translocation is likely to be a critical factor defining the soybean seed yield.

Soybean has three N sources: fertilizer N, soil N and symbiotically fixed N. Of the three N sources, the most practical option for enhancing N absorption by plants is increasing application of N fertilizers, either as basal dressing or top dressing during growth (Yoshida, 1979; Watanabe et al., 1983). However, a high input of N fertilizer increases the potential for groundwater contamination and the cost of production, and the improvement of the capacity of biological N fixation is preferable. Genetic improvement of symbiotic N fixation is an option for enhancement of soybean N-absorption capability. In the past two decades, several supernodulating soybean genotypes that form many more nodules than normally-nodulating cultivars have been isolated through attempts to induce mutations in genes that control nodulation capabilities (Carroll et al., 1985a, b; Day et al., 1986; Gremaud and Harper, 1989; Akao and Kouchi, 1992). Most supernodulating lines bred so far, however, have recorded inferior growth and yields compared with their normally-nodulating ancestral cultivars. For that reason, supernodulating lines have failed to produce practical benefits (Herridge and Rose, 2000; Sinclair, 2004).

Recently, a new supernodulating cultivar "Kanto 100" (previously named Sakukei 4) which was derived from high-yielding cultivars Enrei and Tamahomare, was released (Takahashi et al., 2003a). The yield and agronomic performance of this genotype are equal or superior to those of its ancestral cultivar Enrei under certain conditions (Takahashi et al., 2003b). Kanto 100 is the first supernodulating soybean cultivar with the yield potential to be grown profitably by farmers. Previous studies (Maekawa et al., 2003; Takahashi et al., 2005) characterized Kanto 100 as having superior ability to maintain high leaf N and photosynthesis, irrespective of the application rate and type of N fertilizer. This high photosynthetic capability of Kanto 100 depends on high leaf Rubisco and chlorophyll contents (Maekawa and Kokubun, 2005).

These previous studies were conducted under favorable growing conditions. No data are available to describe the performance of Kanto 100 under...
common unfavorable conditions. Nitrogen fixation by leguminous bacteria is extremely sensitive to changes of chemical and physical conditions in soil. Especially, soil-water conditions (deficit or excess) critically restrict nodulation and N fixation ability and thus markedly reduce seed yield (Harper, 1987). The majority of N in supernodulating soybeans is derived from symbiotic fixation (Takahashi et al., 1995; Sato et al., 1999). These lines of evidence raise a hypothesis: a supernodulating genotype is more susceptible to waterlogging in terms of N acquisition and its related physiological traits.

Waterlogging is a major problem limiting the growth of many crops in humid areas in east Asia, especially in fields with poor water tables. Although soybean plants exhibit adaptive responses to flooding stress by forming adventitious roots (Bacanamwo and Purcell, 1999a) or secondary aerenchyma (Shimamura et al., 2002), prolonged rainy periods and heavy rainfall hinder vegetative growth, thereby reducing soybean yields (Sugimoto et al., 1989; Sugimoto and Satou, 1993). In addition, many previous studies have demonstrated that physiological functions such as photosynthetic activity, N fixation and mineral absorption are harmed by water-induced stresses (Bennett and Albrecht, 1984; Sojka, 1985; Buttery, 1987; Sallam and Scott, 1987; Griffin and Saxton, 1988; Scott et al., 1989; Oostshuis et al., 1990; Sung, 1993; Bacanamwo and Purcell, 1999b). In our preceding study, we evaluated growth and yield of Kanto 100 under the flooding condition, in comparison with its ancestral cultivar Enrei and non-nodulating genotype En 1282, and found that Kanto 100 exhibited a marked decrease in dry matter production by waterlogging, but yield decrease was compensated to a level similar to that of Enrei. The objective of this study was to verify the hypothesis that the supernodulating genotype Kanto 100, in which N acquisition is largely dependent on N fixation, can be more susceptible to waterlogging in terms of N acquisition and photosynthesis.

Materials and Methods

1. Plant materials and waterlogging treatments

Plant materials and flooding treatments imposed were the same as described in our preceding report (Matsunami et al., 2007). Briefly, Kanto 100 and its ancestral cultivar Enrei were grown in pots placed outdoors, and waterlogging treatments were imposed by submerging the pots in a water pool to the soil surface. In 2003 and 2004, three separate waterlogging treatments were imposed each for 10 days at different growth stages, S1, S2 and S3. In 2003, S1 started 10 days after sowing (DAS) (V1 according to Fehr et al. (1971)), S2 began 24 DAS (V3), and S3 began 38 DAS (V8). In 2004, S1 began 21 DAS (V3), S2 began 31 DAS (V6), and S3 began 41 DAS (V8). In 2004, S1 was imposed 10 days later than in 2003, because the later stages appeared to be more susceptible to flooding.

2. Measurement of nodulation and acetylene reduction activity (ARA)

For measurement of ARA and nodulation, three or four plants of average size were selected from treated and control plants and separated into shoots and roots. Immediately after separation, the nodulated root system was enclosed in a 900-mL glass bottle with a rubber cap. Then it was incubated with 30-mL acetylene at 25°C. Gas samples (1 mL) were collected using a syringe after 20 and 30 min of incubation and injected into a gas chromatograph (Hitachi 163; Hitachi Ltd., Tokyo, Japan) equipped with a Porapack N glass column with N2 as carrier. Ethylene produced from the 20th to 30th min of incubation was quantified from the ratio of the peak area of samples to that of control ethylene gas. The two-time-point assays showed that the rate of acetylene reduction was generally linear for all samples. After determination of ARA, nodules were detached from the roots, counted and weighed. For control plants, the measurements of ARA and nodulation were conducted four times in 2003 and six times in 2004, respectively. For waterlogged plants, the measurements were carried out immediately after each waterlogging treatment and at the pod-filling stage (R5 according to Fehr et al. (1971)).

3. Measurement of apparent photosynthetic rate (AP)

The apparent photosynthetic rate (AP) of the recently expanded terminal leaflet in five plants of each treatment was measured using a photosynthesis system (LI6400 Portable; Li-Cor Inc., NE, USA). For each waterlogging treatment, measurements were carried out twice, immediately after the waterlogging treatment, and at the pod-filling stage. For control plants, measurements were conducted four times, after each waterlogging and at pod filling. Measurements were performed between 1000 and 1200 hr. The flow rate of air in the leaf chamber was controlled at 500 μmol s⁻¹, and the CO₂ concentration was maintained at 350 μmol mol⁻¹. The irradiance on the measured leaves (6 cm²) was regulated at 1,500 μmol m⁻² s⁻¹ PPFD. The chamber temperature was maintained at 25°C.

4. Measurement of growth

Four to five plants of medium size were sampled from each treated and control plot. The samples were separated into various plant parts, oven dried at 80°C for three days and weighed.

5. Statistical analysis

The same experimental design was used in 2003 and 2004, except that waterlogging treatment was initiated 11 days later in 2004. Each waterlogging treatment consisted of about 20 pots–15 for the samples (five pots...
× three times), plus five pots to serve as photosynthesis measurements. For control pots, 20 (2003) or 30 pots (2004) were used depending on the frequency of sampling. Each plant (pot) was considered as an experimental unit, and randomly ordered with respect to time within each treatment. Analysis of variance for each physiological trait was performed to evaluate the effects of genotype, waterlogging treatment, year and their interactions using the JMP version 5.1 (SAS Institute Inc., 2002). An LSD means separation test was applied to determine significant differences between the treatment means, when the treatment effect was significant in the analysis of variance ($P<0.05$). Weather data were obtained from the Sendai District Meteorological Observatory website.

**Results**

1. **Climatic conditions**

   Climatic condition in the two years differed (Table 1). Temperature was higher and solar radiation was considerably higher in 2004, but precipitation was greater in 2003. Particularly, lower temperatures and less solar radiation occurred in July and August of 2003, which markedly depressed growth.

2. **Nodulation**

   In 2003, the number of nodules immediately after waterlogging treatment was not markedly depressed by waterlogging in Enrei, but it was significantly lower in plants waterlogged at the S3 stage in Kanto 100 (Fig. 1). At the pod-filling stage, there were fewer nodules in all waterlogging treatments than in the control in both cultivars. The reduction was more pronounced in Kanto 100 than in Enrei.

   In 2004, the flooding-induced depression of the number of nodules, either immediately after waterlogging treatment or at the pod-filling stage, was apparent in most waterlogging treatments in both cultivars. The magnitude of the depression was more pronounced in Kanto 100 than in Enrei. However, the absolute number of nodules was markedly higher in Kanto 100 than in Enrei, irrespective of growth stage or treatment.

3. **Acetylene reduction activity (ARA)**

   In 2003, ARA measured immediately after waterlogging treatment was significantly lower in the plants flooded at the S3 stage in Enrei and Kanto 100 ($P<0.05$) (Fig. 2). At the pod-filling stage, a significant decrease in ARA was observed only in the plants that were flooded at S2 in both cultivars.

   The waterlogging-induced depression of ARA was more marked in 2004 than in 2003; the ARA of each
waterlogged plant was significantly lower than that of the control. Although no marked cultivar difference existed in the magnitude of the depression, the absolute value of ARA per plant tended to be higher in Enrei than in Kanto 100, irrespective of the treatment.

4. Photosynthetic rate

In both years, the apparent leaf photosynthetic rate (AP) was significantly reduced by waterlogging at the S2 and S3 stages, irrespective of cultivar (Fig. 3). The difference of AP between the waterlogged plants and control plants was, however, not significant at the pod-filling stage.

5. Growth

In 2003, the top dry weight of the plants flooded at the S2 and S3 stages was lower than that of control plants, and the magnitude of the reduction was more pronounced in Kanto 100 than Enrei (Table 2). In 2004, no marked cultivar difference was observed in the magnitude of waterlogging-induced reduction of top growth.

The root weight was reduced by waterlogging treatment like the top weight (Table 2).

Discussion

The contrasting weather conditions of the two years apparently caused a difference in physiological functions and growth between 2003 and 2004; the higher temperature and abundant sunshine in 2004 during the growth period might have resulted in greater nodule number and more vigorous growth of control plants (Fig. 1, Table 2).

Table 2 summarizes the magnitude (%) of waterlogging-induced depression of nodulation, activities of N fixation and photosynthesis, and growth. As shown in Table 2, top dry weight at the pod-filling stage was reduced significantly by the 10-day waterlogging during the vegetative growth. Reductions in N fixation and photosynthesis were probably both responsible for the lower growth rates. Immediately after waterlogging treatment, both physiological functions were markedly hindered in a similar manner. At the pod-filling stage, however, the recovery from the immediate impairment was more pronounced in photosynthesis than in N fixation (Figs. 2, 3). Therefore, we conclude that the waterlogging-induced growth reduction was more attributable to impairment of N fixation than to that of photosynthesis in the two cultivars with contrasting nodulation capability. Using normally-nodulating cultivars, Sugimoto and Satou (1990, 1993) demonstrated that depression of growth resulting from excessively moist soil is attributable to the leaf N deficit that was induced by the disabled N-fixation activity by nodule bacteria and N absorption by roots. Reduction of N-fixing activity and nodule numbers in flooded soybean was similarly shown by Sung (1995).

As shown in Table 2, the interaction among year (Y), cultivar (C) and waterlogging treatment (T) was significant, and the C×T interaction was significant only for nodule number in 2004. This interaction is illustrated in Fig. 4. The nodule number measured at the pod-filling stage was much fewer in all waterlogging treatments than in the control in both cultivars. The reduction was more pronounced in Kanto 100 than in
Enrei in terms of percentages of waterlogged plants to control, however, the actual number remained higher in Kanto 100. Thus nodulation of Kanto 100 is more susceptible, depending on year, to waterlogging than its normally-nodulating ancestral cultivar, despite of its genetically superior capability of nodulation.

The waterlogging-induced reduction of growth was more pronounced in the plants flooded at a later stage, and this was in parallel with the reduction of the number of nodules (Fig. 1, Table 2). In 2004, when the magnitude of the reduction was substantial, the period of waterlogging treatment was given 31–41 DAS (S2 stage) and 41-51 DAS (S3 stage). These periods corresponded to the stage during which nodules were actively increasing in number and volume (Fig. 1). Presumably, favorable soil conditions during this stage are extremely important for adequate growth of bacteria and full exhibition of their N-fixing ability. Conversely, flooding during this stage might have hindered an active nodulation, and it was more

Table 2. Selected traits of waterlogged and control plants at the pod-filling stage.

| Year | Cultivar | Waterlogging treatment | Nodule number (plant⁻¹) | ARA (C₂H₄μmol hr⁻¹ plant⁻¹) | AP (μmol m⁻² s⁻¹) | Top dry weight (g plant⁻¹) | Root dry weight (g plant⁻¹) |
|------|----------|------------------------|--------------------------|-----------------------------|-----------------|---------------------------|-----------------------------|
| 2003 | Enrei    | Control                | 371(100)a                 | 128(100)a                   | 16.0(100)a      | 34.9(100)a                | 7.6(100)a                   |
|      |          | S1                     | 272(65)b                 | 110(86)a                     | 18.5(116)a      | 35.2(101)a                | 7.9(105)a                   |
|      |          | S2                     | 193(52)b                 | 86(67)a                      | 14.4(90)a       | 26.0(75)a                 | 6.9(92)a                    |
|      |          | S3                     | 237(64)b                 | 102(80)a                     | 9.0(56)a        | 28.0(80)a                 | 7.0(92)a                    |
|      |          | Average                | 224(60)                  | 99(78)                       | 14.0(87)        | 29.7(85)                  | 7.3(96)                     |
|      | Kanto 100| Control               | 4011(100)a               | 62(100)a                     | 16.6(100)a      | 24.1(100)a                | 9.3(100)a                   |
|      |          | S1                    | 1821(45)b                | 57(92)a                      | 16.6(100)a      | 22.8(95)a                 | 7.0(76)a                    |
|      |          | S2                    | 1434(36)b                | 40(65)a                      | 15.4(93)a       | 11.3(47)a                 | 4.7(50)a                    |
|      |          | S3                    | 2235(56)b                | 52(84)a                      | 12.1(73)a       | 10.0(42)a                 | 6.5(70)a                    |
|      |          | Average               | 1830(46)                 | 50(80)                       | 14.7(89)        | 14.7(61)                  | 6.1(65)                     |
| 2004 | Enrei    | Control                | 605(100)a                | 100(100)a                    | 15.6(100)a      | 39.3(100)a                | 19.5(100)a                  |
|      |          | S1                    | 400(66)a                 | 58(38)ab                     | 16.7(107)a      | 16.0(41)b                 | 12.1(62)b                   |
|      |          | S2                    | 485(80)a                 | 39(39)ab                     | 13.2(85)a       | 5.2(13)c                  | 5.2(27)bc                   |
|      |          | S3                    | 145(24)b                 | 26(26)b                      | 14.3(92)a       | 13.2(34)bc                | 5.1(26)c                    |
|      |          | Average               | 343(57)                  | 34(34)                       | 14.7(94)        | 11.5(29)                  | 7.5(38)                     |
|      | Kanto 100| Control               | 4980(100)a               | 50(100)a                     | 17.4(100)a      | 35.4(100)a                | 24.2(100)a                  |
|      |          | S1                    | 2487(50)b                | 25(49)ab                     | 20.5(118)a      | 16.1(46)b                 | 13.4(56)b                   |
|      |          | S2                    | 1171(24)c                | 14(28)b                      | 18.4(106)a      | 7.2(20)c                  | 10.1(42)bc                  |
|      |          | S3                    | 911(18)c                 | 10(20)b                      | 15.8(91)a       | 8.8(25)bc                 | 7.0(29)c                    |
|      |          | Average               | 1523(31)                 | 16(32)                       | 18.2(105)       | 10.7(30)                  | 10.2(42)                    |

Significance

| 2003 | Cultivar (C) | ** | ** | ** | ** | ns |
|      | Treatment (T) | ** | ns | ns | * | ns |
|      | C × T         | ns | ns | ns | ns | ns |
| 2004 | C             | ** | *  | ns | ns | ** |
|      | T             | ** | ** | ns | ** | ** |
|      | C × T         | *  | ns | ns | ns | ns |
| 2003/2004 | Year (Y) × C | ns | ns | ** | ** | * |
|        | Y × T         | ns | ns | ns | ns | ns |
|        | Y × C × T    | ** | ** | ** | ** | ** |

Waterlogged treatments were imposed for 10 days at S1, S2 and S3 stages in 2003 and 2004. Average: average values in the plants treated at S1, S2 and S3 stages. Figures in parentheses indicate percentages to the value of control plants. * and ** indicate significance at the 5% and 1% level, respectively. ns: not significant at the 5% level. Values followed by the same letter within a column of each cultivar are not significantly different at the 5% level, based on Tukey’s multiple range test.
pronounced in the supernodulating cultivar Kanto 100. Although the absolute values of nodule number remained higher in Kanto 100, this superior capability of nodulation did not reflect the N-fixation capability; ARA of Kanto 100 was generally lower than that of Enrei (Figs. 2, 4). Takahashi et al. (2005) showed that Kanto 100 exhibited higher ARA throughout growth than did Enrei. The different results in our study most likely resulted from two factors. First, there were temperature differences between experimental sites; reporting 25.1°C average temperature in the experiment of Takahashi et al. (2005), and 20.8°C in 2003 and 22.1°C in 2004 in our experiment. The lower temperature in our experiment might have hindered the realization of the potentially superior N-fixation capability of Kanto 100, because significant positive correlations were found to exist between N-fixing activity and soil temperature at temperatures below 30°C (Sinclair and Weisz, 1985). Another explanation might be the different soil types used in the two experiments: low-humic Andosols were used in Takahashi et al.’s study, whereas fine-textured clayey Terrace Yellow soil was used in the present study. Given that root system of Kanto 100 requires more oxygen for respiration (Takahashi et al., 2005), a soil type with good aeration, such as an Andosol, might be more advantageous for enhancement of Kanto 100’s N fixation. Further studies are necessary to examine these possibilities.

A comparison among cultivars and years of waterlogging-induced growth reduction revealed that the cultivar difference in the magnitude of the reduction was negligible in 2004 (average ratio of top dry weight of treated plants to that of control plants were about 30% in both cultivars). In 2003, however, the reduction was more pronounced in Kanto 100 than in Enrei (the corresponding percentage was 85 in Enrei vs. 61 in Kanto 100) (Table 2). The magnitude of the reduction of ARA and AP did not differ significantly between the two cultivars (Table 2). Therefore, physiological functions other than these two parameters appear to be responsible for the difference. Another possibility exists: Does ARA measured in a laboratory (25°C, with adequate supply of air) reflect the actual N-fixing capability of nodules in soil? Lower temperatures and much rainfall occurred in 2003 than in 2004. Under such unfavorable soil conditions in 2003, the N-fixing activity can be more adversely affected than the degree estimated by ARA measured in a laboratory. Soybean plants that are exclusively dependent on N fixation for N resources are more vulnerable to hypoxic conditions than plants that are totally dependent on N in nutrient solution (Bacanamwo and Purcell, 1999b). Kanto 100 was found to be more dependent on N fixation for its N acquisition than Enrei (Nohara et al., 2006). For that reason, Kanto 100, which is strongly dependent on N fixation for its N resources, is likely to be more susceptible than Enrei, which is not as dependent on N fixation as Kanto 100, to excessively wet soil conditions. Although this interpretation needs to be verified by further study, the results obtained in the present study verify the hypothesis that the supernodulating cultivar Kanto 100 may be more susceptible to waterlogging than its normally-nodulating ancestral cultivar.

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