Pod Setting Pattern during and after Low Temperature and the Mechanism of Cold-Weather Tolerance at the Flowering Stage in Soybeans

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Abstract: The effects of short-term exposure to a low temperature [two weeks, at 18°C/13°C (day/night)] and long-term exposure to a low temperature [four weeks, at 18°C/13°C (day/night)] from the beginning of flowering on pod setting were examined in one of the most cold-weather tolerant variety cv. Hayahikari and a standard sensitive variety cv. Toyomusume. The short-term exposure to a low temperature did not decrease the total number of pods even in Toyomusume. However, pod setting ability during the low temperature exposure was higher in Hayahikari than in Toyomusume. After the short-term exposure to a low temperature, pod setting showed recovery from the cold-weather damage by compensative pod setting after the low temperature treatment in both varieties. The long-term exposure to a low temperature significantly decreased the total number of pods in Toyomusume. But Hayahikari showed a greater capacity for pod setting during the low temperature. And the mechanism of tolerance was related to a superior fertilization ability during the low temperature.

Key words: Cold-weather tolerance, Fertilization, Flowering stage, Low temperature, Pod setting pattern, Pollination.

Abscission of flowers and pods due to low temperatures during the flowering stage causes the most serious cold-weather damage in soybean production in Northern and Eastern Hokkaido, Japan. Cold-weather damage occurs once every four years in this area on the average (Tanaka, 1997). Poor growth, abscission of flowers and pods, and insufficient grain filling are the three major factors reducing soybean yields affected by low temperature (Yamamoto and Narikawa, 1966). Although the most common damage by low temperature is poor growth, abscission of flowers and pods causes the most severe damage in soybean yield (Matsukawa, 1994). There is a strong negative correlation between the temperature in July, at the flowering stage of soybean, and grain yield in Hokkaido (Horiguchi, 1980). Many publications have documented that a low temperature around the flowering stage decreases the total number of pods and grain yield of soybean. Cooling treatment before flowering (Toriyama and Toyokawa, 1957; Saito et al., 1970) and during the flowering period (Saito and Takazawa, 1962; Hume and Jackson, 1981; Michailov, et al., 1989) cause severe pod abscission. Goto and Yamamoto (1972) reported that exposure to a low temperature for 15 days before flowering decreased pollen activity and the density of pollen grains deposited on the stigma.

Several researchers documented a varietal difference in the response to low temperature during the flowering stage in field experiments. Szymer (1985) counted the total number of flowers and pods in cold-weather years, and concluded that cold-weather tolerant varieties show higher pod-setting rates. Our previous report (Kurosaki and Yumoto, 2003) showed that the yield components—number of pods per plant, ovules per pod, grains per pod and seed size—of a cold-weather sensitive variety are more susceptible to cold-weather damage than those of a cold-weather tolerant variety. Furthermore, the report clarified that the greatest factor giving tolerance to low temperatures is pod number.

Although pod number is the key factor of cold-weather tolerance, it is important to study the mechanism of the tolerance in detail. In this experiment, we examined the effects of short-term and long-term exposure to a low temperature on pod-setting rate in cold-weather sensitive and tolerant varieties, and found that the pod-setting rate varied with the period of exposure to a low temperature. From the results, we suggest that the fertilization is a key process in acquiring a high pod-setting rate and superior cold-weather tolerance.

Materials and Methods

1. Plant materials

Two determinate cultivars, which have a similar maturity, plant type and seed yield in normal years were chosen for the experiment. Both were bred at the Hokkaido Prefectural Tokachi Agricultural Experiment Station. One was Toyomusume with a yellow seed coat, yellow hilum, gray pubescence and purple flowers (I/I, t/t, r/r, O/O, W/I/WI; genotypes were summarized by Palmer and Kilen (1967) ), which is a standard variety...
sensitive to low temperatures. Toyomusume was released in 1986 (Sasaki et al., 1988) and has been a major variety grown in Hokkaido because of the excellent seed quality.

By contrast, Hayahikari has a yellow seed coat, brown hilum, tawny pubescence and white flowers (i-i, i-i, T/T, T/i, O/O, w1/w1). Hayahikari showed the lowest seed yield reduction among the soybean varieties and lines, in 1993, the year with the most severe cold-weather damage in the last 50 years. Since then, field experiments in cooler locations and experiments at a low temperature in a phytotron were conducted. Hayahikari showed excellent cold-weather tolerance in those experiments, and was released in 1998 as one of the most cold-weather tolerant varieties (Yumoto et al., 2000).

2. Experiment I: Effects of low temperatures on pod setting

Experiments were conducted from May to October in 1996 at the Hokkaido Prefectural Tokachi Agricultural Experiment Station (42°35’N, 143°03’E. 95 m above sea level). On 23 May, twelve seeds were planted in pots (1/2000 a), filled with dry Andosol supplemented with a synthetic fertilizer (0.6 N - 5.3 P₂O₅ - 2.7 K₂O kg⁻¹) and watered regularly. Two weeks after emergence, pots were thinned to two plants per pot.

During experiments, pots were grown in an experimental facility having a plastic roof without walls. The average temperature during the growth period was 15.1°C, which was 1.0°C lower than the average temperature of the last ten years.

Thirty plants of each variety were grown in pots (two plants per pot) in the experimental facility. For cooling treatments, 20 plants were transferred to a phytotron. The air temperature in the phytotron was set at 18°C/15°C (day/night), because in the year with cold-weather damage, the average and minimum temperatures of every ten days during the flowering season were from 16°C to 19°C and from 12°C to 14°C respectively. The cooling treatments started at the beginning of flowering, 19 July in Toyomusume and 22 July in Hayahikari. Since shading during low-temperature treatment is known to stimulate pod abscission (Kurosaki and Yumoto, 2003), the plants were shaded (50% shade) during the cooling treatments. The two low-temperature regimes in the phytotron are shown in Table 1. For the short-term treatment group, the low temperature stress was applied for two weeks. For the long-term treatment group, it was applied for four weeks. Ten plants of each variety were assigned to each group. The remaining ten plants of each variety were used as the control.

From the beginning of flowering for six weeks during which all flowers opened, the number of flowers in four out of the ten plants in each group were counted and marked with different colored strings. The total number of flowers was calculated for each week. Flowers that opened during the 1st, 2nd, 3rd, 4th, 5th and 6th week after the initial flower opened are referred to as F1W, F2W, F3W, F4W, F5W and F6W, respectively. At maturity, the number of pods and weight of seeds were measured for each plant.

Pots in the phytotron and in the experimental facility were randomly rearranged at least once a week. The significance of the difference between the treatments in each variety was determined by the Tukey-Kramer multiple method for the number of pods and weight of seeds. The pod-setting rate each week was calculated as follows:

\[
\text{The pod-setting rate} = \left( \frac{\text{The number of pods}}{\text{The number of flowers}} \right) \times 100
\]

3. Experiment II: Effects of low temperatures on pollination

Toyomusume and Hayahikari were cultured as in Experiment I, from 15 June 1996. The cooling treatment [18°C/15°C (day/night)] was similarly applied for a week from the beginning of flowering.

Flowers were sampled every two days from the beginning of flowering for seven days. Stigmas were cut from the sampled flowers and stained with Cotton-blue solution. Then, the number of stained pollen grains on each stigma was counted using a microscope with magnification ×200.

The flowers were classified according to the method used for Adzuki bean reported by Shimada (1990). Limited research has been done on the number of pollen needed for a successful fertilization in soybeans even under optimal conditions, probably due to the miniscule size of the soybean stigma. Shimada categorized flowers of adzuki bean by the number of pollen grains on stigma, which has several times larger stigma than soybean stigma, and reported that more than 100 pollen grains are required for a successful fertilization.

In this experiment, the flowers of soybean were classified into three categories: flowers having more than 100, those with 50 – 100 and those with less than 50 pollen grains on each stigma. A ratio of the flowers classified into the three categories was calculated.

Since an error was assumed to follow a poisson distribution in this analysis, a logarithmic transformation was used to improve the homoscedasticity. The effect of the low temperature treatment was tested by the likelihood
ratio test. The procedure GENMOD of SAS was used for the calculation.

4. Experiment III: Effects of low temperatures on fertilization

The materials and methods were the same as in Experiments I and II, but the plants were planted on 23 June in 1997. The plants were exposed to short-term low temperatures by the same method as in Experiment I.

Flowers were marked 1, 3, 5 and 7 days after opening. The marked flowers were continuously exposed to low temperature, and very young pods and abscised flowers were sampled during the cooling treatment. The sampled pods and flowers were fixed in formalin-acetate-alcohol (FAA) solution and observed under a microscope with magnification ×30. The pods with one or more fertilized ovules were counted as fertilized flowers, and the pods without fertilized ovules as unfertilized flowers. The rate of fertilization was calculated as follows:

\[ \text{percentage of fertilized flowers} \% = \frac{\text{fertilized flowers}}{\text{total flowers} \times (\text{fertilized flowers} + \text{unfertilized flowers} + \text{abscised flowers})} \times 100. \]

In this analysis, since an error was assumed to follow a binomial distribution, a logit transformation was used to improve the homoscedasticity. The effect was tested by the likelihood ratio test. The procedure GENMOD of SAS was used for this calculation, too.

Results

1. Effects of low temperatures on the number of pods per plant and seed yield

Although the seed yield in the control group was nearly the same in both varieties, Hayahikari showed higher tolerance to the low temperature than Toyomusume (Table 2). The low temperature treatments significantly \((P<0.05)\) decreased the seed yield in

| Variety   | Treatment | First flower opened | Last flower opened | Maturity | Pod number per plant | Seed yield per plant |
|-----------|-----------|---------------------|--------------------|----------|----------------------|---------------------|
|           |           | date                | date               | date     | (g)                  | (g)                 |
| Toyomusume| Control   | 19,July 14,Aug      | 1,Oct              | 29.5 (100)| a 17.4 (100) a       |                     |
|           | Short-term| 19,July 24,Aug      | 5,Oct              | 29.2 (99) | a 14.1 (81) b        |                     |
|           | Long-term | 19,July 28,Aug      | 15,Oct             | 16.1 (55)| b 4.3 (25) c         |                     |
| Hayahikari| Control   | 22,July 16,Aug      | 25,Sept            | 35.1 (100)| ab 17.3 (100) a      |                     |
|           | Short-term| 22,July 21,Aug      | 30,Sept            | 37.9 (108)| a 15.0 (87) b        |                     |
|           | Long-term | 22,July 30,Aug      | 14,Oct             | 32.8 (95)| b 11.9 (69) c        |                     |

See Table 1 for cooling treatments.
Within columns, means followed by the same letter are not significantly \((P=0.05)\) different by the Tukey-Kramer multiple method.
Parentheses are percent of control.

Fig. 1. Total number of flowers and pods per plant in the plants exposed to low temperatures for two weeks (short-term treatment) and four weeks (long-term treatment).

- Pods, - Flowers, Vertical bars: standard error.
2. Effects of low temperatures on flowering and pod setting

The total number of flowers per plant in the control was 77.3 and 76.4 per plant in Toyomusume, in Hayahikari, respectively, and the cooling treatments increased the number in both varieties (Fig 1). The total number of flowers was 86.3 per plant in the short-term treatment, and 92.4 in the long-term treatment in Toyomusume, and 84.8 and 92.6, respectively in Hayahikari.

Fig 2 shows the effects of low temperatures on flowering and pod setting at each week. In the control group, F1W and F2W accounted for a major proportion of the total flowers in both varieties, and flowering was completed within four weeks. Cooling treatment prolonged the period of flowering to six weeks (except for the short-term treatment in Hayahikari), but no plants opened
flowers after the seventh week. In the short-term treatment of both varieties, fewer flowers opened in F1W and F2W than in the control, but the number of flowers increased in the third and fourth weeks in both varieties.

In the control, pod setting completed within two weeks in both varieties, and no pod was set in the third and fourth weeks. The pod-setting rates of F1W and F2W in the control were 60% and 41%, respectively in Toyomusume, and 72% and 36%, respectively in Hayahikari (Fig 3). In the short-term treatment, pods set even in the fourth and fifth weeks, and many pods were set after the cooling treatments in both varieties. Similar to the number of flowers, the cooling treatment decreased the number of pods during the short-term treatment (F1W and F2W) compared to the control in both varieties. For F1W, however, the effect of the low temperature on pod setting was different with the variety. The pod-setting rate of F1W was 33% in Toyomusume and 83% in Hayahikari. The effect of short-term treatment on the pod-setting rate of F2W was almost the same in both varieties. Little damage was observed in F2W, and a similar number of pods developed in both varieties. The pod-setting rate was 45% in Toyomusume, and 51% in Hayahikari. The pod-setting rate of F3W in the short-term treatment was as high as that of F2W in the control in both varieties, i.e., 56% in Toyomusume and 52% in Hayahikari (Fig 3).

In the long-term treatment, the number of flowers in F3W and F4W were higher than those in the control (Fig 2). Many flowers opened in the fifth week after the cooling treatment in both varieties. In the long-term treatment, the number of F3W was less than that in the short-term treatment. However, the number of F4W was similar in both long- and short-term treatments. In the fifth week, the number of flowers in the long-term treatment was much higher than that in the short-term treatment (Fig 2).

The effect of the long-term treatment on pod setting was also greater than that of short-term treatment. In the first two weeks, the effect of the low temperature on pod setting varied with the variety; the pod-setting rates of F1W and F2W were very low in Toyomusume, but those in Hayahikari were approximately equal to those in the control (Fig 3). The pod-setting rate in the long-term treatment was 9% in F1W and 8% in F2W in Toyomusume, while they were 60% and 59%, respectively in Hayahikari.

In F3W, the pod-setting rate was 52% in Hayahikari, but it was only 26% in Toyomusume (Fig 3). In F4W, however, the number of pods and the pod-setting rate in the long-term treatment were approximately the same (about 30%) in both varieties.

Fewer pods were set after the cooling treatment in the long-term treatment than the short-term treatment for both varieties. Hayahikari set pods 93% of those in control by the fourth week, and little pod setting was observed in the fifth or sixth weeks. In contrast, the pod number of Toyomusume by the fourth week was only 45% of that in the control. Toyomusume continued to set some pods in the fifth and sixth weeks. However, the pod-setting rate in Toyomusume was 23% in F5W and 12% in F6W. Thus, the total number of pods in Toyomusume in the long-term treatment was only 55% of that in the control at maturity.

3. Effects of low temperatures on pollination and fertilization

On the first day of the cooling treatment, the ratio of flowers which had more than 100 pollen grains on each stigma was 76% in Toyomusume and 81% in Hayahikari (Fig 4). This ratio decreased with time during the cooling treatment rapidly in Toyomusume, but slowly in Hayahikari (Fig 4). The ratio of flowers with different pollen grain number was significantly different (P = 0.01) between varieties (Table 3). On the seventh day of the cooling treatment, the ratio of flowers which had more than 100 pollen grains on each stigma was 22% in Toyomusume, and 46% in Hayahikari.

The percentage of fertilized flowers on the first day of the cooling treatment was near 100% in both varieties, and it greatly decreased with time in Toyomusume, but only slightly in Hayahikari (Fig 5). A reduction in the percentage of fertilized flowers on the seventh day was 89% in Toyomusume and only 32% in Hayahikari. The difference was significant at the P < 0.01 level (Table 4).

Discussion

1. Effects of low temperatures on pod number

In this research, the number of pods per plant was not significantly decreased by the short-term cooling treatment in either variety. Saito (1972) reported similar results. He found that the total pod number was slightly reduced by two weeks of cooling treatment.

In this study, however, the pod-setting rates of Hayahikari and Toyomusume were greatly different, even in the short-term treatment. The pod-setting rate of F1W in Hayahikari was 50% higher than that in Toyomusume. The high tolerance of Hayahikari to low temperatures could be attributed to the superior pod setting ability under low temperature conditions. The difference in cold-weather tolerance between Hayahikari and Toyomusume was more evident in the long-term treatment, in which the pod number was 93% of the control in Hayahikari, but only 55% of the control in Toyomusume.

2. Effect of short-term cooling treatment on pod setting

The total number of pods was not significantly decreased by the short-term treatment in either variety. The effect of the low temperature on pod setting was nearly the same in F2W in both varieties. However, the pod-setting rate of F1W in Hayahikari was as high as
Fig. 4. Ratio of flowers classified by pollen number on stigma in the plants exposed to a low temperature from the beginning of flowering.

Table 3. Likelihood ratio test for the ratio of flowers classified by pollen number on stigma.

| Source     | df | Chi Square | Probability |
|------------|----|------------|-------------|
| Variety    | 1  | 1.43       | 0.23        |
| Category   | 2  | 48.38      | <0.01       |
| Variety × Category | 2 | 9.20       | 0.01        |

Fig. 5. Effect of the cooling treatment on the percentage of fertilized flowers.

- Hayahikari
- Toyomusume

(•) : Number of examined flowers.

that in the control, although it was apparently lower in Toyomusume. As F1W were exposed to cooling treatment for two weeks, the pod-setting rate varies according to tolerance level even in the case of short-term low temperature exposure.

As described above, the total number of pods was not decreased by the short-term cooling treatment in either variety, because many pods were set after the treatment to compensate for the decreased pod number due to the cooling treatment. Approximately 70% of the total pods in Toyomusume were set after the short-term treatment and 40% in Hayahikari. This phenomenon can be explained by an increased number of flowers after the short-term treatment. Low temperatures damaged flowers which opened during the cooling treatment in Toyomusume but not in Hayahikari.

Suppressed flower development resulted in fewer flowers to bloom during the short-term cooling treatment in both varieties. After the short-term cooling treatment, the plants produced more flowers to compensate for decreased pod setting. As a result, the total number of flowers in the short-term treatment was higher than the control. After the cooling treatment, many flowers were able to set pods because they did not receive severe damage from the short-term exposure to a low temperature. These results indicate that a lower rate of pod setting during short-term treatment can be compensated by pod setting after the cooling treatment.

3. Effect of long-term cooling treatment on pod setting

A greater varietal difference in the number of pods per plant was observed in the long-term treatment than in the short-term treatment. The total pod number in Toyomusume was reduced nearly 50% by the long-term cooling treatment, but that in Hayahikari was reduced only less than 10%. The varietal difference in the total pod number in the long-term treatment was directly related to the difference in pod setting ability at low temperatures. In Toyomusume, the pod-setting rates of F1W and F2W in the long-term treatment were less than one third of those in the short-term treatment. However, in Hayahikari, the pod-setting rates of F1W and F2W in the long-term treatment were only slightly less than those in the short-term treatment. Although the pod-setting rate of F4W in the long-term treatment was
almost the same in both varieties, the pod-setting rate of F3W in the long-term treatment was about 20% lower than that in the short-term treatment in Toyomusume. On the other hand, in Hayahikari, the pod-setting rate of F3W in the long-term treatment was almost equal to that in the short-term treatment. Thus, Hayahikari had a higher pod setting ability, even in the long-term low temperature. In the meantime, the long-term cooling treatment greatly reduced the pod setting ability of Toyomusume.

Toyomusume had poor compensative pod setting after the long-term cooling treatment. In cold-weather sensitive varieties, damage caused by a low temperature for a short period can be compensated by pod setting after the stress. However, damage caused by a low temperature over a long period cannot be effectively compensated. Flowers exposed to a low temperature for a long-term before flowering seem to receive lethal damage and cannot develop into pods even after the removal of stress. The varietal difference in the total pod number in the long-term treatment was attributed to the difference in the pod setting ability of F1W and F2W. F1W and F2W in the long-term treatment were exposed to a low temperature two weeks longer than those in the short-term treatment. The results show that low temperatures for more than two weeks after flowering cause severe flower abscission in cold-weather sensitive varieties such as Toyomusume. On the other hand, Hayahikari showed a high tolerance to low temperature stress in pod setting.

4. Mechanism of tolerance to low temperature

The pod-setting ability of F1W exposed to low temperatures was higher in Hayahikari, than in Toyomusume. This suggests that the varietal difference in cold-weather tolerance is caused by the difference in the pollination and fertilization of flowers opened in the first week during exposure to a low temperature.

It can be considered that the effects of low temperatures on the fertilization are related to two floral organs. One is the low temperature tolerance of a stamen and the other is that of a pistil. In this experiment, we did not investigate the varietal difference in the low temperature tolerance of pistils, because Goto and Yamamoto (1972) reported that a stamen is more susceptible to low temperature than a pistil. We suspected that the varietal difference to low temperature in stamens would be existed.

In the present study, pollination was reduced by low temperature treatment in both varieties, and the effect of low temperature was significantly smaller in Hayahikari than in Toyomusume (Fig. 4). Furthermore, the number of pollen deposited on stigma was closely correlated with the percentage of fertilized flowers (Fig. 5). Hayahikari showed a significantly higher percentage of fertilized flowers than Toyomusume. Since a close relationship was observed between the pollen number per stigma and the percentage of fertilized flowers, we concluded that the effects of low temperatures on pollination are closely related with the fertilization of flowers. A superior fertilization ability at a low temperature is one of the factors required in cold-weather tolerance.

Conclusion

The total number of pods was not significantly decreased by a short-term (two weeks) exposure to a low temperature even in cold-weather sensitive varieties because of the post-stress pod setting. However, the pod setting ability during the low-temperature period varied with the variety. Cold-weather tolerant varieties had a superior pod setting ability, but cold-weather sensitive varieties had a low pod-setting ability under low-temperature conditions. The total number of pods after a long-term (four weeks) exposure to a low temperature was lower in cold-weather sensitive varieties than in tolerant varieties. Cold-weather tolerant varieties showed a greater capacity for pod setting during low temperature stress. On the other hand, cold-weather sensitive varieties sustain damage not only during low temperature stress but also after it. The superior pod setting ability at a low temperature was correlated with an effective fertilization at low temperatures.

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