Roles of Auxin and Cytokinin in Soybean Pod Setting

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Abstract: Soybean plants differentiate abundant floral buds, but most of them fail to grow pods and abort during development. Many studies indicated promotive effects of exogenously applied cytokinin on pod setting, but the effects of auxin application on pod set are ambiguous. In this study, we examined the changes in the concentrations of endogenous auxin and cytokinin in racemes and the effects of application of the two hormones on pod setting to clarify the role of auxin and cytokinin in soybean pod setting. The long-raceme soybean genotype IX93-100 was grown in pots and in the field. The auxin (IAA, indoleacetic acid) concentration in racemes was high for a long period from pre-anthesis to 9 days after anthesis (DAA) of the first flower on a raceme, but the cytokinin concentration was high for a short period, with a peak at 9 DAA. The IAA concentration was higher in distal portions of racemes, but the cytokinin concentration was higher in basal portions of racemes. In pot-grown plants, IAA applied to racemes tended to reduce the number of flowers and pods. In contrast, 6-benzylaminopurine (BA) applied to racemes before anthesis tended to reduce the number of flowers and pods, and that applied around 7 DAA significantly increased the pod-set percentage. However, these effects of IAA and BA application were slight in field-grown plants. These results indicate that the concentration of endogenous auxin and cytokinin in racemes changes in a different manner, and that cytokinins have a positive, and auxin a negative effect on pod setting when respective hormones are applied to racemes after the anthesis stage.

Key words: Auxin, Cytokinin, Flower abortion, Pod set, Sink formation, Soybean.

Previous studies indicated that the grain yield of soybean is determined by the number of pods more decisively than by other yield components (Schou et al., 1978; Kokubun, 1988). Soybean plants differentiate abundant floral buds, but a large proportion of them abort during development (Kato, 1964; Abernethy et al., 1977): under normal conditions, pod set percentage is only 20 to 40% (Wiebold et al., 1981; Antos and Wiebold, 1984; Jiang and Egli, 1993). By reducing this abortion, it might be possible to increase the pod number, thereby increasing the yield (Kokubun, 2004).

Numerous precedent studies showed two putative factors involved in controlling the abortion: availability of photosynthate or nutrients for pod development (Breedan et al., 1978; Antos and Wiebold, 1984; Brun and Betts, 1984; Heitholt et al., 1986a, b) and availability of certain hormones (Huff and Dybing, 1980; Heindel et al., 1982; Spollen et al., 1986a, b; Carlson et al., 1987; Yarrow et al., 1988; Kokubun and Honda, 2000; Yashima et al., 2005).

Within individual racemes, the pod set percentage of basal flowers is considerably higher than that of distal ones (Wiebold et al., 1981; Spollen et al., 1986a; Saitoh et al., 1999): this phenomenon appeared to be associated with the endogenous cytokinin content of flowers; the basal flowers contained a higher percentage of cytokinin than the distal flowers (Kokubun and Honda, 2000). Numerous studies showed the promotive effects of cytokinin (BA, 6-benzylaminopurine) applied to racemes on the pod formation (Crosby et al., 1981; Dyer et al., 1987; Peterson et al., 1990; Mosjidis et al., 1993; Nagel et al., 2001; Yashima et al., 2005), suggesting that cytokinin plays an important role in increasing pod number in soybean. The promotive effect was clear in pot-grown plants, but it was inconsistent in field conditions, suggesting that the promotive effects might be influenced by growing conditions (Cho et al., 2002).

A few investigators suggested the participation of auxin in regulating pod setting in soybean. Huff and Dybing (1980) applied extracts from flowers at different growth stages to growing flowers, and found that the extract from young pods accelerated the abortion of flowers. Among the hormones applied in the same way, indoleacetic acid (IAA) had an abortion promoting effect similar to that of the extract, but abscisic acid (ABA), gibberellin (GA) and 6-benzylaminopurine (BA) had no significant effect. In contrast, Oberholster et al. (1991) showed...
that IAA delayed the abortion. Thus, the views on the role of IAA in soybean pod setting are conflicting. Probably, the differences in genotypes or growing conditions in those experiments might have caused the inconsistency.

These previous studies underscore the necessity of studying the roles of both hormones in pod setting be examined simultaneously using the same genotype and under the same growing conditions. In this study, we examined the endogenous change of auxin and cytokinin in racemes and the effects of these hormones (IAA and cytokinin) applied to racemes on pod setting.

**Materials and Methods**

1. Changes in endogenous concentrations of auxin and cytokinin in racemes (Exp. 1)

   (1) Plant materials

   Soybean (*Glycine max* L. Merr.) genotype IX93-100 was used in this study. This genotype has long racemes (up to ca. 10 cm), which facilitate treatment and sample collection. Within individual racemes, flowers open from the base to the tip at a rate of one or two flowers a day (Kokubun and Honda, 2000). The original seeds were provided by Dr. R.L. Nelson, USDA-ARS, Urbana, IL. The seeds were sown in the experimental field of Tohoku University on 28 May 2004 and 26 May 2005 at a spacing of 70 × 10 cm. Three seeds per hill were sown and thinned to a plant per hill after emergence. Nitrogen fertilizer was applied at a rate of 2 g m⁻² N as LP-70, a coated fertilizer that releases 80% of its total N in 70 days at 25°C in water (Chisso Asahi Fertilizer Co. Ltd., Tokyo, Japan). The other applied fertilizers were 10 g m⁻² of P₂O₅ and 10 g m⁻² of K₂O. The soil type was a fine-textured Terrace Yellow soil, clayey (Classification Committee of Cultivated Soils, 1996). The seeds were inoculated with a strain of *Bradyrhizobium japonicum* (Tokachi-noukyouren, Obihiro, Japan). The rainfall distributions were favorable in both years; consequently, there was no need to irrigate. Weeds were removed by hand and insects were controlled using insecticides.

   (2) Measurement of auxin and cytokinin content

   Racemes on mid-canopy main stems were sampled at successive intervals before and after anthesis of flowers. The samples were collected from 0900 to 1100 h at −3, 0, 3, 6, 9 and 15 days after anthesis (DAA) in 2004, and at 0, 5, 10, and 15 DAA in 2005. The DAA of individual racemes was determined according to the morphological change of control plants that were labeled with the date of anthesis. For each sampling, several racemes were collected from several plants. In 2004, whole racemes were used for analysis, but in 2005, racemes sampled after 5 DAA were divided into basal and distal portions according to the floral position on the rachis (1 - 4, 5 and above, from the basal position). Sampled racemes were weighed immediately, then frozen in liquid nitrogen and stored at −80°C until analysis.

   Sampled racemes (approximately 1 g FW) were homogenized in 10 mL of cold methanol (80%, v/v), then centrifuged at 10,000 g for 15 min. The residues were further extracted twice with 5 mL of 80% (v/v) methanol; the combined supernatants were then concentrated using a rotary evaporator. The condensed extract was dissolved in 30 mL of deionized water, and adjusted to pH 2.5 by adding 1 N HCl. The solution was mixed with the same amount of ethyl acetate in a separatory funnel, and was shaken for 15 min. Subsequently, the mixture was separated into two layers: ethyl acetate in the upper and water in the lower layer. Ethyl acetate was readded to the water portion, followed by shaking and separating into two layers; this procedure was repeated three times. For dehydation, the combined ethyl acetate layer was mixed with an excess of anhydrous magnesium sulfate and kept for approximately 12 h. Then the ethyl acetate layer was separated from the rest using a separatory funnel. After the ethyl acetate solution was concentrated using a rotary evaporator, it was dissolved in 1 mL of 5% (v/v) methanol and then loaded onto a Bond Elut C18 column and eluted successively with 60% (v/v) methanol. The effluent was used for determination of the IAA concentration. The combined water layer was concentrated using a rotary evaporator, then adjusted to pH 8.0 with 1N KOH. The solution was mixed with the same amount of water-saturated butanol and the mixture was separated into two layers: butanol and water. After repeating this procedure three times, the combined butanol layer was concentrated in a rotary evaporator. The condensed extract was dissolved in 1 mL of 5% (v/v) methanol, and loaded onto a Bond Elut C18 column. The effluent was used for determination of the cytokinin concentration. The concentration of IAA and cytokinin (t-ZR (trans-zeatin riboside) equivalent) in the samples were quantified using enzyme immunoassay (EIA) with PhytoTest plant growth regulator immunoassay detection kit t-ZR (Agdia Inc., ID, USA). The EIA procedures were based on the directions provided with the kit. Since t-ZR kit crossreacts with t-Z, the cytokinin concentration shown in the figures are the sum of the content of t-ZR and t-Z.

2. Effects of exogenous IAA and BA (Exp. 2)

   Plants were grown in the field and pots using the same genotype and soil type as Exp. 1.

   (1) Field experiment

   Three seeds per hill were sown in the experimental field of Tohoku University on 20 May 2003 at a spacing of 70 × 10 cm. After emergence, seedlings were thinned to one plant per hill. Nitrogen fertilizer was applied at a rate of 2 g m⁻² of N as LP-70. The other fertilizers applied were 10 g m⁻² of P₂O₅ and 10 g m⁻² of K₂O. The inoculation of seeds with a strain of
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Bradyrhizobium japonicum, and other cultural practices were conducted in the same manner as that described for Exp. 1.

For preparation of the IAA and 6-benzylaminopurine (BA) solutions, a certain amount of IAA or BA was dissolved in 1 N KOH and diluted to 0.1 mM (IAA) or 1 mM (BA) with 16 mM K-phosphate buffer (pH 6.4) containing 0.05% (v/v) Tween 80. Preliminary trials showed that the respective concentrations of IAA and BA were the most effective for flowering and pod setting. The solutions were dropped, using a syringe (0.05 mL per raceme) on racemes on the main stem at the 6th, 7th and 8th nodes from the base at intervals. IAA was applied at -7, 0, 7, 14 DAA, and BA at 0, 7, 14, 21 DAA. Control plants received the solution without IAA and BA.

The flowering, pod initiation and abscission of the treated racemes were monitored every several days until maturity. At maturity, seven plants each with treated racemes were harvested, and the number of pods and seeds, and grain yield of the treated nodes (6th, 7th and 8th nodes) were measured.

(2) Pot experiment

Five seeds per pot (16 cm diameter, 19 cm tall) were sown on 10 May 2004, and thinned to two plants per pot after emergence. Prior to seeding, 0.5 g of N, 2 g of P₂O₅, 2 g of K₂O and 5 g of slaked lime per pot were applied by mixing with soil. The seeds were inoculated with a strain of Bradyrhizobium japonicum. The plants were grown in a greenhouse until the application of IAA and BA, and thereafter grown outdoors. The plants were irrigated adequately.

IAA and BA were applied to racemes on the main stem (7th, 8th and 9th nodes from the base) in the same manner as that for the field experiment. IAA was applied at -7, 0 and 7 DAA, and BA at -7, 0, 7, and 14 DAA. At maturity, seven plants of each treatment were harvested, and the number of flowers and pods were measured along with the grain yield at the treated nodes (7th-9th nodes).

Results

1. Changes in the concentrations of endogenous auxin and cytokinin in racemes (Exp. 1)

Figure 1 shows both the pre-anthesis and post-anthesis change of the concentrations of IAA and
cytokinin in racemes. The IAA concentration was high from $-3$ to 12 DAA with a peak around 3 to 6 DAA. The cytokinin concentration started to increase rapidly after 3 DAA, with a sharp peak at 9 DAA; it then declined rapidly. The cytokinin peak was about one week later than that of IAA. The concentration of cytokinin in racemes was negligible before 3 DAA and after 15 DAA.

Figures 2 and 3 show the concentrations of IAA and cytokinin, respectively, in the basal and distal portions of the racemes. The IAA concentration was higher in the distal portion than in the basal portion, but the peak concentration of cytokinin was higher in the basal portion than in the distal portion. The patterns of the change in the concentrations of IAA and cytokinin in both portions were similar to those in the whole racemes (Fig. 1 - Fig. 3).

2. Effects of exogenous IAA and BA (Exp. 2)

(1) Field experiment

Figure 4 shows the number of flowers, the number of pods, the pod-set percentage, and the grain yield at the nodes treated with IAA and those in control plants. The values of these yield components of IAA-applied plants were not significantly different from those of control plants irrespective of the time of IAA application. IAA applied at $-7$ DAA increased the pod-set percentage, the number of pods and grain yield (compared with control plants) although the effects were not significantly different ($P<0.05$). On the other hand, IAA applied after anthesis slightly decreased or had no effect on the values of these yield components.

Figure 5 shows the effects of exogenous BA on yield components. BA applied at 0 DAA slightly decreased the pod-set percentage and the number of pods, resulting in a significant decrease in the grain yield ($P<0.05$). BA applied later had no marked effect.

(2) Pot experiment

Table 1 summarizes the effects of IAA and BA applied to racemes on yield and yield components in pot-grown plants. In contrast to the results of the
experiment with field-grown plants, significant effects of IAA and BA were observed in the pot experiments; the IAA applied at 0 and 7 DAA reduced the pod-set percentage and the number of pods, reducing the grain yield, whereas BA applied at 7 DAA significantly increased the pod-set percentage and moderately increased the number of pods and the grain yield.

Individual racemes in the mid-canopy of the plants produced 15-20 flowers per raceme. Within each raceme, the pod-set percentage was higher at the basal positions, and most distal flowers abscised. IAA applied to the mid-canopy racemes at 0 or 7 DAA did not markedly affect the pod-set percentage, whereas BA applied at 0 or 7 DAA markedly increased the pod-set percentage of the flowers at position 3 and above of the racemes (Fig. 6).

Discussion

The concentration of endogenous cytokinin in racemes increased rapidly several days after anthesis, with a peak around 9 DAA. The concentration was higher in the basal portion of the racemes than in the distal portion (Figs. 1 and 3), which confirmed our previous observation (Kokubun and Honda, 2000). The amounts of cytokinins in xylem sap exudate from the stem were high at 0-9 DAA (Carlson et al., 1987) and during late flowering or early pod formation stage (Heindl et al., 1982). The pattern of the cytokinin level detected in racemes in the present study resembled that detected in the xylem sap exudate, suggesting that the cytokinins synthesized in roots were transported through the xylem to racemes.

The cytokinin (BA) applied to racemes around 7 DAA significantly increased the pod-set percentage (Table 1). BA applied to the racemes markedly increased the pod-set percentage of flowers at position 3 and above on the rachis (Fig. 6). This positive effect of cytokinin application on pod formation has been found in numerous previous studies (Crosby et al., 1981; Carlson et al., 1987; Dyer et al., 1987; Peterson et al., 1990; Mosjidis et al., 1993; Reese et al., 1995; Nagel et al., 2001). In the present study, each flower in the basal portion (1-4 from the base) of a raceme initiated a pod 5-7 days after anthesis. The growth stage at
which cytokinin application was effective corresponds to the stage at which endogenous cytokinin levels were high (Fig. 1), indicating that whether fertilized zygotes initiate pods or abort is determined several days after anthesis. Kato et al. (1964) observed that abortion occurred most frequently at initial stages of proembryo development (3-7 days after fertilization). They ascribed the abortion to water deficit in plants because the abortion frequency increased under an inadequate water supply. Competition among seeds and organs for photosynthate is also considered an important factor causing abortion (Shibles et al., 1975). In this regard, our previous study demonstrated that the pod-set percentage can be increased by applying cytokinin to racemes at high levels of assimilate availability (Yashima et al., 2005). Although the exact mode of action of cytokinin remains unclear, cytokinin seems to play a pivotal role in enhancing early development of fertilized embryos.

The relationship of structure with activity of the various endogenous cytokinins has been extensively studied by a number of bioassays. Among the cytokinins, t-Z, isopentenyl adenine, and their 9-ribosyl are generally very active (McGaw and Burch, 1995). There is little information on the structure/activity relationship in soybean pod-setting. The cytokinin concentrations peaked at the late flowering and early podding stage both in xylem sap (Heindl et al., 1982; Carlson et al., 1987) and racemes as shown in this study and our previous paper (Kokubun and Honda, 2007).

Table 1. Effects of IAA and BA application on the number of flowers, pod-set percentage, number of pods and grain yield at the applied nodes (Exp. 2, pot, 2004). IAA and BA were applied to racemes at intervals before or after anthesis.

| Time of application (DAA) | Control | −7 | 0 | 7 | 14 |
|--------------------------|---------|----|---|---|----|
| Number of flowers per applied nodes | IAA | 63.0a | 64.3a | 51.3a | 51.3a |
| Pod-set percentage (%) | IAA | 22.8a | 18.7ab | 17.4b | 15.1b |
| Number of pods per applied nodes | IAA | 14.2a | 12.0ab | 8.7bc | 7.7c |
| Grain yield of applied nodes (g) | IAA | 5.4a | 4.1ab | 3.1b | 2.9b |

Values represent the mean values of six plants. Values followed by the same letter within one line are not significantly different at the 0.05 probability level, based on Tukey’s multiple range test.

Fig. 6. Effects of IAA or BA application on pod-set percentage of flowers versus floral position on rachis (Exp. 2, field, 2003). IAA or BA was applied to racemes at different times before and after anthesis (−7 to 7 DAA for IAA, −7 to 14 DAA for BA). C. Control.
suggesting that cytokinins produced in root at this stage were exported to racemes and promoted pod-setting. However, the predominant cytokinins in xylem and racemes were different: they were t-ZR and dihydrozeatin in xylem sap, but e-ZR and isopentenyl adenosine in racemes. More precise monitoring of the various kinds of cytokinins along the transport from root system to racemes is necessary to understand the structure/activity relationship of cytokinin in pod-setting.

The endogenous IAA concentration in racemes was high for a longer period and peaked earlier than the endogenous cytokinin concentration (Fig. 1). The concentration of IAA was higher in distal portions of the racemes than in the basal portion, which was the reverse of the result for cytokinin (Figs. 2 and 3). No reports described the endogenous change in IAA concentrations in soybean racemes during reproductive growth. Therefore, this is the first report that demonstrated a pattern of change in IAA concentrations in soybean racemes.

Contrary to cytokinin application, IAA application to racemes did not promote pod-setting: IAA application to racemes tended to increase the number of flowers when applied before anthesis, but it reduced the number of flowers and pods when applied after anthesis (Table 1). Huff and Dybing (1980) attempted to identify the substances that are responsible for differences in abortion percentage among flower positions on racemes. They applied the extracts from flowers of different growth stages to the basal part (positions 1-3) of racemes, and found that the extract from young pods promoted the abortion. Among the plant hormones tested, IAA was found to show a promotive effect. Beckmann (1981) showed that spraying of IAA on leaves also increased abortion of soybean flowers. The previous results and our present study indicate the possibility that changes in sensitivity to IAA levels depending on the growth stage might engender negative effects on pod-setting of IAA applied after anthesis.

In the application experiments, the significant effects of both hormones were observed only in pot-grown plants, and the effects were obscure in field-grown plants (Table 1, Figs. 4 and 5). Cho et al. (2002) also reported the instability of the effects of cytokinin application in field conditions. Since the plants were grown outdoors in different years, climate conditions may be responsible for the difference. Another possibility is that the pot-grown plants might be more responsive to an external hormone supply, at least to cytokinin, which is mostly produced in roots, because the restricted root zone in pots might limit the capacity of cytokinin synthesis. These possibilities need to be examined in future studies.

Acknowledgements

We thank the Takano Life Science Foundation for their financial support given to part of this study.

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*In Japanese with English abstract.