Control of Soybean Nodule Formation by a Crack Fertilization Technique

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Abstract: Enhancement of nitrogen fixation activities by a cultivation technique is one of the potential targets to improve soybean yield in Japan. A cultivation technique named “crack fertilization” is described and the nodulation control by this technique is analyzed by experiments in two fields with different soil and environmental conditions, and a root box experiment. Crack fertilization is a technique that utilizes the irregularly shaped soil cracks formed by subsoiling just before the flowering stage of soybean, and introduces root nodule bacteria, fertilizers, and so on to the deep subsoil layer. Production of ureide-form nitrogen at six weeks after the crack fertilization (only nodule bacteria application to the subsoil layer) was 1.4 times higher than the control in the Hikone field. Acetylene reduction activity at four or eight weeks after the crack fertilization (application of both nodule bacteria and low level of fertilizer to the subsoil layer) tended to be higher than in the control in both fields. In the root box experiment, nodule number was four times higher than that in the control at the lower portion of the root system, where modified crack fertilization treatment was conducted, and the acetylene reduction activity was increased significantly by the treatment. These results indicated that the soybean nodulation control, i.e., timing and position of nodulation, as well as the enhancement of nitrogen fixation activity could be achieved by this crack fertilization technique.

Key words: Acetylene reduction activity, Glycine max Merr., Nitrogen fixation, Nodule bacteria, Soybean production, Subsoiling, Ureide form nitrogen.

Soybean yield in Japan is significantly lower than the world average, although many studies have been conducted to increase the yield. Improvement of soybean yield by a cultivation technique is thus still important considering the present situation of food production in Japan. For example, enhancement of nitrogen fixation by a cultivation technique, not by the genetic improvement such as the use of supernodulating soybean cultivars (Takahashi et al., 2003, 2005; Nakamura et al., 2010), is one of the potential targets to improve soybean yield in Japan. In this paper, we report a new method of enhancing soybean nodulation by a cultivation technique called “crack fertilization” which is a combination of two existing concepts; deep layer fertilization (Takagi et al., 1963; Takahashi et al., 1991; Kaushal et al., 2004, 2006, 2007; Watanabe et al., 2007) and subsoiling during soybean growth (Hamada et al., 2003; Ochiai et al., 2004; Iijima et al., 2005; Honjo et al., 2007). Deep layer fertilization is an old concept (eg; Takagi et al., 1963) that has been well tested, and subsoiling during soybean growth is a technique that was first tested at the Aichi Agricultural Research Center in 2001 (Hamada et al., 2003).

Crack fertilization is a technique to utilize the irregularly shaped soil cracks formed by subsoiling just before the flowering stage of soybean, and introduce root nodule bacteria, fertilizers, and soil amendments to the deep subsoil layer (Fig. 1). Through this channel of cracks, aeration could be secured to a deep soil layer so that the applied bacteria can form nodules on the soybean roots distributed in the subsoil layer. Subsoiling during the plant growth period will cut a significant amount of soybean roots; hence, newly emerged deep roots will appear after root cutting (Honjo et al, 2007). These deep roots should offer the habitat for the nodule bacteria supplied to the deep soil layer together with the copious amount of air through the cracks during the maturing stage. Because...
nodule growth (Kon’no et al., 1990) and/or nitrogen fixation activity (Pfeiffer et al., 1983; Zapata et al., 1987) usually cease after the flowering stage and/or the early seed development stage, the newly formed root nodule in deep soil after the flowering stage may supply a significant amount of nitrogen to the growing seed resulting in higher soybean yield. However, it is not easy to introduce soybean nodulation in the deep soil layer in the ordinal upland field converted from paddy field in Japan, due to the wet condition in the deeper layer. The crack fertilization technique ensures that the ventilation channel extends to the deep subsoil layer just before the soybean flowering stage; the dry soil condition during mid-summer after the rainy season would most probably help maintain the soil crack structure, in most cases, for a relatively longer period.

In the first paper of the series of crack fertilization studies, we examined whether nodulation can be controlled by crack fertilization or not. Basic agronomic traits of soybean plants together with the nitrogen fixation ability were also analyzed to evaluate the efficiency of crack fertilization technique. The goal of this study was to examine the possibility of soybean nodulation control, i.e. timing and position of nodulation, by the newly developed crack fertilization technique.

Materials and Methods

1. Field Experiments

(a) Study sites

Nodulation control by the crack fertilization was tested at two locations with different soil and environmental conditions in the summer of 2007 and 2008. One is the experimental field of the University of Shiga Prefecture (Hikone; latitude 35º15’N, longitude 136º13’E, altitude 87 m asl), and the other is the experimental farm at the Aichi Agricultural Research Center (Nagakute; latitude 35º10’N, longitude 137º4’E, altitude 90 m asl). In Hikone, the total rainfall during the five-month period from June – October, the main growth season for soybean, was 928 mm (5 mm below the long-term average, LTA) and 627 mm (206 mm below LTA), respectively, in the

Fig. 1. Crack fertilization technique to control nodule formation in the deep soil layer. The depth of subsoiler blade was adjusted to 30 cm. The bottom blade was 6.5 cm in width and 27 cm in length.

Fig. 2. Monthly average temperature and total rainfall during the period of 2007, 2008 and 30 years average (1971–2000) at the experimental sites of Hikone and Nagakute. Sources; Aichi Agricultural Research Center and Local Meteorological Observatories of Hikone and Nagoya.
Table 1. Experimental set up to analyze the nodulation control by the crack fertilization technique.

| Crack formation | Nodule bacteria | Fertilization | Treatment |
|-----------------|-----------------|---------------|-----------|
| Crack           | Application     |               | Ck-FH     |
|                  | High            |               | Ck-FH     |
|                  | Low             |               | Ck-FL     |
|                  | Zero            |               | Ck-F0     |
| No application   | Zero            |               | Ck        |

Crk, Crack; F, Fertilizer; H, High; L, Low; Cnt, Control.

2007 and 2008 cropping seasons, and the average temperature was 23.4°C (0.9°C below LTA) and 23.5°C (0.8°C below LTA), respectively (Fig. 2). In Nagakute, the total rainfall in the same period was 863 mm (64 mm below LTA) and 724 mm (205 mm below LTA), respectively, in 2007 and 2008, and the average temperature was 22.9°C (0.5°C below LTA) and 22.8°C (0.4°C below LTA), respectively. In Hikone, the top soil in the field was light clay with pH of 7.02; total N, 1.75 g kg⁻¹; total C, 20.9 g kg⁻¹; CEC, 15.15 cmol kg⁻¹ (Zegada-Lizarazu et al., 2006). In Nagakute, the soil was also characterized as light clay with total N, 1.10 g kg⁻¹; total C, 12.5 g kg⁻¹; CEC, 15.5 cmol kg⁻¹.

(2) Treatments and field management

In Hikone, the field used in this experiment was for upland cropping, and soybean and rape (Brassica napus L.) were grown as summer and winter crops, respectively, in the previous year of the experiment. In Nagakute, the field was for paddy rice field and rice was grown as a previous year cropping. One day before sowing, the land was prepared and leveled with a rotary plough to a depth of 0.24m and 0.12m in Hikone and Nagakute, respectively. Before sowing, 20, 60, and 80 kg ha⁻¹ of each of N, P₂O₅, and K₂O were broadcasted and incorporated into the soil in Hikone. Fertilizer was not applied to the soybean cropping in Nagakute following the recommendation of Aichi Prefecture.

The effects of crack formation, nodule bacteria application, and fertilization were analyzed under the conditions shown in Table 1 in both fields. Crack formation (Crack treatment) was conducted during the soybean growth by subsoiling, and nodule bacteria were applied to the subsoil layer as the nodulation treatments. Compound synthetic fertilizer was applied at three levels; no fertilization treatment, low level of fertilization (N:P₂O₅:K₂O=20:20:20 kg ha⁻¹) and high level of fertilization (N:P₂O₅:K₂O=80:80:80 kg ha⁻¹) to the subsoil layer. Five different combinations from the 12 possible combinations of the above treatments (2×2×3) were chosen for the present field studies to test the validity of nodulation control by the crack fertilization technique: Ck-FH, high level of fertilizer and nodule bacteria were applied to the deep soil layer during subsoiling; Ck-FL, low level of fertilizer and nodule bacteria were applied to the deep soil layer during subsoiling; Ck-F0, only nodule bacteria were applied to the deep soil layer during subsoiling; Ck, nothing is applied to the deep layer during subsoiling; Cnt, no subsoiling was done as a control treatment. The five treatments were arranged with the completely randomized block design replicated for three times in both fields. The size of each plot was 11.3 m² in Hikone, and 23 m² (2007) and 31 m² (2008) in Nagakute, respectively. The total experimental area was 250 m² in Hikone, and 500 m² in Nagakute, respectively. The recommended cultivar in each prefecture was chosen as the test cultivar; Tamahomare in Hikone and Fukuyutaka in Nagakute. The seeds were sown on 20 June and 19 June in 2007 and 2008, respectively in Hikone, and on 21 June and 10 July in 2007 and 2008, respectively, in Nagakute. The row and intra-row distance were 0.6 and 0.2 m for Hikone and 0.75 and 0.16m in Nagakute, respectively. After the plant emergence and/or establishment, thinning and complementary planting were done to adjust the planting density to 8.33 plants m⁻² in both fields. Flowering in 2007 and 2008 started on 27 and 31 July in Hikone, and 12 and 15 August in Nagakute, respectively. Insect control was done by spraying MPP (Emulsifiable Concentrate; EC) or Etofenprox (EC) against stink bug and Fenvalerate plus MEP (Wettable Powder), Chlorfluazuron (EC), or Indoxacarb (Suspension Concentrate) against common cutworm. Harvesting in 2007 and 2008 was done at 80 (29 Oct) and 100 (31 Oct) days after the crack fertilization treatment (DAT), respectively, in Hikone, and at 99 and 93 DAT in Nagakute. Ten to 15 plants which showed representative growth in each replicate and treatment were harvested, and the yield and shoot biomass production were estimated.

(3) Crack fertilization

A test machine (Sukigara nouki Co Ltd) attached to a tractor (Yanmer CT 540 in Hikone and Kubota MZ75 in Nagakute) was used for the crack fertilization in both fields. The test machine was developed by using the subsoiler (S-28-1S; Matsuyama Co. Ltd) and fertilizing machine (VR-10; Jounishi Co. Ltd). The mixture of
fertilizer, soil, and soybean nodule bacteria was applied to approximately 25–30 cm deep soil layer along the blade of subsoiler. The mixture was applied to the center line of every other furrow. Soil cracks were mostly formed to the depth of the subsoiler blade. The shape of the cracks was not uniform but mostly irregular depending on the local difference of soil mechanical impedance and soil moisture content. These cracks will act as ventilation as well as drainage channels, but the extent of the function is still unknown due to the difficulty of quantification. The detail of the test machine structure will be reported elsewhere. Crack fertilization treatment was conducted on 10 August and 23 July in Hikone, and on 8 and 20 August in Nagakute in 2007 and 2008, respectively. Crack fertilization in Hikone 2007 and in Nagakute 2008 was done during early flowering time due to the delay caused by the rainfall and mechanical problem of the test machine. Nodule bacteria (both the commercial bacteria (Mame-zo; Tokachi agricultural cooperation) and cultured nodule bacteria originating from both field soils) were mixed with Akatama soil and used as the nodule bacteria inoculants. Akatama soil was applied at a rate of 33.3 kg ha$^{-1}$, and the commercial bacteria, Mame-zo, at a rate of 10 bags (each bag contains approximately 35 g of bacteria-soil mixture) per ha$^{-1}$. Isolation of nodule bacteria from the soil was conducted according to the general procedure using YM medium (Somasegaran and Hoben, 1985). Akatama soil inoculated with the nodule bacteria and compound fertilizer were applied to the subsoil layer in Ck-FH and Ck-FL and Ck-F0 treatments.

(4) Analysis of Nodule Activity

Soybean nodule activity was evaluated based on both acetylene reduction activity and ureide translocation rate. Soybean shoot, nodule, and xylem sap were sampled at different timing in each location and year as indicated in tables 2, 3, and 4. Soybean shoot was cut at 5 cm above the soil surface and xylem sap was collected for 1 h for the analysis of ureide translocation rates by Young–Conway method (Young and Conway, 1942). Then, root system was sampled with a 15 cm diameter core sampler for 2007 and by digging the soil, approximately 0.2 × 0.2 × 0.2 m cubic region around the soybean plant, at a hand shovel in 2008 in both fields. The root system was washed under tap water and a root nodule was carefully sampled for the analysis of acetylene reduction activity (Hardy et al., 1968). In 2007, only 10 representative fresh root nodules were selected from the root system inside the 15 cm core for the analysis of the acetylene reduction activity, and therefore, nodule formation was not analyzed. In 2008, nodules from the whole root system were used for the analysis of both acetylene reduction activity and nodule formation. Photosynthetic and transpiration rates were measured as a plant physiological parameter by the portable photosynthetic analyzer (LI-COR Biosciences, LI-6400) at different times in each location and year as indicated in table 5. Terminal leaflet of uppermost fully expanded leaf was chosen for the measurement, and three to five plants from each replicate field were measured from 1000 to 1100 h.

2. Root box study

The root box study was conducted at Nagoya University (latitude 35°9’N, longitude 136°58’E, altitude 55 m asl) in the summer of 2007. A loamy sand soil mixed with powdered synthetic fertilizer (N:P$_2$O$_5$:K$_2$O = 0.32:0.32:0.32 g kg$^{-1}$ soil) was filled in a root box (400 × 240 × 20 mm in length, width and thickness, respectively) at the bulk density of 1.50 g cm$^{-3}$ and placed under a vinyl shade. Four replicate boxes in each treatment, 20 boxes in total were used for the statistical evaluation. Pre-germinated soybean seed (Fukuyutaka) was directly planted to the root box on 27 June, and modified crack fertilization treatment (see Fig. 3) was conducted on 18 August (52 days after sowing). The front panel of the root box was carefully removed, and the lower half of the soybean root system was cut along the line indicated in Fig. 3 with a knife. Then nodule bacteria and fertilizers, which were the same as the treatments used in the field experiments, were applied to the cut line so that an effect similar to that of crack fertilization could be expected. Root cutting and application of bacteria and fertilizers to the lower root system were conducted just before the soybean flowering time. At 23 DAT (10 September), the root system was sampled following the
Nodule growth was generally enhanced by the crack fertilization treatments (Table 2). Nodule dry weight at 28 DAT in Nagakute was significantly increased in Ck-FL treatment as compared with the control. The maximum increment of nodule dry weight by the crack treatment at three to four weeks after the treatment was 2.3 and 1.7 times higher than the control in Hikone and Nagakute, respectively. Effects of nodule bacteria application can be evaluated by the comparison between Ck and Ck-F0 treatments. Nodule DW and number were slightly higher in Ck than in Ck-F0 at 19 DAT. At 28 and 40 DAT, however, opposite trend was found; Ck-F0 showed higher values than those of Ck. This indicated that nodule application was effective to enhance nodulation at four to six weeks after the treatment. Some of the crack fertilization treatment increased the nitrogen fixation activity as compared with the control. Ureide translocation rate was increased 1.4 times by Ck-F0 treatment in 2008 in Hikone at around six weeks after the treatment (Table 3). Acetylene reduction activity was also increased 1.9 (Ck 23 DAT in 2007) and 1.4−1.5 (Ck-F0 except for 23 DAT in 2007) times in Hikone and 1.9 (in 2007) and 3.3 (in 2008) times by Ck-FL treatment in Nagakute at three to eight weeks after the treatment although there was no significant difference from the control treatment (Table 4).

Photosynthetic rate was increased by the crack fertilization treatments, at one to three weeks after the method described elsewhere (Iijima and Kono, 1991; Iijima et al., 1991), and root nodules on the root system were sampled from both the upper and lower half of the root box. Thereafter, the acetylene reduction activity was measured to evaluate the nitrogen fixation activity and root dry weight of the two portions was measured after oven drying at 80°C for three days.

### Field experiments

In Hikone, year 2007 was a relatively cool year especially after August and significant rainfall was recorded in June (Fig. 2). Year 2008 showed more or less average trend. In Nagakute field, less rainfall was recorded in August and September although significant rainfall was received in July for 2007. Little rainfall was recorded in July 2008. Temperature in both years was near the average of the last 30 years.

### Table 2. Nodule formation of soybean as affected by crack fertilization during 2008 growing season.

|                  | Hikone      | Nagakute   |
|------------------|-------------|------------|
|                  | 19 DAT  | 40 DAT  | 28 DAT  | 19 DAT  | 40 DAT  | 28 DAT  |
| Cnt              | 0.42 (0.03)| 1.97 (0.29)| 3.49 (0.36)| 38 (5)  | 241 (15) | 683 (73) |
| Ck               | 0.78 (0.23)| 1.56 (0.34)| 3.62 (0.31)| 62 (13) | 201 (39) | 749 (125) |
| Ck-F0            | 0.71 (0.09)| 2.46 (0.19)| 4.45 (0.41)| 60 (5)  | 314 (32) | 914 (212) |
| Ck-FL            | 0.47 (0.16)| 1.71 (0.30)| 6.07 (1.60)| 45 (11) | 272 (37) | 1004 (327) |
| Ck-FH            | 0.99 (0.21)| 1.29 (0.23)| 3.84 (0.59)| 82 (21) | 204 (43) | 760 (124) |
| F Value          | 2.03     | 2.60     | 1.87     | 1.89    | 1.90    | 0.54     |
| Probability      | 0.17     | 0.10     | 0.19     | 0.19    | 0.19    | 0.71     |
| Ratio to Cnt     | −        | −        | −        | −       | −       | −        |
| Ck               | 1.84     | 0.79     | 1.04     | 1.63    | 0.84    | 1.10     |
| Ck-F0            | 1.67     | 1.25     | 1.27     | 1.58    | 1.31    | 1.34     |
| Ck-FL            | 1.10     | 0.87     | 1.74 *   | 1.18    | 1.13    | 1.47     |
| Ck-FH            | 2.33     | 0.65     | 1.10     | 2.15 *  | 0.85    | 1.11     |

|                  | Hikone      | Nagakute   |
|------------------|-------------|------------|
| DW, Dry weight; DAT, Days after treatment (crack fertilization); Cnt, Control; Ck, Crack; F, Fertilizer; H, High; L, Low. Values in parentheses indicate standard error of mean. * indicates significant difference from control at P<0.05 by Dunnett’s multiple comparison test.

3. Statistical analysis

In both experiments, one-way analysis of variance (ANOVA) was used for the statistical evaluation at first. F values, probability levels, and standard error of means were indicated in all the parameters. Degree of freedom was 10 and 15 for the experiment 1 and 2, respectively. In this study four similar treatments with subsoiling were conducted together with the control without subsoiling. Because of the similarity of the four treatments, paired comparison with control under a multiple comparison procedure was necessary for comparing several treatments simultaneously with a control. Therefore, Dunnett’s multiple comparison test (Dunnett, 1964) were used for the evaluation of the differences from the control at the level of P<0.05 using a software of Ekusuru-Toukei 2008 (Social Survey Research Information Co., Ltd.).

Results

### Field experiments

In Hikone, year 2007 was a relatively cool year especially after August and significant rainfall was recorded in June (Fig. 2). Year 2008 showed more or less average trend. In Nagakute field, less rainfall was recorded in August and
treatment; 1.2 times higher than control in Ck-F0 and Ck-FH at 25 DAT in Hikone (Table 5). The enhancement of photosynthetic rate was diminished at 47 DAT in Hikone 2007, and Ck-F0 was significantly lower than the control at 51 DAT in Hikone 2008. Transpiration rate was slightly higher in Ck-F0 at 33 DAT in Nagakute 2007. In Hikone,
similar trend of time course changes in transpiration rate were observed, although there were no significance differences. The shoot dry weight, in most of the crack fertilization treatments were generally lower than control at three weeks after the treatments, similar at four to seven weeks, and finally similar or lower at harvesting, although no statistical difference was observed (Table 6). Seed yield was not significantly influenced by the treatments at either location or year; at maximum, however, 15% higher yield in Ck-FL, as compared with the control, in Nagakute 2007.

**Table 5. Photosynthetic rate and transpiration rate of soybean as affected by crack fertilization.**

|                | Photosynthetic rate (μmol m⁻² s⁻¹) | Transpiration rate (mmol m⁻² s⁻¹) |
|----------------|-----------------------------------|----------------------------------|
|                | 2007                               | 2008                             |
|                | Hikone                             | Nagakute                         | Hikone                           | Nagakute |
|                | 6 DAT                              | 47 DAT                           | 6 DAT                           | 47 DAT |
| Cnt            | 4.4 (1.9)                          | 19.8 (0.9)                       | 12.5 (1.0)                      | 19.5 (1.4) |
| Ck             | 8.9 (2.3)                          | 22.7 (1.0)                       | 10.4 (2.6)                      | 19.3 (1.7) |
| Ck-FL          | 6.3 (3.3)                          | 22.7 (0.8)                       | 9.3 (1.4)                       | 21.3 (0.5) |
| Ck-FH          | 4.6 (2.3)                          | 23.7 (1.4)                       | 11.9 (2.4)                      | 19.0 (1.6) |
| F value        | 0.58                               | 2.64                             | 0.57                            | 0.92 |
| Probability    | 0.69                               | 0.10                             | 0.69                            | 0.49 |
| Ratio to Cnt   |                                   |                                 |                                 | 0.76 |

DAT, Days after treatment (crack fertilization); Cnt, Control; Ck, Crack; F, Fertilizer; H, High; L, Low. Values in parentheses indicate standard error of mean. * indicates significant difference from control at P < 0.05 by Dunnett’s multiple comparison test.

**Table 6. Shoot dry weight of soybean as affected by crack fertilization.**

|                | Shoot dry weight (g plant⁻¹) |
|----------------|------------------------------|
|                | 2007                         | 2008 |
|                | Hikone                       | Nagakute |
|                | 23 DAT                       | 47 DAT | 17 DAT | 49 DAT |
| Cnt            | 43.9 (6.9)                   | 67.8 (6.2) | 98.6 (10.2) |
| Ck             | 41.2 (5.0)                   | 69.1 (4.8) | 100.9 (8.2) |
| Ck-FL          | 37.0 (6.8)                   | 69.6 (9.3) | 99.2 (7.0) |
| Ck-FH          | 37.0 (7.0)                   | 77.1 (13.5) | 101.8 (6.8) |
| F value        | 0.47                         | 0.17                             | 0.34                            | 0.24 |
| Probability    | 0.76                         | 0.95                             | 0.84                            | 0.91 |
| Ratio to Cnt   |                            |                                  |                                 | 0.91 |

DAT, Days after treatment (crack fertilization); Cnt, Control; Ck, Crack; F, Fertilizer; H, High; L, Low. Values in parentheses indicate standard error of mean. No significant difference from the control was found at P<0.05 by Dunnett’s multiple comparison test.
and 8% higher in Nagakute 2008 were observed (Table 7). In Hikone, the seed yield was even lower in all the treatments as compared with the control in 2008.

2. Root box experiment

In the root box experiment, root dry weight in upper region was 1.4 times heavier in Ck-F0 than in the control although nodule number and size (average dry weight per single nodule) were not significantly modified by the treatment (Table 8). In contrast, the growth in the lower region of the root was modified by the treatment. Nodule number in the lower portion of the root box was significantly increased by most of the modified crack fertilization treatments (root cutting) except for Ck-FH. It was 4.5 and 4.3 times higher in Ck and Ck-F0, respectively, as compared with the control. The nodule size judging from the average dry weight per single nodule was significantly decreased by 70% in Ck-F0. The acetylene reduction activity in Ck treatment was decreased by 67% in upper region but was increased to 2.3 times in lower part of the root box as compared with the control. Although no statistical difference was observed, root growth in the lower region in Ck-FL treatment was 1.5 times greater than in the control. All the plants of the four replicate showed the enhanced root growth (from 1.2 – 2.0 times higher than the average value of control; data is not shown) after root cutting by adding the lower concentration of NPK to the soil layer. In the growth-enhanced root system, 3.4 times more root nodules grew newly. Their sizes, however, were only half of that in the control, and therefore, the young nodule can produce a 1.7 times larger amount of nitrogen although no significant difference was observed. These results indicated that crack fertilization treatment significantly increased the nodule number in the lower root system and the young nodules had accelerated acetylene reduction activity.

Discussion

This study conducted two-year field experiments in two different locations and a root box study to find out whether crack fertilization could control soybean root nodulation or not. In the field experiments soybean nodule formation and nitrogen fixation activities were evaluated at different timing after the crack fertilization treatments. The root box experiment was conducted to

| Table 7. Seed yield of soybean as affected by crack fertilization. |
|-------------------|------------------|------------------|------------------|------------------|
|                  | 2007             | 2008             |                  |
|                  | Hikone | Nagakute | Hikone | Nagakute |
| Cnt              | 314 (28) | 292 (35) | 430 (11) | 496 (23) |
| Ck               | 312 (26) | 273 (20) | 377 (23) | 475 (39) |
| Ck-F0            | 300 (18) | 323 (9)  | 359 (60) | 538 (6)  |
| Ck-FL            | 303 (9)  | 337 (35) | 347 (15) | 520 (25) |
| Ck-FH            | 313 (27) | 276 (13) | 392 (77) | 537 (56) |
| F value          | 0.09     | 1.02     | 0.49     | 0.50     |
| Probability      | 0.98     | 0.44     | 0.74     | 0.74     |

Cut, Control; Ck, Crack; F, Fertilizer; H, High; L, Low. Values in parentheses indicate standard error of mean. No significant difference was found in any yield data.

| Table 8. Effects of the crack fertilization on nodule formation in different soil layers in soybean plants grown in a root box. |
|-------------------|-------------------|-------------------|-------------------|-------------------|
|                  | Root Dry Weight (g) | Nodule number | Average dry weight per single nodule (mg) | Acetylene reduction activity (μmol h⁻¹ plant⁻¹) |
|                  | Upper | Lower | Upper | Lower | Upper | Lower | Upper | Lower |
| Cnt              | 1.70 (0.21) | 1.12 (0.25) | 25.3 (4.2) | 28.0 (10.2) | 19.8 (4.1) | 7.9 (2.3) | 0.280 (0.051) | 0.167 (0.058) |
| Ck               | 2.09 (0.18) | 1.04 (0.28) | 14.8 (4.6) | 125.8 (38.6) | 21.5 (2.4) | 3.0 (1.4) | 0.092 (0.036) | 0.377 (0.080) |
| Ck-F0            | 2.36 (0.21) | 1.22 (0.24) | 27.2 (3.2) | 120.0 (23.0) | 23.8 (4.5) | 2.2 (0.4) | 0.282 (0.060) | 0.209 (0.024) |
| Ck-FL            | 2.27 (0.08) | 1.66 (1.16) | 33.0 (9.6) | 95.4 (17.9) | 18.2 (4.4) | 3.7 (1.8) | 0.199 (0.030) | 0.275 (0.052) |
| Ck-FH            | 2.14 (0.24) | 1.59 (0.30) | 40.0 (13.2) | 20.5 (1.0) | 12.6 (3.7) | 7.6 (2.4) | 0.153 (0.055) | 0.064 (0.018) |
| F value          | 1.66     | 1.32     | 1.46     | 5.00     | 1.14     | 2.35     | 2.76     | 5.05     |
| Probability      | 0.21     | 0.30     | 0.26     | 0.01     | 0.37     | 0.10     | 0.06     | 0.01     |

Cut, Control; Ck, Crack; F, Fertilizer; H, High; L, Low. Values in parentheses indicate standard error of mean. * indicates significant difference from control at P<0.05 by Dunnett’s multiple comparison test.
evaluate the control of nodule position by the modified crack fertilization treatment.

1. Nodulation and nitrogen fixation activity in the field experiments

Field experiments indicated that nodule formation was enhanced by crack fertilization three to seven weeks after the treatment. Since it is quite humid and hot in both Hikone and Nagakute at this stage (mid-August to mid-September), the newly formed root nodules would grow rapidly and will produce fixed nitrogen. In fact, nitrogen fixation activity estimated by the ureide production rate and acetylene reduction activity was enhanced by some crack fertilization treatment. The fixed nitrogen can be used for the soybean seed formation during the early maturing stage. In this study five different treatments were selected out of the 12 possible combinations of crack formation, nodule bacteria and fertilizer application to the deep soil layer. By these five treatments, agricultural inputs desirable for applying to the subsoil layer were analyzed. Generally speaking Ck-F0 treatment in Hikone and Ck-FL treatment in Nagakute tended to increase acetylene reduction activity. The field in Hikone was an upland field supplied with basal fertilizer, but the field in Nagakute was converted from paddy field without any fertilization during soybean growth. This may be the reason for the difference in the activity; it was not necessary to apply fertilizer for nodule growth after the flowering stage in Hikone, but a low level of fertilizer was needed in Nagakute. Negative relationships have been widely observed between nitrogen fertilization rate and nitrogen fixation when nitrogen was supplied with basal fertilizer, but the field in Nagakute was converted from paddy field without any fertilization during soybean growth. In Nagakute 2008) are comparable with those of the root box experiment (sampling at 23 DAT) in terms of acetylene reduction activity because of the similarity of the timing after the treatment. In both experiments, Ck-FL always enhanced the nodulation as compared with the control. In the lower portion of the root system, where modiffied crack fertilization (except for Ck-FH) treatment was conducted, the number of newly formed root nodules with smaller size was higher than in the upper position. This indicated that the crack fertilization technique can control the nodulation position of the soybean.

2. Nodule position examined in the root box study

The root box method used in this study enabled us to acquire the in situ information on the root nodule distribution along the soil depth from the two-dimensionally developed root system (see the limitation and advantage of this technique in the following citations; Kono et al., 1987; Iijima and Kono, 1991; Iijima et al., 1991). In real field conditions, it is not easy to acquire reliable soil depth data by sampling the root nodules within a limited time. Therefore, we used the root box method to analyze the nodulation position. The results of the field experiment (23 DAT in Hikone 2007 and 28 DAT in Nagakute 2008) are comparable with those of the root box experiment (sampling at 23 DAT) in terms of acetylene reduction activity because of the similarity of the timing after the treatment. In both experiments, Ck-FL always enhanced the nodulation as compared with the control. In the lower portion of the root system, where modified crack fertilization (except for Ck-FH) treatment was conducted, the number of newly formed root nodules with smaller size was higher than in the upper position. This indicated that the crack fertilization technique can control the nodulation position of the soybean.

3. Biomass and seed production in the field experiments

Seed production of soybean by the crack fertilization was also evaluated by the present study. Although nitrogen fixation activities were enhanced by the crack fertilization, shoot growth and yield were not significantly different from the control treatment. At present, even though the nitrogen fixation activities were enhanced during the flowering stage and/or early seed maturing stage in some treatments, it did not contribute significantly to the seed and biomass production of soybean. This may be because the enhanced nodulation at the later growth stage competed with the seed for the photosynthetically fixed carbon supply. In fact, supernodulation studies indicated that the supernodulation itself does not promise the high yielding of the soybean due to the higher competition between nodule and seed. Nakamura et al. (2010) recently showed that the supernodulating Sakukei 4 (presently Kanto 100) could not exceed Enrei or Tamahomare in seed productivity, which was consistent with previous reports (Maekawa et al., 2005; Takahashi et al., 2005).

Moreover, waterlogging tolerance, which is one of the major stresses restricting the high yielding of Japanese soybean cultivation, in supernodulating cultivar was analyzed by Jung et al. (2008). They suggested that the supernodulating cultivar Kanto 100 was more susceptible to waterlogging than its normally-nodulating ancestral cultivar Enrei. Waterlogging or very wet soil condition is a common stress in the soybean field converted from paddy field during the rainy season just before the flowering stage, and thus the use of supernodulating cultivars to
acquire high yielding also needs more research works like the crack fertilization studies.

Measurement of photosynthetic activities at different timing after the crack fertilization would indicate the trend of physiological status of the soybean plants. It was enhanced at first, but the effect declined gradually thereafter. This may be caused by the new root growth in the deep soil layer after subsoiling (Iijima et al., 2005) and carbon competition between nodules and soybean plants. At first, even a week after the treatments, e.g., Hikone at 6 DAT in 2007, Ck treatment showed the best value of photosynthetic rate. 2.0 times higher average value was recorded although no statistical difference was observed. This may be explained by the air supply to the deep soil layer by crack-formation and subsequent new root growth to explore the new region of soil where valuable nutrients and/or water for photosynthetic activities exist (Data is not shown). At 51 DAT in Hikone in 2008, the photosynthetic rate in all the crack fertilization treatments was lower than in the control. This implies that higher nodulation required a larger amount of carbon and other nutrients to sustain nodule growth at the maturing stage. The nutritional imbalance may accelerate aging of the leaf resulting in lower photosynthetic activity (Abu-Shakra et al., 1978; Sinclair and de Wit 1975). To achieve the higher yielding by the crack fertilization technique, photosynthetic activity should be sustained at a later seed maturing stage, so that the competition between the nodule and seed does not occur at this stage.

4. Significance of the crack fertilization technique

Crack fertilization is the technique to apply agricultural inputs to the deep soil layer through the subsoiling crack formed during the plant growth period. As indicated in the introduction section, crack fertilization is an application of the traditional concept of deep layer fertilization (Takagi et al., 1963). Takahashi and his co-workers (eg., Takahashi et al., 1991; Kaushal et al, 2004, 2006, 2007) reported a series of experiments on deep layer fertilization. The major difference between the current deep layer fertilization (although not really adapted in the present soybean cultivation) and crack fertilization is whether the roots are cut during plant growth or not. When the roots are cut by the vibration of the subsoiler blade during soil crack formation, root emergence will be enhanced near the cut end, and the emerged roots may be infected with nodule bacteria which were applied through the cracks. The crack fertilization may also be useful to modify the hard soil condition during the mid-summer dry period and/or wet soil condition during the short-term rainy season after the dry summer. The technique can be used for midstream drainage during plant growth in the wet field condition of upland field converted from paddy field. As described in the previous section, anaerobic condition of the soybean field is one of the most important targets to be improved in Japan. Further studies on the crack fertilization will give the way to modify the cultivation technique of hard and/or wet prone upland field. The crack fertilization can be modified to various ways; not only soybean nodule bacteria but also any other agricultural inputs can be applied to the deep soil layer during the plant growth period; e.g. organic manures such as barnyard manure and compost, and/or soil conditioner such as charcoal and plant ash. The technique should be studied further not only for soybean growth but also other crop species such as wheat.

5. Conclusion

We concluded that the soybean nodulation control, i.e. timing and position of nodulation, as well as the enhancement of nitrogen fixation activities were possible by the newly developed crack fertilization technique. In the two field experiments and two years, the crack fertilization was suggested to enhance the nitrogen fixation activity from the flowering to early seed maturing stage; especially Ck-F0 treatment in Hikone and Ck-FL treatment in Nagakute were effective. The root box study revealed growth of a significant number of small and young nodules in the lower half of the root system indicating that the nodule position could be controlled by crack fertilization.

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