Continuous Application of Biochar Inoculated with Root Nodule Bacteria to Subsoil Enhances Yield of Soybean by the Nodulation Control using Crack Fertilization Technique

Morio Iijima¹, Koji Yamane¹, Yasuhiro Izumi², Hiroyuki Daimon³ and Takayuki Motonaga¹

¹Graduate school of Agricultural Science, Kinki University, Nara 631-8505, Japan; ²School of Environmental Science, The University of Shiga Prefecture, Hikone 522-8533, Japan; ³Graduate school of Life and Environmental Sciences, Osaka Prefecture University, Sakai 599-8531, Japan

Abstract: Crack fertilization is a soybean cultivation technique for nodulation control in which midterm subsoiling is used to supply fertilizing materials to deep soil just before the flowering stage. This study examined the effects of fertilizing materials and the continuous application of nodulation control, on soybean yield enhancement in two field experiments. The survival of nodule bacteria in deep soil was also evaluated by a bioassay of nodule bacteria in a root box. When the nodule bacteria on biochar were continuously applied without any other chemical fertilizers for three successive years, seed weight was significantly heavier being up to 1.34 times that of the control. The application of nodulation control in the previous year but not in the experimental year did not have residual effects on seed weight. The enhancement of seed weight in a farm field converted from a paddy was much lower. This may be partly attributed to the midterm tillage practice, which destroys the crack structure after the nodulation control, together with soil water status and cultivar differences. Nodule growth and nitrogen fixation activities significantly increased in the soybean plants grown on the soil collected from the subsoil to which nodule bacteria on biochar had been applied the previous year. This suggests that nodule bacteria in the subsoil survived in the biochar habitat for at least a year after application. These results indicate that nodulation control by the crack fertilization technique leads to yield enhancement when nodule bacteria on biochar are continuously applied.

Key words: Biochar, Crack fertilization, Glycine max Merr, Inoculation, Midterm tillage, Root nodule bacteria, Soil amendment.

Increasing the nitrogen supply to maturing seeds is a key strategy to increase soybean yields due to their high nitrogen content. Chemical fertilizer application, such as top dressing (e.g. Nakano et al., 1989; Salvagiotti et al., 2008), may not be a high yielding technique for soybeans (Glycine max (L.) Merr.) because the fertilizer nitrogen does not accumulate in the soybean seed, in contrast to the fixed nitrogen from nodule bacteria symbiosis (Ohyama, 1983). Nitrogen fixation often ceases after the flowering stage (Pfeiffer et al., 1983; Zapata et al., 1987; Kon’no et al., 1990), and to increase the yield, measures to maintain the nitrogen fixation during the maturing stage may increase the yield. Soybean nodules tend to accumulate in shallow soil layers before the flowering stage. This nodulation habit can be utilized for nodulation control as an agronomic practice.

Iijima et al. (2011) proposed the new agronomic concept of nodulation control through “crack fertilization” based on the hypothesis that there would be enhanced nitrogen supply to maturing seeds if nodulation in deep soil layers after the flowering stage was achieved by a cultivation technique. Young roots would emerge from the cut surfaces of the root system created by the formation of soil cracks during midterm subsoiling in the soybean stands in situ. The cracks create aerobic conditions in the deep soil layer by aeration through the continuous channel formed from the soil surface to the deep soil. The population of aerobic soybean nodule bacteria (Bradyrhizobium japonicum (Kirchner) Jordan) is limited in the anaerobic deep soil environment. Nodulation of newly emerged roots after the flowering stage can be induced once the cultured soybean nodule bacteria are introduced through the crack channel to the deep soil together with other nutrients to support root formation. The new nodules may supply nitrogen to the soybean seeds during the late maturing stage.

A previous study (Iijima et al., 2011) showed that the yield was not increased by crack fertilization of a soybean stand during the early flowering stage. The study used akadama soil, which is a low-nutrient, granular, clay-like
volcanic soil with slight acidity, as the artificial shelter for the cultured soybean nodule bacteria. We hypothesized that the shelter may not persist in the deep soil; once rain water penetrates through the crack channel to the deep soil, the shelter would be easily destroyed. Akadama soil may thus not be favourable for long-term bacterial growth. Therefore, there is a need for further research to find a stable and suitable artificial shelter for soybean nodule bacteria to test its soybean yield enhancement. Recently, charcoal in soil has attracted worldwide attention as a sustainable carbon sink to mitigate the anthropogenic greenhouse effect by reducing concentrations of atmospheric CO2. Biochar is a relatively new term for the charcoal used for soil amendment, which includes plant biomass-derived materials contained within the black carbon continuum, but excludes fossil products or geogenic carbon (see e.g. Warnock et al., 2007). Biochar may be a stable artificial shelter for soybean nodule bacteria supplied to the deep soil layer by the present crack fertilization technique. In our early trials, chemical nitrogen was applied together with nodule bacteria on akadama soil to the deep soil layer for the enhancement of new root formation; however, the nitrogen may have disturbed the nodulation (Streeter, 1985a and b; Nakano et al., 1989; Salvagiotti, 2008). Therefore, fertilizing materials, including akadama soil, should be further examined to test the yield enhancement of the present crack fertilization technique. Because most soybeans in Japan are grown in upland fields converted from paddies, field experiments should be conducted in both an upland field and an upland field converted from a paddy to discuss the yield enhancement of a new cultivation technique.

After the soybean nodule bacteria are introduced to the deep soil, the bacterial infection area may spread year by year. A continuous supply of soybean nodule bacteria may cause the expansion of the bacterial colonies in the subsoil. Once the nodule bacteria persist in the deep subsoil layer of the soybean field, they may contribute to nodulation in deep soil. Therefore, the survival of nodule bacteria in the subsoil is important for yield enhancement with the present crack fertilization technique. The purpose of the present paper is, therefore, to examine the effects of fertilizing materials, including biochar and the continuous application of nodulation control, on the yield enhancement of soybean. The survival of nodule bacteria in subsoil by the present crack fertilization technique was analysed by a bioassay method using the root box pin-board method (Kono et al., 1987; Iijima and Kono, 1991). Successive and residual effects are also discussed in order to make the present experimental technique a practical method.

**Materials and Methods**

1. **Experiment 1: Field study in an experimental upland field**

Nodulation control was tested in an experimental
upland field of the University of Shiga Prefecture (latitude 35°15′ N, longitude 136°13′ E) for three successive cropping seasons from 2009 to 2011. The upper part of Figure 1 shows the temperature and rainfall during the experimental period together with the 30 year average (the long-term average, LTA). The average temperature in the 2009, 2010, and 2011 cropping seasons was 22.8°C (0.1°C below LTA), 24.3°C (1.4°C above LTA), and 24.1°C (1.2°C below LTA), respectively. The total rainfall from June to October, the main growth season for soybeans, was 645 mm (157 mm below, LTA), 1007 mm (205 mm above LTA), and 847 mm (45 mm above LTA), in the 2009, 2010, and 2011 cropping seasons, respectively. The top soil in the field was light clay with a pH (H₂O) of 7.02, total N of 1.75 g kg⁻¹, and total C of 20.9 g kg⁻¹ (Zegada-Lizarazu et al., 2006).

Wheat was grown annually as a winter crop in the previous year of the experiment. Before sowing, the land was prepared and levelled with a rotary plough to a depth of 15 cm in both fields. Basal fertilizer was applied to each field at concentrations of 20, 60, and 80 kg ha⁻¹ of N, P₂O₅, and K₂O, respectively, every year. The seeds were sown on 19 June, 17 June, and 21 June in 2009, 2010, and 2011, respectively. After seeding, trifluralin granular formulation was surface broadcast for weed control. The row and intra-row distances were 0.6 and 0.15 m, respectively. After plant emergence and/or establishment, thinning and complementary planting were done to adjust the planting density to 11.11 plants m⁻².

Crack fertilization and the preparation of nodule bacteria were done according to the method of Iijima et al. (2011). In brief, a test machine (Sukigara Nouki Co Ltd) attached to a tractor (Yanmar CT 340) was used for the crack fertilization. The mixture of fertilizer and nodule bacteria was applied to the approximately 25–30 cm deep soil layer along the blade of the subsoiler. Granular chemical fertilizers of N, P₂O₅, and K₂O were applied to the subsoil layer at rates of 20, 20, and 20 kg ha⁻¹, respectively (Table 1). Coated fertilizer (LP40, N = 42%), superphosphate of lime (P₂O₅ = 17.5%), and potassium sulphate (K₂O = 50%) were used as nitrogen, phosphate, and potassium fertilizer, respectively. Although there are many reports investigating the response of soybeans or

| Crack formation | Nodule bacteria | Fertilization | Treatment | Exp. 1. | Exp. 2. |
|-----------------|----------------|--------------|-----------|--------|--------|
| Ck              | As·Bc          | –            | Control   | ○      | ○      |
| Ck              | As             | P·K          | Ck·PK     | ○      | –      |
| Ck              | As·Bc          | P·K          | Ck·PKBc   | ○      | ○      |
| Ck              | As             | N-P·K        | Ck·PK     | ○      | –      |
| Ck              | As·Bc          | N-P·K        | Ck·NPKBc  | ○      | ○      |

As, akadama soil; Be, Biochar, Ck, Crack; K, Potassium; N, Nitrogen; P, Phosphorus.
nodule activity to N fertilization (reviewed in Salvagiotti et al., 2008), the effects of other chemical fertilizers such as P and K on nodule inoculant are still unclear. In the present study, we fertilized the deep soil layer not only with N, but also P and K to confirm whether the combination of fertilizers affect the inoculation of nodule bacteria. Nodule bacteria (both commercial bacteria (Mamezo; Tokachi agricultural cooperation) and cultured bacteria originating from the field soil) were mixed with either akadama soil or biochar, and these materials were used as the nodule bacteria inoculants. Both 42g of dry powder for commercial bacteria and 21 g of YM agar medium for cultured bacteria were mixed with 10 L of deionized water and the supernatant solutions were sprayed on the soil or biochar applied to 1,000 m² of the experimental field. The granular size of these particles was adjusted to 2-5 mm by sieving through meshes. Both akadama soil and biochar were applied at a rate of 33.3 kg ha⁻¹. Commercially available akadama soil (the red soil from Kanto loam layer without fertilizer, with bulk density of 0.824 g cm⁻³) was used as the experimental material. Biochar used in the present study was produced by heating broad-leaf trees at 600°C for 2 hr in low oxygen conditions, and its bulk density was 0.434 g cm⁻³.

Crack fertilization in 2009, 2010, and 2011 was conducted at 45 (8 Aug), 35 (22 July), and 31 (22 July) days after sowing (DAS), respectively. The crack fertilization for both 2010 and 2011 was done just before the flowering stage. The treatment in 2009, however, was done during the early flowering stage. Crack fertilization during the early flowering stage significantly damaged bud formation, and therefore yield evaluation in 2009 was not conducted.

Insect control was done by spraying MPP (Emulsifiable Concentrate; EC) or Etofenprox against stink bugs and Fenvalerate plus MEP (Wettable Powder) against common cutworms. In 2010, Enrei, Ootsuru, Sachiyutaka, and Tamahomare were harvested at 113 (8 Oct), 134 (29 Oct), 141 (5 Nov), and 141 (5 Nov) DAS, respectively. In 2011, Tamahomare was harvested at 128 (27 Oct) DAS. Five plants from each replicate plot were harvested for the measurement of air-dried shoot weight and yield components in 2010. In 2011, five plants were harvested for the measurement of seed number, and 20 plants for both air-dried shoot weight and seed yield evaluation per replicate plot.

2. Experiment 2: Field study in a farm field converted from a paddy

Nodulation control was tested at a farmer’s upland field that was converted from a paddy in Sakurai city of Nara prefecture (latitude 34°31’N, longitude 135°50’E), and the experiment was conducted for two successive cropping seasons from 2010 to 2011. The bottom of Figure 1 shows the temperature and rainfall during the experimental period at Oouda, which is located near Sakurai. The average temperature was 22.1°C (1.2°C above LTA) and 21.5°C (0.6°C above LTA) during the experimental period in 2010 and 2011, respectively. The total rainfall of the same period in 2010 and 2011 was 886 mm (40 mm below LTA) and 1424 mm (498 mm above LTA), respectively. The top soil in the field was sandy clay loam with a pH (H₂O) of 6.38, total N of 1.3 g kg⁻¹, and total C of 13.6 g kg⁻¹.

In this experiment, four treatments (Control, Ck-Bc, Ck-PKbc, and Ck-NPKbc) were chosen among the combinations of crack formation, materials for nodule bacteria inoculation, and chemical fertilizer (Table 1). The crack fertilization was conducted in the same position for two years to investigate the successive effects in an upland field that had been converted from a paddy. In the final year of the experiment, the effects of successive crack fertilization on soybean yields were evaluated. Twelve plots (4 different combinations × 3 replication fields) were arranged in a complete randomized block design. The size of each plot was 2.1 m × 12.6 m, and the total planting area was 7.0 m × 50.4 m (352.8 m²) out of the total area of the field of 1167 m². Sachiyutaka, the recommended cultivar in Nara prefecture, was used for the experiment.

In this farm field, soybeans and wheat were grown as the previous year’s summer and winter crops, respectively. Before sowing, the land was prepared and levelled with a rotary plough to a depth of 15 cm. Basal fertilizer (N:P₂O₅:K₂O = 10:10:10 kg ha⁻¹) and top dressing (N:P₂O₅:K₂O = 20:20:20 kg ha⁻¹) were applied to the field during the growth of wheat. For soybean cultivation, basal fertilizer was not applied, but top dressing was applied except for the control and Ck-Bc treatment. Granular chemical fertilizers of N, P₂O₅, and K₂O were used for Ck-PKbc and Ck-NPKbc with the same application rate as the upland field in Shiga. The seeds were sown on 28 June and 30 June in 2010 and 2011, respectively, with a seeding machine (Agritecno Yazaki Co Ltd). The row and intra-row distances were 0.7 and 0.15 m, respectively. After plant emergence and/or establishment, thinning and complementary planting were done to adjust the planting density to 9.52 plants m⁻². Crack fertilization in 2010 and 2011 was done by the same test machine attached to a tractor (Yanmar US261) as in the experimental upland field of Shiga, and was conducted at 28 (26 July) and 26 (26 July) DAS, respectively. Insect control was done by the same manner as mentioned above. Pest management was done by spraying thiophanate methyl plus MEP against purpura or crown rot.

The midterm tillage was conducted once in early August after the nodulation control in both years because weeding by conventional midterm tillage is a compulsory farm practice in the field where the experiment was conducted. The stomatal conductance of the topmost fully developed leaf from the 6 replicated plants was measured by a
porometer (AP4 Leaf Porometer, Delta-T Devices Co Ltd) at the pod-filling stage (48 days after crack fertilization in the 2010 experiment, first year of the experiment). Following which, the shoot was cut at 5 cm above the soil surface and xylem sap was collected for approximately 2 hours to analyse the ureide concentration in xylem sap by the Young–Conway method (Young and Conway, 1942). Due to the uncertainty of the time record, xylem sap exudation rates was not calculated. Harvesting in 2011 was done at 124 (1 Nov) DAS. Five plants were harvested from each replicated field for the measurements of yield components. For the 2010 cropping season, seed weight was not analysed because the seeding machine was not working well during the planting in this field. Thus, the planting error among the replications and/or each plot affected the seed production significantly.

3. Experiment 3: Root box study

The root box study was conducted at Kinki University (latitude 34°40′N, longitude 135°43′E) in the summer of 2012 to evaluate the nodule bacteria survival in deep subsoil by a bioassay method. The subsoil in the experimental upland field (Exp. 1) was filled into a root box (400 × 250 × 20 mm in length, width, and thickness, respectively) at a bulk density of 1.30 g cm⁻³. The root boxes were placed in a concrete pool for regular watering. Three replicate soil blocks (width, 20 cm around the centre of the interrow; length, 30 – 40 cm; depth 25 – 30 cm) were sampled from the control (no crack fertilization) and successive crack fertilization (Ck-Bc) for the three year treatment on 20 April 2012. These soils were passed through a 2 mm sieve and air dried. The biochar in the deep soil was not included in the sieved soil because its size was more than 2 mm diameter. Basal fertilizer and nodule bacteria were not applied to the root box to investigate soybean nodule activity in the soil with or without crack fertilization. Seven replicate boxes in each treatment, 28 boxes in total (2 treatments × 2 sampling × 7 replicates) were used for the statistical evaluation. Pre-germinated soybean seeds (Tamahomare) were directly planted in the root box on 24 July 2012. Insect control was done by spraying Etofenprox against stink bugs and Malathion against spider mites at 30 DAS. Soybeans started to flower at 31 (24 August) DAS. Since subsoil was packed for the limited volume of root box (2,000 cm³), the plants needed topdressing to avoid nutrient deficiency at the later growth stages. Thus, at 43 DAS, powdered synthetic fertilizer (N:P₂O₅:K₂O = 0.1:0.1:0.1 g kg⁻¹ soil; in total 2.6 g of each element per plant) was applied to the surface of root boxes. Soybeans were sampled at 37 (30 August) and 58 (20 September) DAS, which correspond to the R2 (beginning flowering) and R4 (beginning pod) stages, respectively, and separated into shoots, roots, and root nodules. Soybean shoots were cut at 5 cm above the soil surface and xylem sap was collected for two hours for the analysis of ureide translocation rates by the Young–Conway method (Young and Conway, 1942). The root system was sampled according to the method described elsewhere (Kono et al., 1987; Iijima and Kono, 1991), and root nodules on the root system were sampled and passed through a 2 mm sieve. Nodules larger than 2 mm were counted as large and nodules less than 2 mm were counted as small.

4. Statistical analysis

One-way analysis of variance (ANOVA) was first used for statistical evaluation using Excel 2012 for Windows (SSRI Japan, Co. Ltd.). The ratios to the control of the respective values of each cultivar in Table 2 were subjected to Box-cox transformation prior to statistical analysis. Degrees of freedom, mean squares, F-values, probability levels, and standard error of means were indicated in all the parameters. If an ANOVA was significant, post-hoc analyses were conducted using Dunnett’s multiple comparison test, with the level of statistical significance taken as P < 0.05. The statistical difference in the root box study between the control and crack fertilization was determined by a Student’s t test. Differences with P < 0.05 were considered significant.

Results

1. Experiment 1: Field study in an experimental upland field

Table 2 shows the effects of the combination of nodule bacteria materials and chemical fertilizers by crack fertilization on the growth and yield components of four soybean cultivars in 2010 (the second season of crack fertilization) at the experimental upland field. For the four cultivars overall, crack fertilization tended to increase the number and total weight of seeds, whereas the increment of air-dried shoot weight and 100 seed weight were lower than the control. In order to evaluate the effects of biochar application on the crack fertilization technique, the treatments with biochar application were statistically compared with those without biochar application. Notably, the biochar application into the crack was much more effective than other combinations. No statistical significance was found, however, in traits for seed production except for shoot biomass (P = 0.024). As the values of each cultivar in Table 2 were expressed as the ratio to the control, a statistical comparison of cultivars cannot be done in Table 2. Therefore, only the trend of the treatment effect for each cultivar can be described here. Tamahomare may be the most profitable cultivar for nodule bacteria inoculation among the cultivars tested as
shown in the trends of increases in the number and weight of seeds in Ck-Bc and Ck-NPKBc. Sachiyutaka may also benefit by this treatment, but not as much as Tamahomare. Enrei, however, showed smaller increases than Tamahomare or Sachiyutaka. The results for Ootsuru, were only reference values because of the lack of a control treatment from one replicate field, as described in the materials and method section.

In 2011, the third season of crack fertilization, the successive and residual effects were investigated (Table 3). Air-dried shoot and seed weight significantly differed with the treatment at $P < 0.05$ by one-way ANOVA. The single

| Cultivar       | Treatment | Air-dried shoot weight (g plant$^{-1}$) | Seed number (plant$^{-1}$) | One hundred seed weight (g) | Seed weight (g plant$^{-1}$) |
|----------------|-----------|----------------------------------------|-----------------------------|-----------------------------|-------------------------------|
| Enrei          | Control   | 57.9                                   | 69.3                        | 29.9                        | 20.6                          |
|                | Ck-Bc     | 0.90                                   | 1.17                        | 0.90                        | 1.07                          |
|                | Ck-PK     | 0.89                                   | 0.88                        | 0.99                        | 0.87                          |
|                | Ck-PKBc   | 0.99                                   | 1.05                        | 1.03                        | 1.09                          |
|                | Ck-NPK    | 0.91                                   | 1.02                        | 0.97                        | 0.99                          |
|                | Ck-NPKBc  | 1.15                                   | 1.19                        | 1.05                        | 1.26                          |
| Ootsuru        | Control   | (68.3)                                 | (68.0)                      | (33.3)                      | (22.5)                        |
|                | Ck-Bc     | 1.31                                   | 1.04                        | 1.04                        | 1.09                          |
|                | Ck-PK     | 0.98                                   | 0.92                        | 0.97                        | 0.92                          |
|                | Ck-PKBc   | 0.80                                   | 0.64                        | 0.98                        | 0.63                          |
|                | Ck-NPK    | 0.99                                   | 0.86                        | 0.97                        | 0.85                          |
|                | Ck-NPKBc  | 0.94                                   | 0.85                        | 1.00                        | 0.86                          |
| Sachiyutaka    | Control   | 55.3                                   | 64.4                        | 33.2                        | 21.6                          |
|                | Ck-Bc     | 1.18                                   | 1.18                        | 1.04                        | 1.21                          |
|                | Ck-PK     | 0.97                                   | 1.05                        | 1.03                        | 1.07                          |
|                | Ck-PKBc   | 1.01                                   | 1.07                        | 1.03                        | 1.10                          |
|                | Ck-NPK    | 0.91                                   | 0.93                        | 1.01                        | 0.94                          |
|                | Ck-NPKBc  | 1.10                                   | 1.23                        | 1.07                        | 1.31                          |
| Tamahomare     | Control   | 61.9                                   | 90.2                        | 28.0                        | 25.4                          |
|                | Ck-Bc     | 1.22                                   | 1.32                        | 1.05                        | 1.40                          |
|                | Ck-PK     | 0.82                                   | 0.84                        | 0.98                        | 0.82                          |
|                | Ck-PKBc   | 1.06                                   | 1.18                        | 1.03                        | 1.21                          |
|                | Ck-NPK    | 0.97                                   | 1.02                        | 1.01                        | 1.03                          |
|                | Ck-NPKBc  | 1.11                                   | 1.36                        | 1.04                        | 1.42                          |
| Average of four cultivars | Control | 58.4                                   | 74.6                        | 30.4                        | 22.5                          |
|                | Ck-Bc     | 1.11                                   | 1.18                        | 1.02                        | 1.20                          |
|                | Ck-PK     | 0.96                                   | 0.98                        | 1.02                        | 1.00                          |
|                | Ck-PKBc   | 0.99                                   | 1.04                        | 1.04                        | 1.06                          |
|                | Ck-NPK    | 0.98                                   | 1.02                        | 1.01                        | 1.02                          |
|                | Ck-NPKBc  | 1.08                                   | 1.18                        | 1.07                        | 1.24                          |

| Mean squares | Biochar | $7.25 \times 10^{-2}$ | 0.144  | $7.15 \times 10^{-3}$ | 0.191  |
|--------------|---------|-----------------------|--------|-----------------------|--------|
| Fertilizer   | 4.78 $\times 10^{-2}$ | $6.98 \times 10^{-2}$ | 4.96 $\times 10^{-4}$ | $7.37 \times 10^{-2}$ |

| One-way ANOVA | Biochar | $5.399$ | $5.369$ | 5.494 | 5.690 |
|---------------|---------|---------|---------|-------|-------|
| F (1, 18)     | Fertilizer | 3.721  | 2.444  | 0.285 | 1.940 |
| $P$ (2, 17)   | Biochar | 0.032*  | 0.033*  | 0.031* | 0.028* |
| Fertilizer    | 0.046*  | 0.117  | 0.755  | 0.174 |

Bc, Biochar; Ck, Crack. Akadama soils were supplied in all treatments except for control as a nodule bacteria inoculant. Enrei, Ootsuru, Sachiyutaka and Tamahomare were harvested at 113, 134, 141 and 141 days after sowing (DAS). The values except for control are the ratio to the control. The data of Ootsuru in control are taken from only one plot data. Ratio to the average of four cultivars in each treatment was also calculated. The effects of biochar and fertilizer were analyzed by ANOVA. * indicates the significant difference at $P < 0.05$. 

| Mean squares | Biochar | $7.25 \times 10^{-2}$ | 0.144  | $7.15 \times 10^{-3}$ | 0.191  |
|--------------|---------|-----------------------|--------|-----------------------|--------|
| Fertilizer   | 4.78 $\times 10^{-2}$ | $6.98 \times 10^{-2}$ | 4.96 $\times 10^{-4}$ | $7.37 \times 10^{-2}$ |
Iijima et al. — Nodulation Control by Crack Fertilization Technique

Year treatment with Ck·Bc in 2011 tended to increase seed weight \((P = 0.078 \text{ by F-test})\) as compared to the control. The successive Ck·Bc and Ck·PKBc treatments for three years induced much greater increases in the air-dried shoot and seed weight; A statistical difference from the control by Dunnett’s multiple comparison test was observed only in the seed weight in the Ck·Bc treatment. The seed weight was 1.34 times that of the control. Seed number and 100 seed weight tended to increase under successive crack fertilizations. Notably, the successive Ck·Bc treatments were the most effective treatments. On the other hand, the residual effects of the treatment on the air-dried shoot weight and yield components were not obtained.

### 2. Experiment 2: Field study in a farm field converted from a paddy

In an upland farm field that was converted from a paddy, the effects of successive crack fertilization on yield

---

**Table 3.** Successive or residual effects of crack fertilization on the yield components of Tamahomare grown in an experimental upland field (Exp. 1.).

| Treatment | Succession (Treated year) | Air dried shoot weight \((g \text{ plant}^{-1})\) | Seed number \((\text{plant}^{-1})\) | One hundred seed weight \((g)\) | Seed weight \((g \text{ plant}^{-1})\) |
|-----------|---------------------------|-----------------------------|-------------------------------|---------------------------|-----------------------------|
| Control   |                           | 58.9 (4.74)                 | 101 (5.60)                    | 26.8 (0.62)               | 29.5 (1.34)                 |
| Ck·Bc     | One year (2011)           | 66.7 (3.58)                 | 118 (8.26)                    | 27.1 (0.75)               | 34.4 (1.89)                 |
| Ck·Bc     | Successive (2009 – 2011)  | 76.5 (9.47)                 | 148 (21.6)                    | 29.5 (0.59)               | 39.6* (3.09)               |
| Ck·PK     |                           | 60.6 (5.43)                 | 114 (2.11)                    | 27.2 (1.17)               | 32.0 (2.29)                |
| Ck·PKBc   | Residual (2009 – 2010)    | 63.8 (5.60)                 | 108 (14.4)                    | 28.1 (0.58)               | 32.6 (3.28)                |
| Ck·NPK    |                           | 60.3 (2.80)                 | 116 (9.10)                    | 27.1 (0.45)               | 31.2 (1.44)                |
| Ck·NPKBc  |                           | 74.4 (6.26)                 | 141 (11.4)                    | 28.4 (0.63)               | 37.8 (3.17)                |
| Ck·Bc     | One year (2011)           | 61.4 (6.73)                 | 103 (5.28)                    | 28.4 (0.61)               | 30.5 (3.21)                |
| Ck·PK     |                           | 58.7 (5.88)                 | 118 (12.7)                    | 26.8 (1.09)               | 32.4 (1.98)                |
| Ck·PKBc   | Residual (2009 – 2010)    | 55.6 (2.51)                 | 106 (2.61)                    | 28.1 (0.55)               | 28.6 (1.43)                |
| Ck·NPK    |                           | 53.5 (3.16)                 | 100 (5.26)                    | 26.2 (0.61)               | 27.4 (1.37)                |
| Ck·NPKBc  |                           | 60.9 (2.98)                 | 113 (11.0)                    | 29.7 (1.28)               | 31.2 (2.03)                |

**Mean squares**

\(F(11, 36)\) 2.257 2.017 1.988 2.189

\(P\) 0.033* 0.056* 0.060* 0.038*

Bc, Biochar; Ck, Crack. Akadama soils were supplied in all treatments except for control as a nodule bacteria inoculant. Plants were harvested at 128 DAS. Data are taken from the result of 2011 experiment. The data are means of four replicated plots. The values in small parenthesis and large parenthesis (adjacent to the value of seed weight) indicate standard error of mean and the ratio of control, respectively. When ANOVA was significant, post-hoc analyses were conducted using Dunnett’s multiple comparison test, with the level of statistical significance taken as \(P < 0.05\). * indicates significant differences from control at \(P < 0.05\).

**Table 4.** Successive effects of crack fertilization on the yield components of Sachiyutaka grown in an upland farm field converted from paddy (Exp. 2.).

| Treatment | Ripened pod number \((\text{plant}^{-1})\) | Seed number \((\text{plant}^{-1})\) | One hundred seed weight \((g)\) | Seed weight \((g \text{ plant}^{-1})\) |
|-----------|---------------------------------------|---------------------------------|-----------------------------|-----------------------------|
| Control   | 44.7 (3.73)                           | 67.8 (2.94)                     | 32.5 (0.76)                 | 21.2 (0.92)                 |
| Ck·Bc     | 45.0 (6.17)                           | 69.5 (6.56)                     | 32.7 (0.69)                 | 22.8 (1.93)                 |
| Ck·PKBc   | 42.9 (9.00)                           | 69.6 (11.2)                     | 33.4 (0.61)                 | 22.3 (3.73)                 |
| Ck·NPKBc  | 45.4 (4.62)                           | 75.1 (4.50)                     | 33.6 (1.54)                 | 23.4 (0.71)                 |

**Mean squares**

\(F(3, 8)\) 0.033 0.202 0.299 0.184

\(P\) 0.991 0.892 0.826 0.904

Bc, Biochar; Ck, Crack. Akadama soils were supplied in all treatments except for control as a nodule bacteria inoculant. Plants were harvested at 124 DAS in 2011. Data are means of four replicated plots and the values in parenthesis indicate standard error of mean. ANOVA was used to compare means of groups of treatment.
components were also investigated (Table 4). In this experiment, no statistical differences between treatments were observed by one-way ANOVA in any of the parameters tested. The seed weight was heaviest in Ck·NPKBc among the four treatments. As observed in the experiment on upland field (e.g. successive treatment in Table 3), seed number and weight in the Ck-Bc treatment had an increasing trend as compared with the control in the two-year successive treatment (Table 4). The effect of crack fertilization on 100 seed weight, however, was quite small. The ureide concentration in xylem sap and stomatal conductance in the first year (2010) are shown in Figure 2. At the pod-filling stage (48 days after crack fertilization), Ck-PKBc and Ck-NPKBc had significantly higher ureide concentrations than the control. Although stomatal conductance did not show statistical differences by ANOVA, Ck-Bc showed an increased trend compared with the control (P = 0.005 by F-test).

3. Experiment 3: Root box study

As described in section 1, the successive Ck-Bc treatment (only biochar application during crack fertilization) for three years in the experimental upland field induced a significant increase in seed weight as compared with the control. Thus, we investigated the nitrogen fixation activities of the soybean plants grown in a root box filled with subsoil from successive crack fertilization with biochar application (Ck-Bc). Shoot and root growth of the Ck-Bc treatment were not statistically different from the control (Fig. 3). Long, branched first-order lateral roots grown on the tap root axis and root nodules on a root system were traced to evaluate successive crack fertilization (Fig. 4). Overall root nodules in the crack fertilization grew widely within the rooting zone, especially at the R4 stage, as compared with the control. Figure 5 shows the small, large, and total nodule numbers on the root system. Nodule growth in the crack fertilization was similar to that in the control at the R2 stage. As growth progressed, the number of small nodules (< 2 mm in diameter) nearly doubled from 37 to 58 DAS in both crack fertilization and control. The number of large nodules (> 2 mm in diameter) in the control did not increase during the three-week growth period, however, that in the crack fertilization treatment increased around 1.7 times. This indicated that the small nodules continued to grow well with crack fertilization even though their growth

![Fig. 2. Ureide concentration in xylem sap and stomatal conductance in the plants grown in an upland field converted from paddy (Exp. 2.). Data are taken from the result of 2010 experiment, first year of the experiment. Data are means ± SE (n = 6 replicated plants). When ANOVA was significant, post-hoc analyses were conducted using Dunnett’s multiple comparison test, with the level of statistical significance taken as P < 0.05 and P < 0.01. * and ** indicate significant differences from control at P < 0.05 and P < 0.01, respectively.]

![Fig. 3. Shoot and root growth of soybean as affected by successive crack fertilization (Exp. 3.; Root box study). Subsoil of three-years successive crack fertilization was packed into the root box, and the soybean was grown. Plants were harvested at 37 (R2, left side graph) and 58 (R4, right side graph) DAS. Data are means ± SE (n = 7 replicated plants). The comparison of the means and SE of data were performed using Student’s t-test.]

in the control nearly stopped. As a result, the total nodules in both the control and crack fertilization treatment at 58 DAS (Fig. 5, lower right side) increased to 1.5 and 2.0 times higher than those at 37 DAS, respectively, and the numbers of large and total nodules in the crack fertilization treatment increased significantly as compared with the control. Figure 6 shows the ureide translocation rate in the soybeans grown in the root boxes. The translocation rate in the crack fertilization treatment at 37 DAS (R2 stage) was significantly higher than that in the control (Fig. 6, left side). However, the rate in the crack fertilization treatment at 58 DAS was similar to the control (Fig. 6, right side). These results indicated that the nitrogen fixation activity of each nodule at the R2 stage was significantly increased by the crack fertilization. Crack fertilization increased the large and total nodule numbers, whereas it did not improve nodule activity at the R4 stage. This implied that the large and developed nodules at the R4 stage were not as effective as increasing nitrogen fixation activity.

Discussion
This study examined whether nodulation control by the proposed cultivation technique of crack fertilization enhanced soybean yield. Continuous nodulation control treatments were conducted for three years and two years in an experimental upland field and a farmer’s upland field that was converted from a paddy, respectively. A bioassay of nodule bacterial survival was also evaluated using a root box filled with the subsoil from the experimental upland field.

1. Experiment 1: Field study in an experimental upland field
The crack fertilization technique, which induces nodulation in deep soil after the flowering stage, increased the seed weight when biochar was used as an inoculant (Tables 2 and 3). Overall, application of the nodule bacteria on biochar (Ck-Bc), but no other chemical fertilizer, tended to be as effective as that with NPK.
As for the successive crack fertilization treatment, three years of continuous treatment yielded the heaviest seed weight among the treatments (Table 3). When the nodule bacteria on biochar were continuously applied without any other chemical fertilizers for three successive years, a significantly higher seed weight was obtained, up to 1.34 times greater than in the control. This implies that the colonization of nodule bacteria in the deep soil may be enhanced by the annual supply of biochar. In fact, biochar additions to soil mostly enhance mycorrhizal root colonization (as reviewed by Warnock et al., 2007). The application can also modify soil nutrient availability by affecting soil physico-chemical properties (Warnock et al., 2007). Recently, LeCroy et al. (2013) demonstrated that biochar application promoted mycorrhizal root colonization on sorghum under high nitrogen content. This suggests that the combination of plant nutrition and biochar application is important for the symbiotic microbial relationships between soybean plants and nodule bacteria.

The effect of biochar application on seed weight was analyzed using Student’s t-test. The results showed that the mean seed weight in biochar-amended treatments was significantly higher than in the control treatment. The increase in seed weight was observed in most of the cultivars tested, indicating the potential of biochar for improving soybean yield.

2. Experiment 2: Field study in a farm field converted from a paddy

The enhancement of seed weight in the farm field converted from a paddy was much lower than that in the experimental upland field (Tables 3 and 4). The highest seed weight was obtained in the treatment with all fertilizers, which differed from that obtained in the experimental upland field. The lower seed weight could be partly attributed to the farm practice of midterm tillage after crack fertilization. Because the experiment was conducted in part of a farm field, midterm tillage was required for weed control for the local farmers in Nara prefecture. The midterm tillage must have destroyed the...
crack channels formed by crack fertilization. Once the surface cracks were disturbed by tillage, aeration to the deep soil layer would also be restricted, causing damage to the nodule bacteria in the biochar habitat. Midterm tillage was not conducted in the experimental upland field because of the risk of destroying the cracks in the top soil.

Another possible reason for the difference in seed weight may be the water environment of the subsoil layers of both upland fields. In fact, rainfall in September 2011 was significantly higher than the 30 year average, especially in Oouda district near the farm field (Exp. 2). The >500 mm of rainfall may have disturbed the biochar habitat in the subsoil layer. A farm field converted from a paddy usually forms a hardpan layer that restricts water flow in a downward direction. Paddy fields usually require a high water table so that rice, a wetland-adapted crop, can grow well. Thus, the upland field that had been converted from a paddy most probably had a much wetter environment, especially during the heavy rainfall of September 2011, than the experimental upland field. During the autumn rainy season, the soybean maturity period, the deep soil in the converted field should contain water for a much longer period, which may cause anaerobic conditions for soybean nodule bacteria inside the biochar habitat. The cultivar difference, with Tamahomare in the experimental upland and Sachiyutaka in the upland converted from a paddy, might also have affected the difference in seed weight. These are the most probable reasons for the lesser yield enhancement in this field.

The ureide nitrogen concentration of xylem sap at the pod-filling stage (R6) in the upland farmer’s field converted from a paddy (Exp. 2), increased in all the nodulation control treatments as compared with the control (Fig. 2). The previous paper also reported that crack fertilization tended to increase the nitrogen fixation activities such as ureide content, ureide translocation rates, and acetylene reduction activity in soybean plants at the pod-filling stage (Iijima et al., 2011). In the former study, not only the enhancement of nitrogen fixation activities but also that of nodule development in terms of the number and dry weight was observed in both fields. The results of the present study and former study (Iijima et al., 2011) confirm that nodulation control was achieved by the present crack fertilization technique, resulting in enhanced nitrogen fixation activities.

3. Experiment 3: Root box study

Nodule formation was evaluated using the soybean bioassay method to prove the survival of nodule bacteria in deep soil when biochar was supplied in the preceding year. Nodule bacteria in deep soil most probably survive in the biochar habitat for at least a year after the application of the materials judging from the results of enhanced nodule growth (Fig. 4 and 5) and nitrogen fixation activities (Fig. 6) of the soybean plants grown in the soil collected from the deep field soil. Nodule bacteria are released to the rhizosphere soil from senescent nodules during plant maturity (Pau et al., 1980; Ishii et al., 1985; Denison and Kiers, 2011), although the process has not been studied in detail. The population of nodule bacteria in soil increases temporarily in soybean fields, and then decreases gradually after harvesting (e.g. Reyes and Schmidt, 1979). Although the changes in the soil rhizobia population have been studied in some reports (Reyes and Schmidt, 1979; Ishii et al., 1985; Bottomley and Dughri, 1989), little is known about the lifestyle when host plants are absent from the fields. Nodule bacteria are heterotrophic obligate microaerophiles and need oxygen for respiration. Nodule bacteria can assimilate a wide range of rhizosphere carbon and nitrogen sources, and the assimilation product is mainly metabolized by the tricarboxylic acid (TCA) cycle, which is the central metabolism in rhizobia to extract energy (Fuhrer et al., 2005). The biochar habitat most probably guarantees air supply to nodule bacteria adhering to the surface of biochar because of its stable porous structure in the soil. Therefore, improved air permeability from crack formation and air supply in biochar would allow rhizobia to respire, leading to the higher survival rate in the next year.

4. Implications for the nodulation control concept

The research strategy of the nodulation control is partly similar to that for the breeding of supernodulating cultivars; higher nodulation will cause higher nitrogen fixation, which may reduce the nitrogen shortage in maturing soybean seeds. Nakamura et al. (2010), however, indicated that the supernodulating Sakukei 4 (presently Kanto 100) could not exceed Enrei (parental cultivar) or Tamahomare in seed productivity, despite the inconsistencies of former reports (Maekawa et al., 2003; Takahashi et al., 2005). Moreover, Jung et al. (2008) suggested that the supernodulating cultivar Kanto 100 was more susceptible to waterlogging than its normal-nodulating ancestral cultivar, Enrei. These results indicated that nodulation control by breeding was not successful in term of high yielding technique. The crack fertilization concept also has many issues to be solved in order to become a practical technique for soybean production in Japan.

As discussed previously, the continuous application of crack fertilization is desirable for effective yield enhancement. Thus, it is necessary to develop a practical technique for use in soybean production. Soybeans are often cultivated as part of a crop rotation in Japan, and continuous soybean cropping usually causes yields to decline. Therefore, a technique for enhancing the survival of nodule bacteria in deep soil without a soybean stand would be necessary for practical soybean nodulation control as part of a crop rotation scheme in Japan.
Another issue is the incorporation of mid-term tillage into crack fertilization, as it is a common weed control practice in many parts of Japan. After crack fertilization, the crack should be maintained for good aeration to the subsoil layer. Once the structure is destroyed, nodulation in deep soil may not contribute to yield enhancement. The practical inclusion of nodulation control as part of weed control should be re-designed to incorporate nodulation control into real soybean cultivation practices in Japan. The amount of biochar application and/or more effective nodule habitat materials and continuous crack formation during rotation periods should be further examined to establish this new cultivation technique.

Acknowledgements

We thank Ogura, Y., Kono, Y. Takeuchi, K. and other members of crop science laboratory of faculty of agriculture, Kinki University, and Shibahara, T., Inoue, H. and Kadono, H. of The University of Shiga Prefecture, and Nakatani, Y. of Japan Agricultural Cooperatives for their support to the project work. This study was supported by a Grant-in-Aid for Scientific Research (No. 19380010, 25929016) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Atkinson, C.J., Fitzgerald, J.D. and Hipps, N.A. 2010. Potential mechanisms for achieving agricultural benefits from biochar application to temperate soils: a review. Plant Soil. 337: 1-14.

Bottomley, P.J. and Dughri, M.H. 1989. Population size and distribution of Rhizobium leguminosarum bv. trifolii in relation to total soil bacteria and soil depth. Appl. Environ. Microbiol. 55: 959-964.

Denison, R.F. and Kiers, E.T. 2011. Life histories of symbiotic rhizobia and mycorrhizal fungi. Can. J. Bot. 21: R775-R785.

Fuhrer, T., Fischer, E. and Sauer, U. 2005. Experimental identification and quantification of glucose metabolism in seven bacterial species. J. Bacteriol. 187: 1581-1590.

Iijima, M. and Kono, Y. 1991. Interspecific differences of the root system structures of four cereal species as affected by soil compaction. Jpn. J. Crop Sci. 60: 130-138.

Iijima, M., Honjo, H., Izumi, Y., Daimon, H., Tani, T., Hayashi, M. and Suzuki, T. 2011. Control of soybean nodule formation by a crack fertilization technique. Plant Prod. Sci. 14: 201-212.

Ishii, T., Iwabuchi, H. and Matsuhiro, H. 1985. Number and nitrogen fixing ability of indigenous Rhizobium japonicum in field soils in Hokkaido. Jpn. Soc. Soil Sci. Plant Nutr. 56: 43-48**.

Jung, G., Matsunami, T., Oki, Y. and Kokubun, M. 2008. Effects of waterlogging on nitrogen fixation and photosynthesis in supernodulating soybean cultivar Kanto 100. Plant Prod. Sci. 11: 291-297.

Kon’no, T., Saito, M. and Ishii, K. 1990. Relationship among nodulation, shoot growth, and nutrient status of soybean. Jpn. J. Soil Sci. Plant Nutr. 61: 396-403**.

Kono, Y., Tomida, K., Tatsumi, J., Nonoyama, T., Yamauchi, A. and Kitano, J. 1987. Effects of soil moisture conditions on the development of root system of soybean plant (Glycine max Merr.). Jpn. J. Crop Sci. 56: 597-607.

LeCroy, C., Masiello, C.A., Rudgers, J.A., Hockaday, W.C. and Silberg, J.J. 2013. Nitrogen, biochar, and mycorrhizae: Alteration of the symbiosis and oxidation of the char surface. Soil Biol. Biochem. 58: 248-254.

Maekawa, T., Takahashi, M. and Kokubun, M. 2005. Responses of a supernodulating soybean genotype, Sakukei 4 to nitrogen fertilizer. Plant Prod. Sci. 6: 206-212.

Nakamura, T., Nakayama, N., Yamamoto, R., Shimamura, S., Kim, Y., Hiraga, S., Ohyama, T., Komatsu, S. and Shimada, S. 2010. Nitrogen utilization in the supernodulating soybean variety “Sakukei 4” and its parental varieties, “Enrei” and “Tamahomare”. Plant Prod. Sci. 13: 123-131.

Nakano, H., Watanabe, I. and Tabuchi, K. 1989. Supplemental nitrogen fertilizer to soybeans. III. Effects on nitrogen fixation. Jpn. J. Crop Sci. 58: 192-197*.

Ohyama, T. 1983. Comparative studies on the distribution of nitrogen in soybean plants supplied with N, and NO₃⁻ at the pod filling stage. Soil Sci. Plant Nutr. 29: 133-145.

Paau, A.S., Bloch, C.B. and Brill, W.J. 1980. Developmental fate of Rhizobium meliloti bacteroids in alfalfa nodules. J. Bacteriol. 143: 1480-1490.

Pfeiffer, N.E., Torres, C.M. and Wagner, F.W. 1983. Proteolytic activity in soybean root nodules. Activity in host cell cytosol and bacteroids throughout physiological development and senescence. Plant Physiol. 71: 797-802.

Reyes, V.G. and Schmidt, E.L. 1979. Population densities of Rhizobium japonicum strain 123 estimated directly in soil and rhizospheres. Appl. Environ. Microbiol. 37: 854-858.

Salvagiotti, F., Cassman, K.G., Specht, J.E., Walters, D.T., Weiss, A. and Dobermann, A. 2008. Nitrogen uptake, fixation and response to fertilizer N in soybeans: a review. Field Crop. Res. 108: 1-13.

Streeter, J.G. 1985a. Nitrate inhibition of legume nodule growth and activity. I. Long term studies with a continuous supply of nitrate. Plant Physiol. 77: 321-324.

Streeter, J.G. 1985b. Nitrate inhibition of legume nodule growth and activity. II. Short term studies with high nitrate supply. Plant Physiol. 77: 325-328.

Takahashi, M., Nakayama, N. and Arihara, J. 2005. Plant nitrogen levels and photosynthesis in the supernodulating soybean (Glycine max L. Merr.) cultivar ‘Sakukei 4’. Plant Prod. Sci. 8: 412-418.

Warnock, D.D., Lehmann, J., Knup, T.W. and Rillig, M.C. 2007. Mycorrhizal responses to biochar in soil – concepts and mechanisms. Plant Soil. 300: 9-20.

Young, E.G. and Conway, C.F. 1942. On the estimation of allantoin by the ninhydrine-schiff reaction. J. Biol. Chem. 142: 839-853.

Zapata, F., Danso, S.K.A., Hardarson, G. and Fried, M. 1987. Time course of nitrogen fixation in field-grown soybean using nitrogen-15 methodology. Agron. J. 79: 172-176.

Zegada-Lizarazu, W., Izumi, Y. and Iijima, M. 2006. Water competition of intercropped pearl millet with cowpea under drought and soil compaction stresses. Plant Prod. Sci. 9: 123-132.

* In Japanese with English abstract.
** In Japanese. English title was given by the present author.