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Research Article

Effect of the Nitrification Inhibitor 3,4-Dimethylpyrazole Phosphate on the Deep Placement of Nitrogen Fertilizers for Soybean Cultivation

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The deep placement of urea fertilizer (DMU) containing 1% (W/W) of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on soybean growth and seed yield was as effective as those of the coated urea (CU) and lime nitrogen (LN) in a field research. The average seed yields were high in LN ($464\,g{\cdot}m^{-2}$) and DMU ($461\,g{\cdot}m^{-2}$) and relatively low in CU ($405\,g{\cdot}m^{-2}$), U ($396\,g{\cdot}m^{-2}$), and Cont ($373\,g{\cdot}m^{-2}$) treatments. The accumulations of dry matter and nitrogen in soybean shoots were higher in the plants with deep placement of CU, LN, and DMU than U and Cont. The daily nitrogen fixation activity and daily nitrogen absorption rate were calculated based on the relative ureide method. Both nitrogen fixation activity and nitrogen absorption rate were higher in DMU, CU, and LN compared with control treatment, suggesting that the deep placement of DMU did not repress nitrogen fixation. Soil incubation test was performed using the same field soil with DMU, U, LN, and urea with DMPP 1%, 2%, and 4%. DMU inhibits nitrification similar to the pattern of LN until 8 weeks. The increasing DMPP concentration did not markedly increase the nitrification inhibition. From these results, it was concluded that urea fertilizer with 1% DMPP is efficient for deep placement of N fertilizer for soybean cultivation due to its lower price compared with CU and LN.

1. Introduction

The legumes provide important sources of protein, oil, and carbohydrate for human diets and livestock feeds. Most of the leguminous grain crops and legume forage crops can fix atmospheric dinitrogen (N_2) by symbiosis with soil microorganisms such as rhizobia. The supply of N by symbiotic N_2 fixation by legume-rhizobium symbiosis is still the most important N source in agroecosystems [1]. The world population is rapidly increasing especially in developing countries, and world food production needs to be increased with the demand for human consumption [1]. However, the increase in the cultivated area is difficult, so we need to promote crop production per area and the efficient and sustainable use of croplands. The wide use of chemical nitrogen fertilizers promoted crop production by 4–10 times in the past one hundred years and could support the food production for increasing world population [1]. However, the cost of chemical N fertilizers is expensive for small farmers in developing countries, and the fertilizer production requires a lot of fossil fuels. In addition, it was noticed that an inappropriate or excess application of chemical N fertilizers causes environmental problems, such as groundwater contamination by nitrate leaching, air pollution, and global warming by nitrous oxide emission [1].

Soybean (*Glycine max* (L.) Merr.) seed is one of the most important protein sources for human and livestock all over the world [2]. Soybean is widely cultivated in tropical, subtropical, and temperate regions with the optimum mean temperature between 20 and 30°C [2]. World production of soybean has been increasing in recent decades [2, 3]. The world average seed yield was 2.76 $t\cdot ha^{-1}$ in 2016, and the yield is high in the USA (3.50 $t\cdot ha^{-1}$), Brazil (2.90 $t\cdot ha^{-1}$), Argentina (2.76 $t\cdot ha^{-1}$), Paraguay (2.72 $t\cdot ha^{-1}$), and Canada (2.66 $t\cdot ha^{-1}$) compared with China (1.80 $t\cdot ha^{-1}$), Japan (1.59 $t\cdot ha^{-1}$), and India (1.22 $t\cdot ha^{-1}$) [3]. Board and Kahlon reported that recent yield increases in the USA are 50% due to genetic improvement and 50% to improve cultural practices [4]. They suggested that the yield increase in Asian countries would be much greater since their yield levels are substantially low due to many biotic and abiotic stresses in Asia [4].

Soybean plants obtain N from two underground organs, the fixed atmospheric N_2 by their root nodules and the absorbed inorganic nitrogen by the roots from soil or fertilizers. To obtain a high yield of soybean, good nodulation and high and long-lasting nitrogen fixation activity are very important because the availability of soil N is limited in many regions. Nodule formation and nitrogen fixation activity are influenced by various soil conditions and climatic conditions [3]. However, it is well known that a high concentration of mineral N represses nodule formation and nitrogen fixation activity, especially, nitrate, the most abundant inorganic nitrogen in upland fields, severely inhibits nodulation and nitrogen fixation activity of soybean plants [5–7].

Efficient and appropriate fertilizer application is critical to current crop production, economic benefit, and ecological advantages for sustainable agriculture. The chemical formula, amount, size of a fertilizer, and timing and placement have effects on the fertilizer use efficiency and consequently crop yield. For soybean cultivation in Japan, a basal dressing of N, P, and K compound fertilizer containing ammonium sulfate or urea at the rate of 20–40 $kgN\cdot ha^{-1}$ is generally applied as the "starter N," which supports the initial vegetable growth before starting nitrogen fixation.

Recently, soybeans are cultivated in rotated paddy rice fields in Japan, due to the decrease in rice consumption and to avoid the excess rice production. Takahashi et al. [8–11] developed a new fertilization technique of deep placement of coated urea (CU) for soybean cultivation to promote soybean seed yield in a rotated paddy field in Japan. Field experiments were conducted with a deep placement of 100 $kgN\cdot ha^{-1}$ of CU being applied under seeding line and compared with control plots with the conventional basal dressing of N, P, and K compound fertilizer with 16 $kgN\cdot ha^{-1}$ as ammonium sulfate. The deep placement of nitrogen fertilizer was carried out using the fertilizer injector, and 100-day type CU was applied just under the seed placement lines at a depth of about 20 cm. The 100-day type CU hyperbolically releases urea and 80% of which is released in 100 days in water at 25°C. In 1989, the soybean seed yield by deep placement of CU was 4.24 $t\cdot ha^{-1}$ and 14% higher than the conventional control treatment (3.73 $t\cdot ha^{-1}$). In 1990, the seed yield by deep placement of CU was 5.92 $t\cdot ha^{-1}$ and 23% higher than the conventional treatment (4.80 $t\cdot ha^{-1}$).

Coated urea is suitable for deep placement because it gradually releases urea through the porous plastic coat until reproductive growth stages in accordance with soybean N requirement [12]. However, the price of CU is relatively expensive about 5 times higher than urea in Japanese markets. Tewari et al. compared the effects of deep placement of lime nitrogen (LN) with CU [13–19] due to the lower cost of LN. LN has been initially produced by the artificial N_2 fixation by Frank and Caro in 1901. LN contains 60% of calcium cyanamide ($CaCN_2$) with calcium oxide (CaO) and free carbon (C). The fertilizer grade LN contains ca. 21% N, 11% Ca, 11% C, 5% oil, 2–4% water, and oxides of aluminum, iron, and silicon [20]. $CaCN_2$ is converted to urea in the soil, and then the urea is hydrolyzed to ammonium and carbon dioxide. In the presence of moisture and air, dicyandiamide (DCD) is formed from cyanamide, and this is a potent nitrification inhibitor, which inhibits the oxidation of ammonium to nitrate. Therefore, the ammonium produced by $CaCN_2$ decomposition persists for a long period of time, and the nitrate concentration remains low in the soil.

In 2001, fertilizer experiments were conducted in three sites in Niigata, Japan: a rotated paddy field [13, 19], a newly reclaimed wetland [14], and a sand dune field [15]. Four fertilizer treatments: control without deep placement, deep placements of urea (U), 100-day type CU, and LN, were compared. In the rotated paddy field in Niigata, the same field used by Takahashi et al., a significantly higher yield was observed by deep placement of CU and LN compared with control cultivation without deep placement. Similar results were observed in the newly reclaimed field and the sand dune field. The deep placement of LN tended to give higher seed yields than that by CU, although the data were statistically not significant. The visual quality of harvested seeds was improved by deep placement of CU, in which the percentage of good seeds increased and the percentage of turtle wrinkle and broken seed coat decreased. As a result of three field experiments, it was confirmed that deep placement of CU can be replaced by LN. Sakashita et al. [21] carried out the field trials of deep placement of LN in eight farmers' fields in Niigata and Toyama prefectures in Japan. They confirmed the effect of deep placement of LN on yield promotion and seed quality improvement.

Recently, urea fertilizer containing nitrification inhibitor, 3,4-dimethylpyrazole phosphate (DMPP), has been commercially available in Japan. Nitrification inhibitors are compounds that delay the bacterial oxidation of ammonium to nitrite by inhibiting ammonia monooxygenase of autotrophic ammonia-oxidizing bacteria or ammonia-oxidizing archaea [22, 23]. Although there are many compounds used as nitrification inhibitors, only a few have been used practically: DCD in Europe and nitrapyrin in the USA [22]. DMPP was recently developed in Germany, and this inhibits nitrification at low concentrations of 0.5–1.0 kg active compound ha^{-1}; this amount is less than one-tenth of the DCD applied [22]. Therefore, the price of the urea fertilizer containing 1% (W/W) of DMPP is cheap compared with controlled release nitrogen fertilizers, CU and LN. Akiyama et al. [24] reported that agricultural fields are an important anthropogenic source of atmospheric nitrous oxide (N_2O) and nitric oxide (NO), and nitrification inhibitors were effective in reducing N_2O emission from both chemical and organic fertilizers. DMPP has been widely used together with synthetic N fertilizers (Weiske et al. [25]; Di and Cameron [26]; Liu et al. [27]) and animal manure (Dittert et al. [28]; Hatch et al. [29]; Maienza et al. [30]). Shi et al. [31] reported that DMPP slowed nitrification by inhibiting the growth of AOB, and DMPP application affected the abundance of AOB more than the ammonia oxidizer community composition. Also, Kong et al. [32] suggested that DMPP reduces the activity of ammonia oxidizers without adverse effects on nontarget soil microorganisms and functions.

In this research, we compared the effect of urea fertilizer mixed with 1% DMPP (DMU) and U, CU, and LN on deep placement soybean cultivation in the rotated paddy rice field in Niigata Agricultural Research Institute. In addition, the time course of ammonium and nitrate accumulations was investigated by the soil incubation test comparing U, LN, DMU, and control without nitrogen fertilizer in the same soil as the field experiment in this research. We also investigated the effect of DMPP concentrations on nitrification of urea by incubation test.

2. Materials and Methods

2.1. Field and Planting Conditions. The field experiment was carried out in an upland field located in Nagaoka (Nagakura field) at the Niigata Agricultural Research Institute, Nagaoka, Niigata (37.4388°N/138.8719°E). The field had been converted from a drained paddy rice field in the previous year. The soil was a fine-textured gray lowland soil (gray type). The chemical properties of soil were as follows: pH(H_2O) 6.8, cation exchange capacity 25 cmol (+)·kg^{-1}, total carbon content 10 g·kg^{-1}, total nitrogen content 1.2 g·kg^{-1}, C/N ratio 8.4, mineralized N by the incubation of air-dried soil under upland conditions for 4 weeks at 30°C 38 mgN·kg^{-1}. One plot was 15 m^2 (1.5 m × 10 m) with 2 furrows. Five fertilizer treatments were carried out by 2 replications.

2.2. Fertilizer Application and Planting. Basal application of the compound fertilizer containing 16 kgN·ha^{-1} (ammonium sulfate), 40 kgP_2O_5·ha^{-1} (superphosphate), and 60 kgK_2O·ha^{-1} (potassium chloride) was mixed by a rotary cultivator in the surface layer at about 0–10 cm depth. Deep placement of nitrogen fertilizers was placed just under sowing line at 20 cm depth from soil surface by a newly developed fertilizer injector equipped with rotary cultivator (Figure S1). Deep placement of five treatments was performed as follows: control (Cont) without deep placement; urea (U, N46%); 100-day sigmoidal release type coated urea (CU, N41%); lime nitrogen (LN, N20%); DMPP urea (DMU, N45%) at the rate of 100 kgN·ha^{-1}. Seeds of the soybean cultivar "Satonohohoemi" were sown on 13 June, 2016 by a single stem training, and the seeding rate was 8.9 plants·m^{-2} (75 cm × 15 cm). Seeders were equipped with rotary cultivator just behind the fertilizer injector, and deep placement of fertilizer and sowing was done simultaneously.

Soybean variety "Enrei" has been cultivated in Niigata and Hokuriku region because seed size is relatively large and it is suitable for preparing tofu and soy-foods [33]. However, recently the seed quality of Enrei declined due to turtle wrinkle or side wrinkle, which occurs in the seed coat. These damages do not affect the material quality for food processing, but the grade for trading will be down when the percentage of wrinkle seeds are high. A new variety "Satonohohoemi" was developed in 1996 in Tohoku Agricultural Research Center, National Agriculture and Food Research Organization [34]. Satonohohoemi has several characteristics: later growth stage than Enrei about 7–10 days, resistant against soybean mosaic virus, the main stem is stronger than Enrei and lodging resistant, the seed size is very big (394 mg per a seed) compared with Enrei (331 mg per a seed), and the protein concentration (44%) is as high as Enrei (44%). In 2017, Enrei accounted for 70% of soybean cultivation area in Niigata and Satonohohoemi increased to 20%.

2.3. Plant Sampling and Analysis. A sampling of xylem sap and shoots was carried out at initial flowering stage (R1) on 25 July, R5 on 24 August, and R7 on 26 September, with the reproductive stage described by Ferr et al. [35]. The xylem sap was collected for one h from the cut main stem about 5 cm above soil surface covered by a plastic tube with an absorbent cotton inside [36]. A 5 μl aliquot of xylem sap was subjected to microscale colorimetric analysis of ureide-N, amide-N, and nitrate-N concentrations [37], and the percentage of relative ureide-N (RU%) was calculated by the equation (RU% = 100 × ureide-N/ureide-N + amide-N + nitrate-N). Shoots were dried in a ventilator oven at 80°C for 48 h, and the leaves, petioles, stems, pods, and seeds were separated. The dry weight was measured, and these tissues were ground into a fine powder, and then nitrogen (N) concentration was determined by the Kjeldahl digestion method [37]. The quantitative estimation of daily nitrogen fixation activity and nitrogen absorption rate was analyzed over the growth stage as same as the former method [10, 15].

Soybean plants were harvested on 17 October. All plants grown in 2 m long in each furrow were harvested and air-dried in a plastic mesh bag. Seeds were collected and classified to good seeds, side wrinkle, turtle wrinkle, broken coat, small seeds, and damaged seeds based on naked eye examination [16]. Then, the seeds' weight was measured.

2.4. Soil Incubation Test. The effect of DMPP on the nitrification inhibition was investigated by soil incubation test. The soil was obtained from the same experimental field as above. The 10 mgN of each fertilizer or urea plus DMPP were incubated with a fresh soil equivalent to 20 g air-dried soil with 60% of field capacity water. Treatments are as follows: Cont: only soil without fertilizer; U: 21.7 mg urea; UD1: 21.7 mg urea with 1% DMPP (0.22 mg); UD2: 21.7 mg of urea with 2% DMPP (0.44 mg); UD4: 21.7 mg of urea with 4% DMPP (0.88 mg); DMU: 22 mg of commercial urea fertilizer containing 1% DMPP; LN: 50 mg of lime nitrogen. Incubation periods were 0, 1, 2, 4, and 8 weeks at 25°C, and the soils were extracted with 80 mL of 2 M KCl solution. The concentrations of ammonium and nitrate were determined by the indophenol method [37] and modified Cataldo's method as follows [38]: 0.5 mL of KCl extract was put into a Pyrex test tube and dried completely in an aluminum block at 150°C. 0.4 mL of 5% salicylic acid in sulfuric acid was added and KCl was dissolved. After cooling to the room temperature, 10 mL of 2 M NaOH was added and the absorbance at 410 nm was determined.

2.5. Statistical Analysis. Statistical analysis (Tukey's test among five treatments and Student's *t*-test between control and each treatment) was done by MEPHAS Osaka University (http://www.gen-info.osaka-u.ac.jp/MEPHAS/).

3. Results

3.1. Field Experiment. Figure 1 shows the dry weight of shoot tissues in soybean plants cultivated with various forms of nitrogen fertilizers. On 25th July, the average total shoot dry weight was high in DMU, LN, CU, and U in this sequence, although statistically not significant at $P < 0.05$ level. The total dry weights of the shoot in the deep placement of LN and DMU were significantly higher than the control plants based on Student's *t*-test. On 24th August, the similar trends as those on 25th July were shown, and the total dry weights of shoots with LN and DMU were significantly ($P < 0.05$) higher than those with control, and those were higher than CU and U (not statistically significant). On 26th September, the total dry weight of shoot in deep placement of LN ($1504\,g{\cdot}m^{-2}$) and DMU ($1502\,g{\cdot}m^{-2}$) was higher than control plants ($1201\,g{\cdot}m^{-2}$). The total dry weights of CU ($1405\,g{\cdot}m^{-2}$) and U ($1334\,g{\cdot}m^{-2}$) were in between control and LN and DMU.

Figure 2 shows the changes in nitrogen accumulation in the shoot tissues. The total nitrogen accumulation was higher in DMU and LN than Cont, U, and CU treatments at all sampling dates. The total nitrogen accumulation in the shoot with deep placement of DMU showed statistical significance ($P < 0.05$) against control plants based on Student's *t*-test. On 26th September, the average total N accumulation in shoots was $21.1\,g{\cdot}m^{-2}$ (Cont), $25.6\,gN{\cdot}m^{-2}$ (U), $27.6\,gN{\cdot}m^{-2}$ (CU), $26.8\,gN{\cdot}m^{-2}$ (LN), and $28.1\,gN{\cdot}m^{-2}$ (DMU), respectively. The average total N accumulations in CU, LN, and DMU were almost the same and not significantly different. On 24th August, more than 80% of total N in the shoot was located in the vegetative organs: stems, petioles, and leaves, but on 26th September, about 80% of N in a shoot was in the reproductive organs: seeds and pods, irrespective of the fertilizer treatment.

Figure 3 shows the concentrations of ureide-N, amide-N, and nitrate-N in the xylem sap collected for 1 h in the morning on 25th July, 24th August, and 26th September. On 25th July, the ureide-N concentrations were higher than that of amide-N and nitrate-N, irrespective of fertilizer treatment and sampling date. The ureide-N concentration was highest in CU ($415\,mgN{\cdot}L^{-1}$) followed by Cont ($404\,mgN{\cdot}L^{-1}$), although it was slightly low in LN ($306\,mgN{\cdot}L^{-1}$), U ($348\,mgN{\cdot}L^{-1}$), and DMU ($367\,mgN{\cdot}L^{-1}$) treatment. The amide-N concentration was relatively higher in DMU ($190\,mgN{\cdot}L^{-1}$) and LN ($168\,mgN{\cdot}L^{-1}$) than in Cont ($155\,mgN{\cdot}L^{-1}$) and low in U ($135\,mgN{\cdot}L^{-1}$) and CU ($135\,mgN{\cdot}L^{-1}$). On the other hand, the nitrate concentration was the highest in U ($38\,mgN{\cdot}L^{-1}$) and lowest in Cont ($19\,mgN{\cdot}L^{-1}$). On 24th August, the trends were similar to that on 25th July. On 26th September, the concentrations of ureide-N decreased to about $200\,mgN{\cdot}L^{-1}$, irrespective of fertilizer treatments. This may be due to the decline in nitrogen fixation activity in R7 stage. The concentrations of amide-N were relatively constant among treatments about $150\,mgN{\cdot}L^{-1}$ on 24th August and decreased about $100\,mgN{\cdot}L^{-1}$ on 26th September. The concentrations of nitrate-N were as low as 10–$20\,mgN{\cdot}L^{-1}$, irrespective of the sampling date.

Figure 4 shows the percentage of relative concentrations of ureide-N, amide-N, and nitrate-N in various fertilizer treatments on 25th July, 24th August, and 26th September. The percentages of the relative ureide-N were similar among fertilizer treatments on 25th July (60–73%) and on 24th August (67–73%). It decreased slightly on 26th September (59–69%). Among treatments, the percentages of relative ureide-N were high in CU (73%) and Cont (70%) compared with U (67%), DMU (63%), and LN (59%) on 25th July. The statistic significance at $P < 0.05$ based on Tukey's test was detected only between CU and LN treatments.

The daily N gain ($mgN{\cdot}m^{-2}{\cdot}d^{-1}$) was calculated from the increase of N content in soybean shoots between sampling periods divided by the number of days between sampling date (Figure 5(a)). During the first 42-day period from planting to 25th July, the total N gain per day was relatively higher in LN ($98\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$) and DMU ($96\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$) treatments compared with Cont ($61\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$), U ($75\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$), and CU ($76\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$). During the second 30-day period from 25th July to 24th August, daily N gain was relatively high in CU ($273\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$), LN ($270\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$), and DMU ($258\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$) than that in Cont ($236\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$) and U ($235\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$) treatments. During the third 33-day period from 24th August to 26 September, the daily N gains were high in DMU ($492\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$), CU ($491\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$), U ($466\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$), and LN ($440\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$) than those in Cont ($345\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$). The daily N gains were highest in the third period from R5 to R7 stage, followed by the second period from R1 to R5 stage, and the lowest in the first period from planting to R1 stage in all treatments.

Figure 5(b) shows the daily N_2 fixation activity calculated from the daily N gain and relative ureide-N ratio during the first, second, and third periods. During the first 42-day period from planting to 25th July, daily N_2 fixation activities were relatively higher in DMU ($60\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$) and LN ($59\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$) treatments compared with Cont ($43\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$), U ($50\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$), and CU ($56\,mgN{\cdot}m^{-2}{\cdot}d^{-1}$). During the second 30-day period from

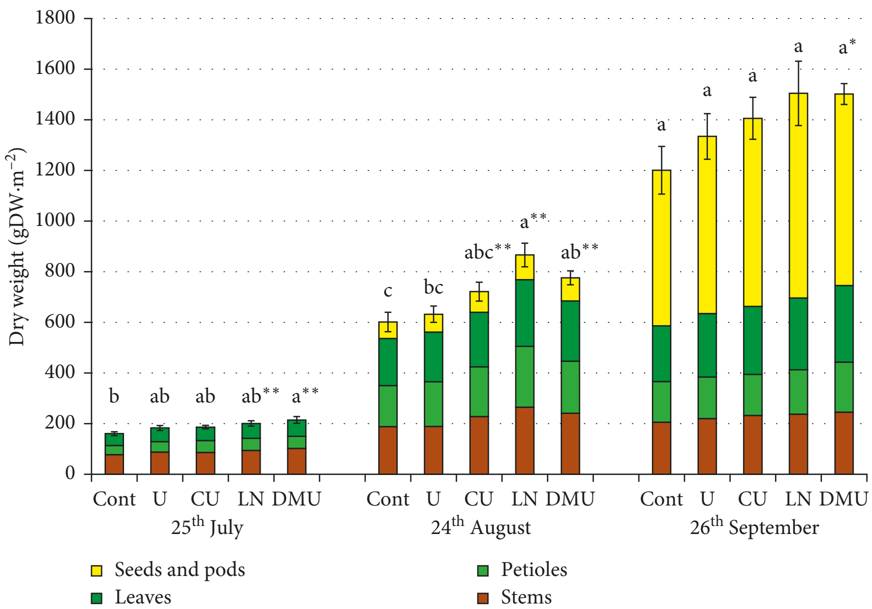

Figure 1: Changes in dry weight of shoot tissues of soybean cultivated with deep placement of various nitrogen fertilizers. Cont: control without deep placement of nitrogen; U: deep placement of urea; CU: deep placement of coated urea; LN: deep placement of lime nitrogen; DMU: deep placement of coated urea fertilizer with DMPP. The error bar indicates the standard error for total shoot weight. Different alphabet on the column indicates a statistical difference ($P < 0.05$) on each sampling day based on Tukey's test. $*$ and $**$ indicate statistical difference ($P < 0.05$, $P < 0.01$) on each sampling day between Cont and nitrogen treatment by Student's t-test.

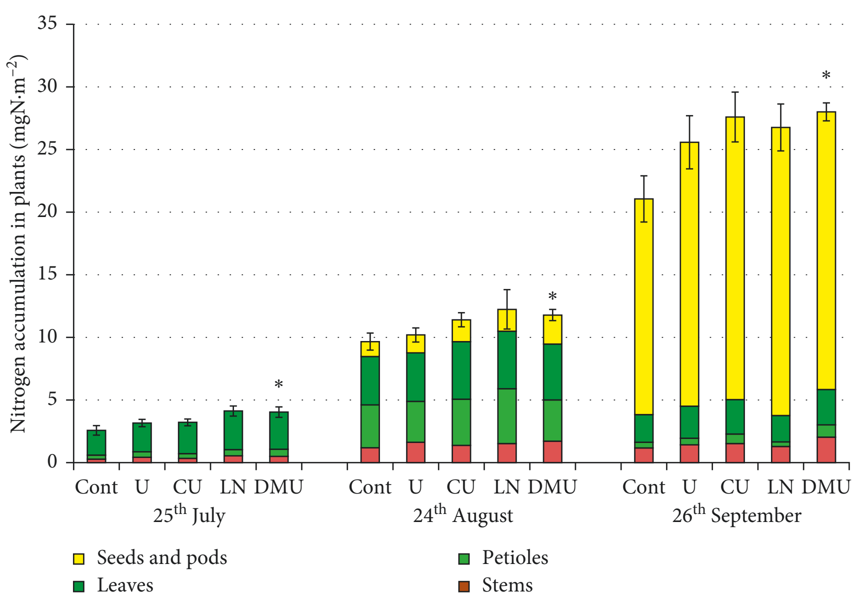

Figure 2: Changes in the nitrogen content of shoot tissues of soybean cultivated with deep placement of various nitrogen fertilizers. The footnotes are the same as in Figure 1. There is no statistical significance on each sampling day based on Tukey's test, so the alphabet is not shown in this figure.

25th July to 24th August, daily N_2 fixation activities were relatively high in CU (195 $mgN{\cdot}m^{-2}{\cdot}d^{-1}$) than those in LN (174 $mgN{\cdot}m^{-2}{\cdot}d^{-1}$), Cont (169 $mgN{\cdot}m^{-2}{\cdot}d^{-1}$), DMU (167 $mgN{\cdot}m^{-2}{\cdot}d^{-1}$), and U (160 $mgN{\cdot}m^{-2}{\cdot}d^{-1}$) treatments. During the third 33-day period from 24th August to 26th September, the daily N_2 fixation activities were high in CU (325 $mgN{\cdot}m^{-2}{\cdot}d^{-1}$), U (322 $mgN{\cdot}m^{-2}{\cdot}d^{-1}$), DMU (317 $mgN{\cdot}m^{-2}{\cdot}d^{-1}$) than those in LN (280 $mgN{\cdot}m^{-2}{\cdot}d^{-1}$) and Cont (232 $mgN{\cdot}m^{-2}{\cdot}d^{-1}$). The daily N_2 fixation activities were highest in the third period from R5 to R7 stage, followed by the second period from R1 to R5 stage, and the lowest in the first period from planting to R1 stage in all treatments.

Figure 5(c) shows the daily N absorption rate calculated from daily N gain minus daily N_2 fixation activity during first, second, and third periods. During the first 42-day

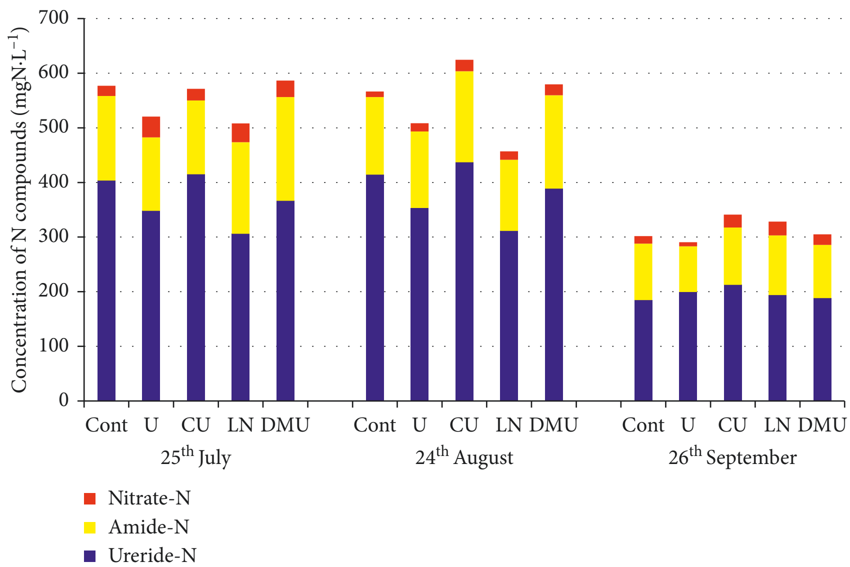

Figure 3: Concentrations of ureide-N, amide-N, and nitrate-N in xylem sap of soybean plants. The footnotes are the same as in Figure 1.

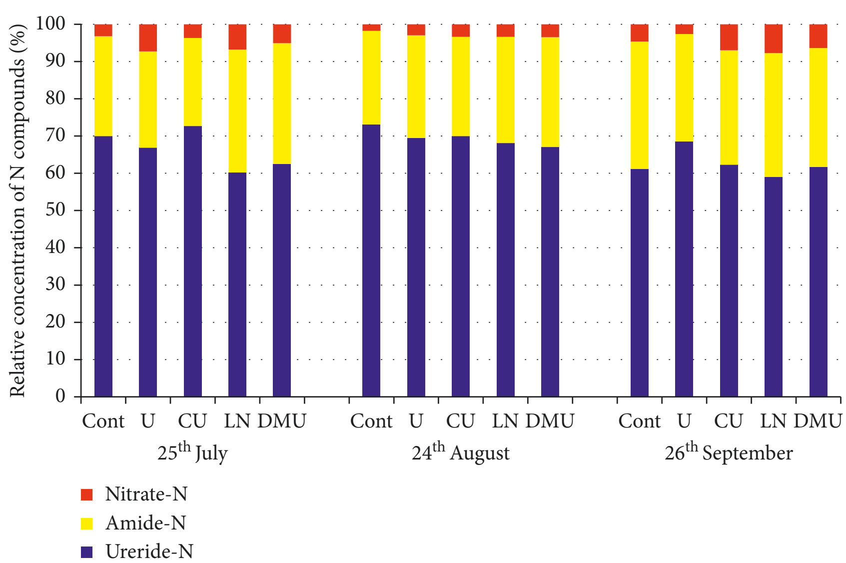

Figure 4: Percentages of ureide-N, amide-N, and nitrate-N in xylem sap of soybean plants. The footnotes are the same as in Figure 1.

period from planting to 25th July, daily N absorption rates were relatively higher in LN (39 mgN·m^{-2}·d^{-1}) and DMU (36 mgN·d^{-1}) treatments compared with U (25 mgN·m^{-2}·d^{-1}), CU (21 mgN·m^{-2}·d^{-1}), and Cont (18 mgN·m^{-2}·d^{-1}). During the second 30-day period from 25th July to 24th August, daily N absorption rates were relatively high in LN (97 mgN·m^{-2}·d^{-1}), DMU (90 mgN·m^{-2}·d^{-1}), CU (78 mgN·m^{-2}·d^{-1}), and U (75 mgN·m^{-2}·d^{-1}) than those in Cont (67 mgN·m^{-2}·d^{-1}) treatments. During the third 33-day period from 24th August to 26th September, the daily N absorption rates were high in DMU (175 mgN·d^{-1}), CU (166 mgN·d^{-1}), and LN (161 mgN·d^{-1}) than those in U (144 mgN·m^{-2}·d^{-1}) and Cont (113 mgN·m^{-2}·d^{-1}). The daily N absorption rates were highest in the third period from R5 to R7 stage, followed by the second period from R1 to R5 stage, and the lowest in the first period from planting to R1 stage in all treatments.

Figure 6 shows the seed yields of soybean among fertilizer treatments. The average seed yield was highest in LN (463 g·m^{-2}) and DMU (461 g·m^{-2}), medium in CU (405 g·m^{-2}) and U (396 g·m^{-2}), and lowest in Cont (373 g·m^{-2}). The seed yield in DMU ($P < 0.05$) and LN ($P < 0.01$) against Cont treatment was statistically significant based on Student's *t*-test.

Figure 7 shows the percentage distribution of seed quality of harvested seeds. In this experiment, the percentage of good seeds was between 40 and 45% and relatively low. The percentage of broken coat accounted for 30–40% in all the fertilizer treatments. The percentage of side wrinkle was relatively low in DMU (6%), U (9%) and LN (11%) compared with Cont (15%). The percentages of turtle wrinkle and small seeds were about 2–3% and 1–2%, respectively. The percentage of damaged seeds due to abortion, insect fed,

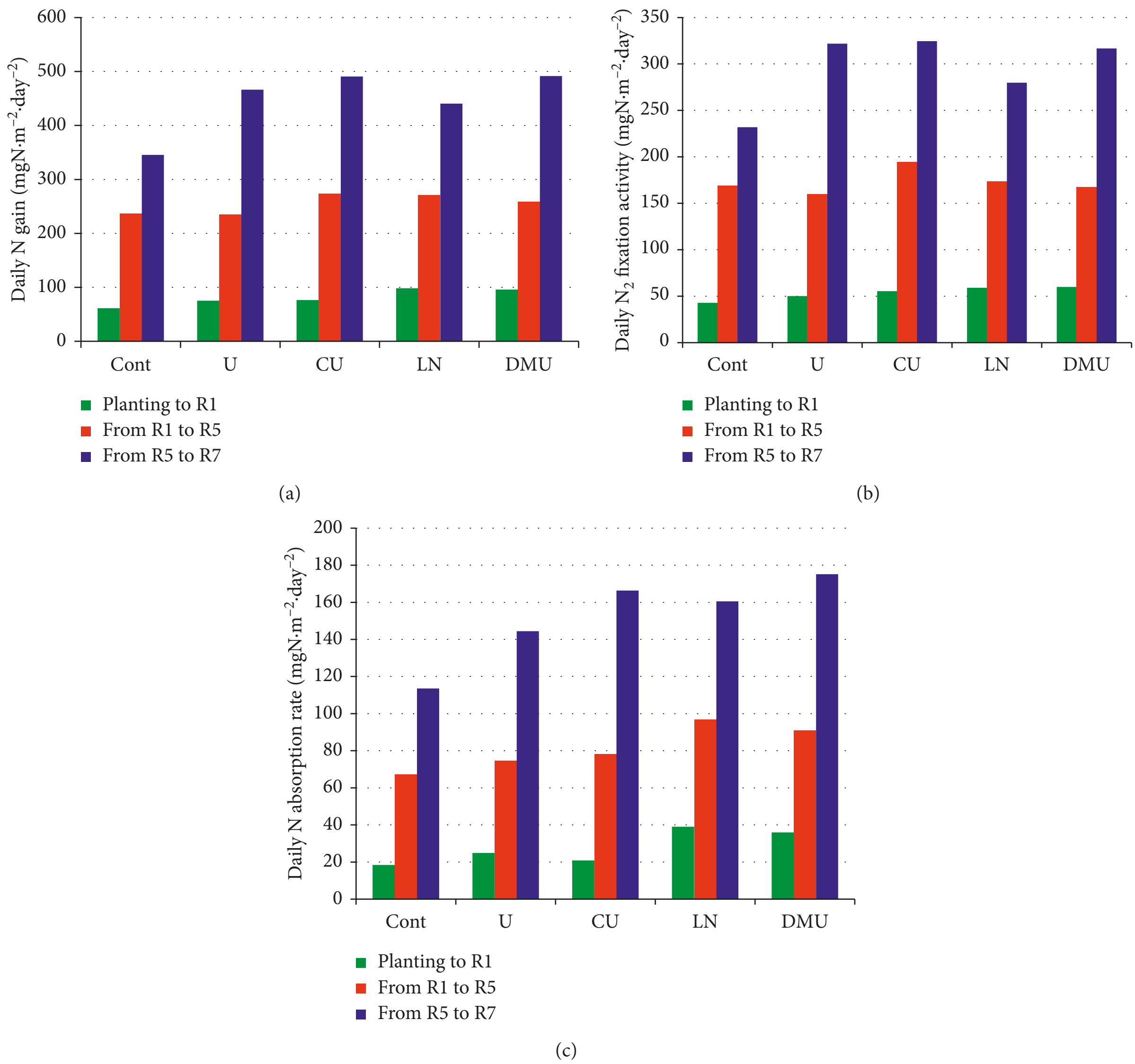

FIGURE 5: Changes in total nitrogen accumulation and daily nitrogen fixation activity and daily nitrogen absorption rate of soybean plants. The footnotes are the same as in Figure 1. (a) Daily total N accumulation. (b) Daily nitrogen fixation activity. (c) Daily nitrogen absorption rate.

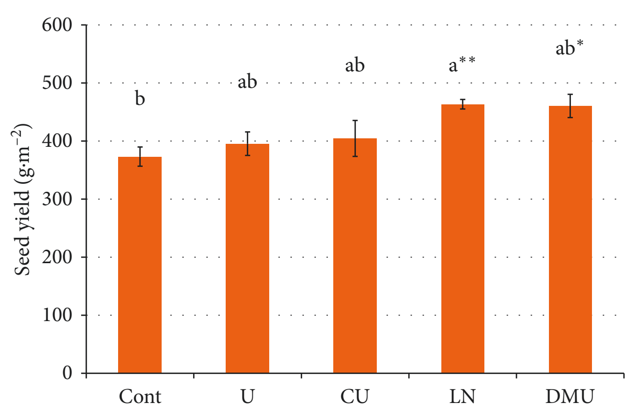

FIGURE 6: Seed yield of soybean plant with different deep placement treatments. The footnotes are the same as in Figure 1.

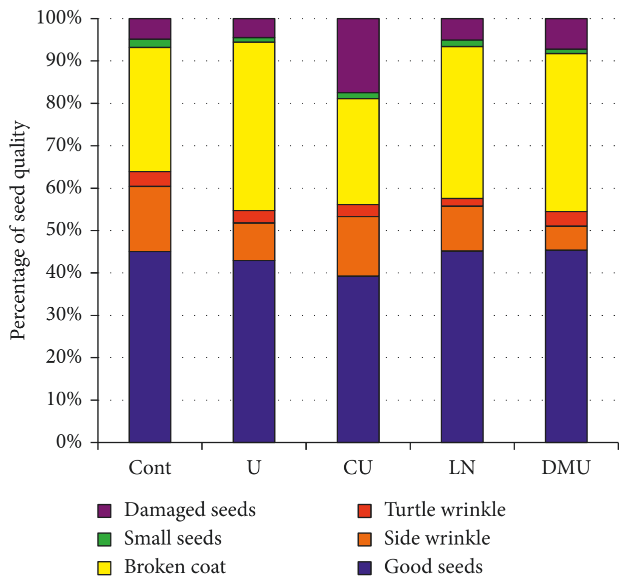

Figure 7: Percentage distribution of seed quality of soybean plant with different deep placement treatments. Cont: control without deep placement of nitrogen; U: deep placement of urea; CU: deep placement of coated urea; LN: deep placement of lime nitrogen; DMU: deep placement of coated urea fertilizer with DMPP.

disease, and dirty seeds was high in CU (17%) compared with other treatments (5–7%).

The average one seed weights were not significantly different among fertilizer treatments: average seed weight (standard error) $n = 16$, Cont 390 (6.6) mg, U 392 (4.4) mg, CU 390 (6.4) mg, LN 386 (4.4) mg, and DMU 412 (6.0) mg.

3.2. Soil Incubation Test. Figure 8 shows the changes in the amounts of ammonium-N and nitrate-N during incubation periods with different fertilizer treatments. Cont indicates that soil was incubated without N fertilizers, so the accumulation of ammonium and nitrate was derived from degradation of soil organic matter. The initial concentrations of ammonium-N and nitrate-N in the soil at 0 weeks were 0.08 mgN and 0.21 mgN, respectively. During incubation for 8 weeks at 25°C, the concentrations of ammonium-N and nitrate-N in Cont increased to 0.18 mgN and 0.59 mgN, respectively. When 10 mgN of urea was added (U), urea was rapidly degraded to ammonium (7.63 mgN) and nitrate (1.71 mgN) after the 1-week incubation period, suggesting that most of the urea was rapidly degraded to ammonium in one week, and some ammonium was further oxidized to nitrate. After 1 week to 8 weeks, the amount of ammonium-N decreased and nitrate-N increased and the final concentrations were 4.0 mgN and 7.2 mgN, respectively. The incubation of soil with the commercial DMU shows rapid urea degradation to ammonium in the 1-week incubation period, and the amount of ammonium-N and nitrate-N was 7.64 mgN and 0.88 mgN, respectively. The amounts of nitrate in DMU treatment were about a half of U treatment from 1- to 8-week period of incubation. The final concentration of ammonium-N was 5.46 mgN and that of nitrate-N was 3.73 mgN. The slow rate of nitrate accumulation may be due to the nitrification inhibition by DMPP. The incubation of LN resulted in the lower ammonium accumulation (5.45 mgN) at 1-, 2-, and 4-week period of incubation compared with DMU. This may be due to the lag-time to hydrolyze calcium cyanamide to cyanamide, to urea, and then to ammonium. The accumulation of nitrate-N was similar to the DMU treatment, and the final concentration of nitrate-N at 8 weeks was 3.54 mgN. When 10 mgN urea was mixed with increasing concentrations of DMPP at 1, 2, 4% of urea (DMPP1, DMPP2, and DMPP4), the amount of nitrate-N was relatively constant among different concentrations of DMPP added.

4. Discussion

Soybean seeds contain a high proportion of protein about 40% based on dry weight. Therefore, the total amount of assimilated N, either from N_2 fixation or from absorbed N from roots, is proportional to the seed yield. About 80 kgN is required for 1 t of soybean seed yield [12]. In addition, soybean plants accumulate only about 20% of total N until the initial flowering stage, and they require about 80% of N assimilated during the reproductive stage. So, the N assimilation during the reproductive stage is very important to obtain high seed yield. The application of a large amount of chemical fertilizer for soybean is generally repressing nodule formation and the N_2 fixation activity, and the seed yield cannot be increased. Salvagiotti et al. reviewed 637 data sets of soybean field experiment from 1966 to 2006 and indicated that a negative exponential relationship was observed between N fertilizer rate and N_2 fixation activity when N was applied on the surface or incorporated in the topmost soil layers [39]. Takahashi et al. developed a deep placement of 100-day type coated urea for soybean cultivation, and soybean seed yield was higher than control conventional cultivation [8–11]. Tewari et al. confirmed the similar effect of lime nitrogen and coated urea for this technique and revealed that coated urea can be replaced by lime nitrogen [13–18].

In this research, the application of a newly developed fertilizer (DMU) containing 1% DMPP showed significantly higher dry matter accumulation in the shoots compared with Cont without deep placement of nitrogen fertilizers, and the dry weight was almost the same levels as LN and CU treatments (Figure 1). The same was true for the nitrogen accumulation in the shoots (Figure 2). The concentrations (Figure 3) and percentages (Figure 4) of ureide-N, amide-N, and nitrate-N in the xylem sap were similar among DMU, LN, and CU treatments and higher than the Cont and U treatments. Seasonal changes of daily nitrogen fixation activity (Figure 5(b)) and daily nitrogen absorption rate (Figure 5(c)) were always higher in DMP, LN, and CU compared with Cont treatment. These results indicate that the deep placement of DMU did not inhibit nitrogen fixation activity but promoted the nitrogen fixation activity. The seed yield was significantly higher in DMU than control treatment (Figure 6), and the seed damage by side wrinkle decreased by DMU and LN deep placement (Figure 7).

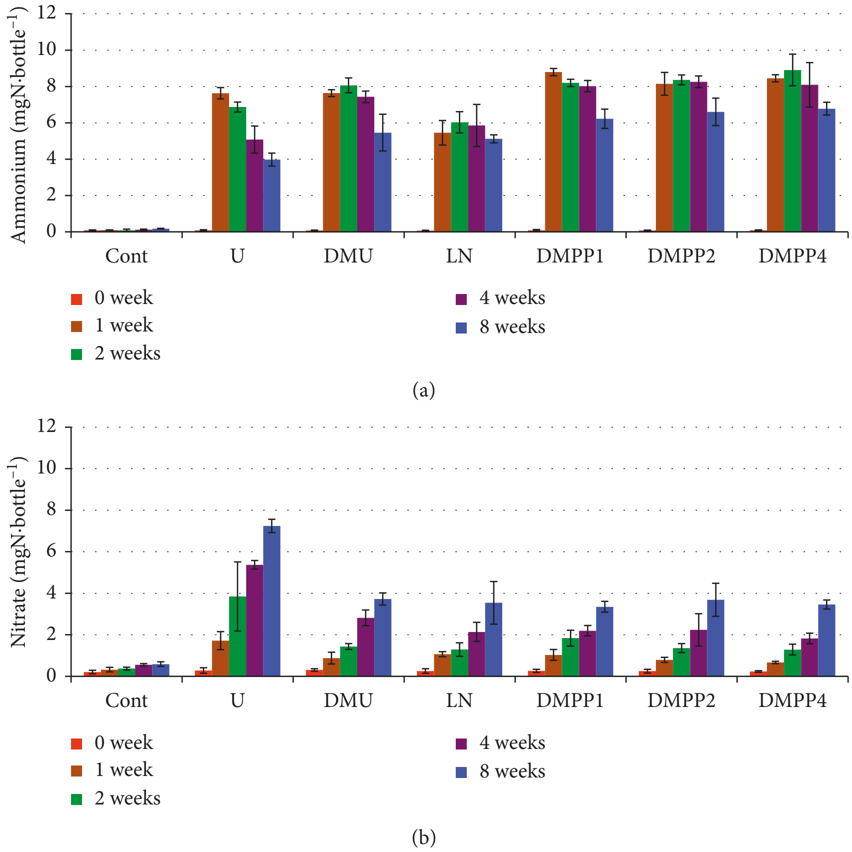

FIGURE 8: Changes in the amount of ammonium and nitrate during incubation period with different fertilizer treatments. The footnotes are the same as in Figure 7.

The nitrogen fixation activity during R5 to R7 was higher than the previous periods from R1 to R5. Former results using soybean cultivar "Enrei" indicated that the maximum nitrogen fixation activity was observed from R1 to R5 and then decreased in the period of R5 to R7 [10, 15, 19]. This discrepancy may be attributed to the different characteristics of growth in "Enrei" and "Satonohohoemi." The growth stage of Satonohohoemi is 7–10 days later than Enrei, and it has larger leaf area and stem diameter as well as a larger seed size.

From the results obtained, DMU can be replaced for CU or LN for deep placement of nitrogen fertilizer. Urea in DMU was rapidly hydrolyzed into ammonium and carbon dioxide, and DMPP might inhibit the conversion from ammonium to nitrite. Takahashi et al. analyzed the accumulation of ammonium-N, nitrate-N, and urea-N in surface soil from 0–10 cm and deep soil from 15–25 cm in the field at R1, R3, R5, and R7 stages [10]. A high accumulation of ammonium-N was observed in the deep layer (15–25 cm) of soil at R3 and R5 stages by deep placement of 100 day-type CU, although ammonium and nitrate accumulation was not observed in surface layer at any stage with deep placement of CU. The result indicated that deep placement of CU slowly released urea, and urea outside the particle was rapidly degraded to ammonium but it remains in the deep layer of soil relatively long time. The slow nitrification rate might be due to low oxygen concentration and low nitrification activities by microbes in the lower layer in rotated paddy fields in Niigata [11]. Using the DMU, the urea is rapidly dissolved in soil water and hydrolyzed to ammonium, but the ammonium remained for a while by nitrification inhibition by DMPP.

From the soil incubation test using the same field (Figure 8), the nitrate accumulation was similar between DMU and LN and about a half of U. By increasing DMPP concentrations to 2% and 4% of urea, the nitrate accumulation was almost the same as the treatment with 1% DMPP. The urea fertilizer with 1% DMPP may be practical from the point of cost.

Application of DMPP has several advantages, such as an increase in N-use efficiency and promote crop yield, decreasing the nitrate leaching to underground and river. Kou et al. [23] investigated the effects of DMPP and DCD on N_2O emission in a greenhouse vegetable soil. DMPP is recognized to have not toxicological or ecotoxicological side effects so far according to the European legislation test and does not give toxic damages to plants [22]. The use of DMU in the deep placement of nitrogen fertilizer for soybean production is promising due to a low price, although more field experiments are needed.

The use of DMU for a deep placement may be useful not only for soybean but also for other crops, the growth period of which is long. Kaneta et al. [40] reported that the basal deep placement of lime nitrogen on winter wheat in a heavy clay upland field converted from rice cultivation resulted in the same yields as the conventional cultivation method with five times top dressing of N fertilizers.

5. Conclusions

A urea fertilizer (100 kgN·ha^{-1}) mixed with 1% (W/W) 3,4-dimethylpyrazole phosphate (DMU) was injected about 20 cm depth under seeding line of soybean. The effect of DMU on soybean growth and seed yield was as effective as the deep placement of coated urea (CU) and lime nitrogen (LN) in the rotated rice field. The accumulations of dry matter and nitrogen in shoots were higher in the plants with deep placement of CU, LN, and DMU compared with control treatment without deep placement. The daily nitrogen fixation activity and daily nitrogen absorption rates were also higher in DMU, CU, and LN compared with control treatment, suggesting that the deep placement of DMU did not repress nitrogen fixation, but increased it similar to the previous results with CU and LN. Soil incubation test was performed using the same field soil with DMU, U, LN, and urea with 1%, 2%, and 4% DMPP. DMU inhibited nitrification similar to the pattern of LN until 8 weeks. The increased DMPP concentrations did not markedly increase the nitrification inhibition. From this result, it was concluded that urea fertilizer with 1% DMPP is efficient for deep placement of N fertilizer for the sustainable cultivation of soybean.

Data Availability

All data generated or analyzed during this study are included within the article as the supplementary information files.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Figure S1: a rotary cultivator with deep placement of N fertilizers, basal dressing of N, P, K combined fertilizers, pesticide, and soybean seeds. (*Supplementary Materials*)

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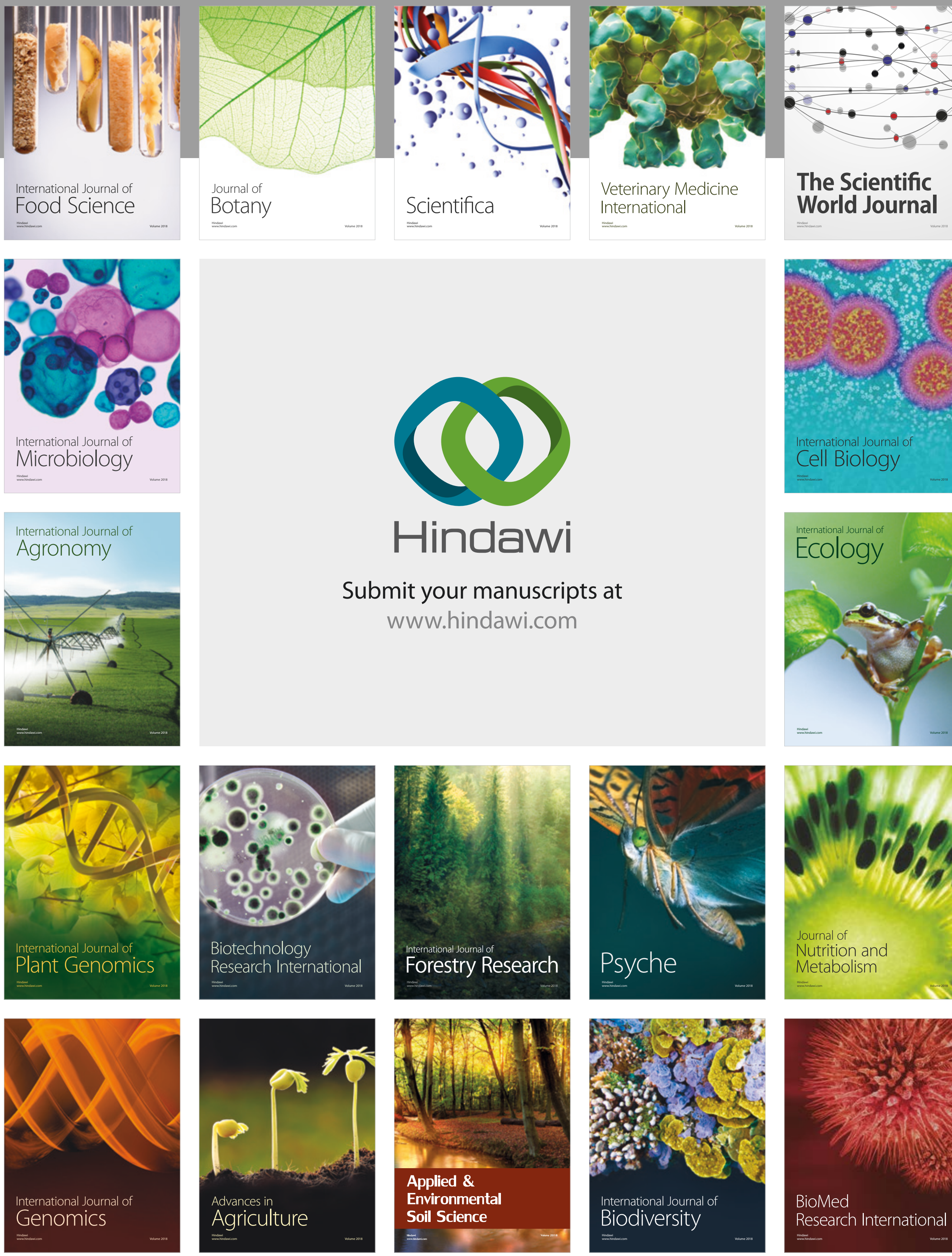
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