Effect of Winter Crop Species on Arbuscular Mycorrhizal Fungal Colonization and Subsequent Soybean Yields

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Abstract: We evaluated how the cultivation of arbuscular mycorrhizal fungi (AMF) host (wheat) and non-host (rapeseed) crops affects the subsequent soybean crop by assessing AMF spore density and AMF colonization, phosphorus (P) uptake by soybean and yields of soybean over a 4-year period. Every year wheat or rapeseed was cultivated from autumn to spring and soybean from spring to autumn. From the first to fourth year, AMF spore density was higher in the plot after the cultivation of wheat (wheat plot) than in the rapeseed plot. From the second to fourth year, the AMF colonization ratio was higher in the wheat plot than in the rapeseed plot. In the first year, there was no difference in the AMF colonization ratio, growth, and P uptake by soybean plants between the rapeseed plot and wheat plot. However, from the second to fourth year, AMF colonization ratio, plant growth, and P uptake by soybean in the wheat plot were higher than those in the rapeseed plot. The soybean yields in both plots gradually decreased from the first to fourth year, but, in the second and the fourth year, soybean yields were higher in the wheat plot than in the rapeseed plot. Soybean yield was significantly correlated with the AMF colonization ratio, but not with AMF spore density. Therefore, we concluded that AMF colonization is not determined by AMF spore density alone, and other factors influence the AMF colonization in subsequent soybean plants. It is important to increase the AMF colonization ratio to increase soybean yield.

Key words: Arbuscular mycorrhizal fungi, Colonization ratio, Cropping system, Preceding crop, Soybean (Glycine max (L.) Merr.), Yield.

At present, all phosphoric acid fertilizer materials is obtained from imported phosphate rock, but the global phosphate rock reserve is decreasing (Kuroda et al., 2005; Gilbert, 2009), alternative techniques to supply the plant phosphorus (P) must be developed. Arbuscular mycorrhizal fungi (AMF) symbiotically inhabit the cortical cells of vascular plant roots. These fungi absorb P and other nutrients from the soil facilitating the uptake of these nutrients by host plants. Utilization of this natural relationship may be beneficial for crop production especially that of leguminous crops whose roots absorb P poorly as compared with gramineous crops (Isobe and Tsuboki, 1998).

The maintenance of AMF spore density in field soil is an important criterion for enhancing crop production. AMF spore density and colonization have been shown to increase after the cultivation of AMF host crops. For example, after a winter crop of barley (Hordeum vulgare L.) or pea (Pisum sativum L.), the density of AMF spore, AMF colonization ratio and the growth of succeeding kidney bean (Phaseolus vulgaris L.) were greater as compared with bare ground (Isobe and Tsuboki, 1999). On the other hand, after a crop that is not an AMF host (non-host crop), the density of AMF spore and AMF colonization ratio in the succeeding crops were decreased (Arihara and Karasawa, 2000; Usuki and Yamamoto, 2003). Moreover, wheat (Triticum aestivum L.) and barley are effective winter crops for maintenance of AMF spore density and increase of colonization ratio in succeeding crops (Isobe and Tsuboki, 1999; Uchida et al., 2011). However, the results concerning AMF spore density, AMF colonization ratio, and successive crop growth varied with the number of years after the host or non-host crops was introduced (Hamel et al., 1996; Isoi, 1997; Usuki et al., 2004; Oyetunji and Osonubi, 2007). For example, the AMF spore density after cultivation of an AMF host increased from the first to third year of a field experiment (Hamel et al. 1996), but only in the first year in another study, and the difference in AMF spore density between the AMF host plot and non-host plot was not clear (Isoi, 1997; Usuki et al., 2004).

Previous studies indicated that an AMF host crop should be cultivated every year to increase the soil AMF spore...
density. Then, AMF colonization, P absorption, and plant growth of subsequent crops are expected to increase with repeated introduction of an AMF host crop. In this study, we evaluated the effect of repeated introduction of AMF host crops versus non-host crops on the growth of soybean, a subsequent leguminous crop by analyzing soil AMF spore density, soybean AMF colonization, P content, and yield of soybean over a 4-year period.

**Materials and Methods**

1. **Experiment site**
   The field experiment was conducted from 2007 to 2011 at the experimental farm of Nihon University in Fujisawa, Kanagawa Prefecture, Japan. The soil type of this field was Andosol. Before this experiment, soybean (cultivar Enrei) was cultivated in the field from May to November 2007, without using any fertilizers.

2. **Plots and cultivation system**
   In November 2007, 6 plots (4.5 m \( \times \) 2.0 m) were established in this field. Every year, from autumn to spring, rapeseed cultivar Michinokunatane (rapeseed plot) and wheat cultivar Bando-wase (wheat plot) were cultivated in 3 plots each. From spring to autumn, soybean cultivar Enrei was cultivated in all 6 plots. Cultivation in each plot was replicated 3 times. Plots were placed randomly in the field. Rapeseed and wheat seeds were sown on 19 November 2007, 19 October 2008, 22 October 2009 and 18 November 2010. The shoot of these crops was cut close to ground on 10 June 2008, 1 June 2009, 5 June 2010 and 8 June 2011 and removed from the field. Subsequently, soybean seeds were sown on 11 June 2008, 9 June 2009, 11 June 2010 and 11 July 2011 in all 6 plots. The field soil was not tilled when sowing any of the crops. All of the crops were cultivated without phosphate (\( \text{P}_2\text{O}_5 \)) fertilizer. The rates of nitrogen (N) and potassium (K) fertilizer application, seeding density, and planting density of each crop are shown in Table 1. The control of weeds, diseases, and insects were done as required. Irrigation consisted of natural rainfall alone from sowing to harvest.

3. **Soil sampling for chemical and AMF spore density analysis**
   After cultivation of the winter crops of rapeseed and wheat, soil was randomly sampled at 5 points (depth, 15 cm; diameter, 20 cm) in each plot on 10 June 2008, 1 June 2009, 5 June 2010 and 8 June 2011. All samples from each plot were combined. A portion of the gathered soil samples was air-dried to measure the chemical properties, while the remaining soil samples were used to determine the number of AMF spores.

   The pH (\( \text{H}_2\text{O} \)), electrical conductivity, total P, and available P were measured using the glass electrode method, the 1:5 water extraction method, wet ashing followed by molybdenum yellow colorimetry, and Bray 2 method (Bray and Kurtz, 1945), respectively. A wet sieving method was used to obtain AMF spores, by sieving 10 g soil through 500 \( \mu \)m and 53 \( \mu \)m meshes (Nishio, 1987). The spores were then recovered by a sucrose density gradient centrifugation, and counted under a microscope, while also confirming their morphological characteristics (Gerdemann and Nicolson, 1963).

4. **Winter crop shoot dry weight**
   The shoots of cultivated winter crops, rapeseed and wheat, the winter crop shoots in 0.5 m \( ^2 \) area were cut at the soil surface on 22 June 2009 and 8 June 2011. Above ground plant biomass were determined after samples were oven-dried at 80ºC for 48 hr.
5. Measurement of AMF colonization and growth of soybean

The AMF colonization ratio in the roots and the shoot growth of soybean were measured at the flowering stage. The flowering stage was 28 July in 2008, 23 July in 2009, 23 July in 2010 and 19 August in 2011. 5 to 10 plants were randomly sampled from each plot and the leaf area and total node number were measured. The leaf area was measured with an Area Meter (LI-3100, LI-COR Inc., USA). Subsequently, the shoots of the soybean plants were cut at the cotyledon node and then the root samples (depth, 15 cm; diameter, 20 cm) were collected. Above-ground plant biomass and P uptake were determined after samples were oven-dried at 80ºC for 48 hr. P content was determined using the molybdenum yellow colorimetric method (Sekiya, 1970).

Soybean root samples were carefully washed with tap water. All the roots from one plant were cut into 1 × 2 cm segments. All root segments from 5 to 10 plants were gathered and mixed. Then, 100 segments were put in a 10% KOH solution, heated at 105ºC for 10 min in an autoclave, and rinsed in distilled water. Subsequently, the samples were bleached in a 10% H₂O₂ solution for 1 min and stained with a 0.05% trypan blue solution. Then, the samples were heated at 105ºC for 5 min in an autoclave to observe AMF colonization (Oba et al., 2006). The AMF colonization ratio was estimated by the grid-line intersect method (Giovannetti and Mosse, 1980). The lowest number of crossings of grid and roots in each repetition was 200.

6. Yield survey of soybean

10 to 25 soybean plants in each repetition (total soybean plants were from 30 to 75 per plot) were cut to measure yield and yield components (i.e., pod number, seed number per pod, and the weight of 100 seeds) at the maturity stage. The maturity stage in each year was 4 October 2008, 22 September 2009, 8 October 2010 and 6 October 2011. In this experiment, seeds with diameter exceeding 5 mm were used for the yield survey.

7. Statistical analysis

All values were expressed as mean values. The data were analyzed statistically and significant differences between the rapeseed plot and wheat plot were determined by F-test.

Results

1. Soil chemistry and AMF spore density after winter crop cultivation

After winter crop cultivation, no significant differences were observed in the soil pH, electric conductivity, or total P content between rapeseed and wheat plots in any of the 4 years. Furthermore, significant differences were not observed for available P content in the rapeseed versus wheat plots in 2008 and 2009; however, in 2010 and 2011, the available P content of the rapeseed plots was higher than that of wheat plots with significant differences at the 5% or 1% level in both plots (Table 2).

| Plots       | 10 June 2008 | 22 June 2009 | 5 June 2010 | 8 June 2011 |
|-------------|-------------|-------------|-------------|-------------|
| Rapeseed pH | 5.8         | 5.7         | 5.4         | 5.7         |
| Wheat pH    | 5.8         | 6.3         | 5.4         | 5.8         |
| F-test      | ns          | ns          | ns          | ns          |
| Rapeseed EC (dS m⁻¹) | 0.10      | 0.13        | 0.20        | 0.21        |
| Wheat EC (dS m⁻¹) | 0.09      | 0.13        | 0.15        | 0.17        |
| F-test      | ns          | ns          | ns          | ns          |
| Rapeseed Available P | 0.5      | 1.3         | 2.0         | 3.9         |
| Wheat Available P | 0.5      | 1.4         | 1.0         | 1.6         |
| F-test      | ns          | ns          | *           | **          |
| Rapeseed Total P (mg 100g⁻¹) | 220       | 235         | 176         | 250         |
| Wheat Total P (mg 100g⁻¹) | 214       | 224         | 144         | 224         |
| F-test      | ns          | ns          | ns          | ns          |

| Plots       | 10 June 2008 | 22 June 2009 | 5 June 2010 | 8 June 2011 |
|-------------|-------------|-------------|-------------|-------------|
| Rapeseed Total P (mg 100g⁻¹) | ns          | ns          | ns          | ns          |
| Wheat Total P (mg 100g⁻¹) | ns          | ns          | ns          | ns          |

ns: no significant difference by F-test. *, **: 5% level, 1% level of significant difference by F-test (n = 3).
rapeseed, there was no significant difference in shoot dry weight between 2009 and 2011, but, in wheat, it was significantly heavier in 2009 at the 1% level (Table 3). The growth of wheat in 2008 and 2010 were similar to those in 2009 and 2011, respectively. In this experiment, above ground plant biomass were not determined in 2008 and 2010.

3. AMF colonization ratio and soybean growth at the flowering stage

After rapeseed cultivation, the AMF colonization ratio in soybean roots at the flowering stage was 12.7, 7.6, 2.9 and 4.0% in 2008, 2009, 2010 and 2011, respectively. After wheat cultivation, the AMF colonization ratio was 15.5, 16.3, 6.7 and 9.1% in 2008, 2009, 2010, and 2011, respectively. The differences between the 2 plots in 2009, 2010 and 2011 were significant at the 5% or 1% level (Fig. 2).

In 2008, there was no significant difference in shoot dry weight, leaf area index (LAI), total node number and P content of the plant shoot at the flowering stage between the rapeseed and wheat plots. However, from 2009 to 2011, these parameters were significantly higher in the wheat plot than in the rapeseed plot at the 5% or 1% level (Table 4, Fig. 3).

4. Yield components and yield of soybean

In 2008, there was no significant difference in pod number between rapeseed and wheat plots. However, from

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**Table 3. Winter crop shoot dry weight in 2009 and 2011.**

| Winter crop | 22 June 2009 | 8 June 2011 | F-test |
|-------------|--------------|-------------|--------|
| Rapeseed    | 1489         | 1276        | ns     |
| Wheat       | 978          | 626         | **     |

ns; no significant difference by F-test. **: 1% level of significant difference by F-test. n = 3.

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**Table 4. Effects of preceding crops on subsequent soybean growth.**

| Plots      | 28 July 2008 | 23 July 2009 | 23 July 2010 | 19 August 2011 |
|------------|--------------|--------------|--------------|----------------|
| Rapeseed   |              |              |              |                |
| Shoot dry weight (g m⁻²) | 129.0 | 59.6 | 58.6 | 72.3 |
| F-test     | ns           | *            | *            | **             |
| Wheat      |              |              |              |                |
| LAI        | 2.1          | 1.2          | 1.2          | 1.7            |
| F-test     | ns           | *            | *            | **             |
| Rapeseed   |              |              |              |                |
| Total node number (node m⁻²) | 304 | 196 | 194 | 191 |
| F-test     | ns           | ns           | *            | *              |
| Wheat      |              |              |              |                |
| Phosphorus content of shoot (mg m⁻²) | 306.5 | 130.2 | 140.6 | 159.1 |
| F-test     | ns           | **           | **           | **             |

ns; no significant difference by F-test. *, **: 5% level, 1% level of significant difference by F-test (n = 5).
not significant in any of the years. There was no significant difference in the 100 seed weight between the rapeseed and wheat plots in 2008, 2009 and 2010. In 2011, it was significantly higher in the wheat plots than in the rapeseed plots at the 5% level. After rapeseed cultivation, the yield of subsequent soybean was 403.1, 277.8, 268.1 and 162.7 g per m² in 2008, 2009, 2010 and 2011, respectively. After wheat cultivation, it was 345.3, 356.5, 297.1 and 233.3 g per m² in 2008, 2009, 2010 and 2011, respectively. Significant differences in yield between the 2 plots were obtained at the 1% level in 2009 and at the 5% level in 2011 (Table 5).

5. Relationship between AMF spore density, colonization ratio, P content of plant shoots and soybean yield

From 2008 to 2011, AMF spore density in both rapeseed and wheat plots was positively correlated with the AMF colonization ratio ($r = 0.433$), P content of the plant shoots ($r = 0.265$) and soybean yield ($r = 0.337$), but the correlation was not significant. In all 4 years, the AMF colonization ratio in the 2 plots was positively correlated with the P content of plant shoots ($r = 0.761$) and soybean yield ($r = 0.762$) at the 5% level (Table 6).

Discussion

1. Effect of AMF spore density and soil chemicals on AMF colonization ratio in successive soybean

We found that cultivation of wheat, an AMF host crop, increased the AMF spore density and the colonization ratio in the subsequent soybean (Fig. 1, 2). This result is in agreement with previous reports (Isobe and Tsuboki, 1999; Arihara and Karasawa, 2000; Usuki and Yamamoto, 2003; Usuki et al., 2007). In general, it is important to increase AMF spore or biomass density to increase the AMF colonization ratio (Isobe and Tsuboki, 1997; Usuki and Yamamoto, 2003), and in this study, a positive correlation ($r = 0.433$) was observed between AMF spore density and the AMF colonization ratio in soybean after cultivation of wheat, an AMF host crop. However, this correlation was not statistically significant (Table 6). This means that the AMF colonization ratio is not determined by AMF spore density alone.

The cultivated crop type and the rate of fertilizer application affect soil chemistry (Tadano and Sakai, 1991; Karasawa, 2004; Takama and Hirosawa, 2008), which in turn may affect AMF spore germination, hyphal growth, and the colonization in plant roots (Daniels and Trappe, 1980; Habte and Soedarjo, 1995; Hamel et al., 1996). We examined whether wheat, an AMF-host crop, had a different effect from rapeseed, a non-AMF-host crop, on the subsequent soybean growth. However, soil pH, electrical conductivity and total P content after cultivation of rapeseed were not significantly different from those after cultivation of wheat (Table 2). In 2010 and 2011, the available P content was higher in the rapeseed plot than in
the wheat plot, but there was no difference between the 2 plots in 2008 and 2009 (Table 2). The reason why the available P content after rapeseed cultivation was higher is that rapeseed roots exude organic acids, resulting in increased P availability under P-deficient conditions (Zhang et al., 1997). Moreover, the reason why the available P content after wheat cultivation was lower than that after rapeseed cultivation is that P in soil was absorbed by mycorrhiza of wheat root. In general, increased available P content of soil lowers the AMF colonization ratio (Tawaraya and Saito, 1994; Isobe and Tsuboki, 1997).

In 2010 and 2011, the available P content was lower in the wheat plot than in the rapeseed plot (Table 2). However, the AMF colonization ratio in soybean was higher in the wheat plot than in the rapeseed plot (Fig. 2). Therefore, we conclude that differences in the available P content might not affect the AMF colonization ratio in subsequent soybean.

### 2. Change in AMF spore density, colonization ratio and soybean growth with the year

In this 4-year field experiment, the AMF spore density and colonization ratio in subsequent soybean in the rapeseed plot differed from those in the wheat plot. The AMF spore density in the rapeseed plot increased in 2009 and 2010, and decreased in 2011, while that in the wheat plot remained stable from 2008 to 2011 the difference between the rapeseed and wheat plots being significant in all 4 years (Fig. 1). The AMF colonization ratio in both wheat and rapeseed plots decreased from 2008 to 2011 (Table 5), with a significant correlation at the 5% level between the AMF colonization ratio and soybean yield over the 4-year period. However, no significant correlation was observed between soybean yield and AMF spore density (Table 6). No P fertilizer was applied to this strong P fixation soil (i.e., Andosol) (Table 1). It is possible that the soybean plants used in this experiment lacked P as a source of nutrition. This lack of P became worse with the year. In general, an increase in soybean growth and yield as a result of AMF colonization is more marked under P-poor conditions (Tawaraya and Saito, 1994; Isobe and Tsuboki, 1997). In this experiment, there was a significant correlation at the 5% level between the amount of P uptake of plant shoots and the AMF colonization ratio (Table 6). Hence, we concluded that the P uptake by soybean plants in this experiment was affected by AMF colonization. Moreover, we concluded that, in this experiment, soybean yield was also affected by AMF colonization. Therefore, it is important to increase the AMF colonization ratio to increase soybean yield in this field. The preceding crop type is known to affect the growth of subsequent crops through several mechanisms, such as changes in water use efficiency, nutrient use efficiency, disease interactions, allelopathy, and soil quality (Liebman and Dyck, 1993; Karlen et al., 1994; Burle et al., 1997). It is possible that differences in the growth and yield of soybean between the rapeseed and wheat plots were influenced by differences in the AMF colonization ratio, as
well as other factors. For example, rapeseed roots contain a large quantity of autotoxic components, and the root exudates inhibit the growth of other crops (Yasumoto et al., 2011). Thus, differences in the growth and yield of soybean between the rapeseed and wheat plots (Table 4, Fig. 3) might have also been affected by the root exudates. Usuki et al. (2004) reported that the growth of subsequent crops through the introduction of an AMF-host crop was stimulated only in the first season. However, this observation was not supported by the current experiment: The growth of soybean in the wheat (AMF-host crop) plot was greater than that in the rapeseed (non-AMF-host) plot from 2009 onwards, though not different in 2008 (Table 4, Fig. 3), which was probably due to the lack of difference in the AMF colonization ratio between the rapeseed and wheat plots in 2008 (Fig. 2). However, Hamel et al. (1996) reported that the introduction of an AMF-host crop enhanced the growth of subsequent crops for 3 successive years. The cause of such differences remains to be elucidated. In conclusion, the current study demonstrates the potential utility of planting wheat prior to soybean to enhance the availability of P nutrients to improve crop yields.

We concluded that AMF colonization in the subsequent soybean plant is not determined by AMF spore density alone. We found that the preceding AMF-host crop increased the colonization ratio and seed yield of the subsequent soybean as compared with a non-AMF-host crop from the second year. Since the soybean yield decreased gradually in parallel with the decrease in the AMF colonization ratio from the first to fourth year cultivation of an AMF host crop could be a useful strategy to improve the AMF colonization ratio and increase soybean yield.

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* In Japanese with English abstract.
** In Japanese with English summary.
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