Nitrogen-Fixing Activity of Root Nodules in Relation to Their Size in Peanut (*Arachis hypogaea* L.)

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Abstract: The nitrogen-fixing activity of root nodules was examined with reference to the nodule size in peanut. Root nodules of field-grown peanut were collected at flowering through harvesting stages and classified into five groups of size using a circle template. Then acetylene reduction activity was measured to evaluate nitrogen-fixing activity for respective size groups. In addition, the diameter of the cross-section of each root nodule and rhizobium-infected areas on the cross-section were measured. The results showed that the nitrogen-fixing activity of root nodules is closely related with their size. In the root nodules in the medium size group (1.5–2.0 mm in diameter), nitrogen-fixing activity per unit fresh weight of nodule was highest at the flowering stage and rapidly decreased thereafter. The nitrogen-fixing activity of root nodules larger than 2.0 mm in diameter did not vary significantly with their size. Colors of rhizobium-infected zones varied with their size: white in small nodules; red in medium-sized nodules; and greenish in larger nodules, which suggests that the concentration of leghemoglobin is highest in the medium-sized nodules. Nitrogen-fixing activities of the medium-sized nodules might determine the amount of nitrogen fixation in the whole root system during leg-ofilling because medium-sized nodules had high activity and were large in number. Classification of root-nodule size based on the circle template is a simple, rapid, and useful method to evaluate nitrogen-fixing activity of root nodules.

Key words: Acetylene reduction activity, Nitrogen-fixing activity, Nodule diameter, Peanut (*Arachis hypogaea* L.), Rhizobia.

Effective utilization of the symbiotic nitrogen fixation in legume crops is expected to contribute to sustainable agriculture (Serraj et al., 2004). Although peanut (*Arachis hypogaea* L.) is an important legume crop in rotation, information on nitrogen fixation in peanut is fewer compared with that in soybean and common bean (Bell et al., 1994; Khan and Yoshida, 1994; Daimon et al., 1999; Daimon and Yoshikawa, 2001). Nodule formation in peanut is known to differ from that in many other legumes: root nodules develop only at the sites of lateral root emergence in peanut, whereas nodulation often occurs at the sites of root-hair formation in other legumes (Uheda et al., 2001). Therefore, more information on the distribution pattern and nitrogen-fixing activity of individual root nodules in peanut is necessary for better understanding and more effective utilization of their nitrogen fixation. The limited number of studies, however, revealed that many root nodules are formed on the first-order lateral roots at the basal part of the taproot (Tajima et al., 2006).

It is almost impossible to measure the nitrogen-fixing activity of all nodules in an entire root system, because the root nodules are numerous and their nitrogen-fixing activity may change with their developmental stages. Therefore, a simple and rapid method to evaluate nitrogen-fixing activity of each root nodule is required. King and Purcell (2001) reported that the nitrogen-fixing activity of a nodule evaluated by acetylene-reduction activity varied with the root nodule diameter, although the volume of nodules infected with rhizobia drastically change with the nodule diameter in soybean. In addition, soybean root systems with many large nodules often exhibit high nitrogen-fixing activity as a whole root system (Sato et al., 2003; Yashima et al., 2003). In light of those results, nodule diameter might be an effective index for nitrogen-fixing activity. Peanut plants have smaller nodules with higher nitrogen-fixing activity than soybean, probably because the anatomy of the peanut nodule differs from that of soybean (Tajima et al., 2004). Peanut nodules are densely filled with rhizobium-infected cells, which suggests a unique relationship between the nodule size and nitrogen-fixing activity in peanut.
We examined the nitrogen-fixing activity of root nodules in relation to the nodule diameter in field-grown peanut to discuss with reference to the rhizobium-infected area.

**Materials and Methods**

1. **Plant materials**

   A leading cultivar of peanut in Japan, Chibahandachi, which is a late-maturing Virginia type of bush plant form, was grown in 2005 in the field at the Field Production Science Center of the University of Tokyo, Tokyo, Japan (35°43′N, 139°32′E). The soil was a Kanto loam (humic Andosol) with a topsoil layer (0−35 cm) of dark humic silty loam (pH 6.2) and a subsoil layer (below 35 cm) of red-brown silty clay loam (Yamagishi et al., 2003). Seeds inoculated with *Bradyrhizobium* ssp. (J2P21; Tokachi Nokyoren, Hokkaido, Japan) were planted in the field by hand on 26 May at 5 cm depth. The row direction was east to west with row spacing of 0.7 m and plant spacing of 0.3 m. Chemical fertilizer including N, P and K, at a rate of 15, 50, and 50 kg ha⁻¹, respectively, was applied as basal dressing. The field was rainfed and manually weeded throughout the growing period. Three plants were collected at three growth stages: 70 (flowering stage), 113 (early maturing stage), and 133 (harvest) days after seeding (DAS). After removing the above-ground parts, root systems were dug out with shovel and the roots in the topsoil layer were carefully washed out. Root nodules were detached from the roots and classified into five size groups, using a circle template, according to their diameter: <1.0, 1.0−1.5, 1.5−2.0, 2.0−2.5 and 2.5−3.0 mm.

2. **Sample analysis**

   Acetylene-reduction activity was measured as an indicator of nitrogen-fixing activity, according to Hardy et al. (1968). Although this method may not be accurate...
for absolute determination of nitrogen-fixing activity (Minchin et al., 1983), this is the only method for absolute determination of nitrogen-fixing activity in field-grown samples and is acceptably reliable (Vessey, 1994). Three nodules from each size group were put into 1.5 ml glass bottles. Five or more replications were measured for each size group. Ten percent of the air in the bottle was replaced with acetylene. After incubation at 28°C for 1 h, the amount of ethylene produced by nitrogenase in 1 mL gas from each bottle was determined using an FID gas chromatograph (GC-8A; Shimadzu Corp., Kyoto, Japan) with a 100 cm stainless steel column packed with Porapak N (Waters Associates Inc. Mass., USA). Immediately after acetylene-reduction activity was measured, the nodules were weighed. Each nodule was cut into two halves with a razor blade and the cross-sections were examined with a digital microscope (VH8000; Keyence Co., Osaka, Japan). The diameter, the whole area of the cross-section, and the rhizobium-infected area identified by its color (Fig. 1) were measured for each nodule using image analysis software (ImageJ, NIH).

Table 1. Diameters and fresh weights of nodules of different size groups, classified using the circle template.

| Class (mm) | 70 DAS | 113 DAS | 133 DAS |
|-----------|--------|---------|---------|
|           | Diameter (mm) | Fresh weight (mg) | Diameter (mm) | Fresh weight (mg) | Diameter (mm) | Fresh weight (mg) |
| <1.0      | 1.5 ± 0.1 a”††† | 5.4 ± 0.3 a | 1.6 ± 0.0 a | 2.9 ± 0.2 a | 1.5 ± 0.1 a | 3.0 ± 0.4 a |
| 1.0−1.5   | 2.1 ± 0.1 b | 6.5 ± 0.3 b | 1.9 ± 0.1 b | 4.8 ± 0.6 a | 1.9 ± 0.1 b | 5.3 ± 0.7 a |
| 1.5−2.0   | 2.4 ± 0.1 c | 10.1 ± 0.6 c | 2.4 ± 0.0 c | 9.1 ± 0.4 b | 2.3 ± 0.1 c | 8.7 ± 0.8 b |
| 2.0−2.5   | 2.7 ± 0.1 d | 15.6 ± 0.7 d | 2.8 ± 0.1 d | 14.3 ± 1.0 c | 2.7 ± 0.0 d | 12.7 ± 0.6 c |
| 2.5−3.0   | 3.2 ± 0.0 e | 24.1 ± 1.2 e | 3.1 ± 0.1 e | 19.1 ± 1.2 d | 3.2 ± 0.1 e | 20.6 ± 1.1 d |

”† Classification using the circle template.
”‖ Diameter measured using microscopy.
”††† Means ±SE. Mean followed by the same letters are not significantly different at the 0.05 level according to Tukey-Kramer’s multiple range test. n ≥ 5.

Results
Classification of root-nodule size using the circle template was a rapid and easy method to rank the root nodule size, compared with time-consuming measurement of their diameter under microscopy (Table 1). As the measured size well corresponded with the fresh weights of root nodules.

Acetylene-reduction activity of root nodules varied with their diameter throughout the growing period. At 70 DAS, acetylene-reduction activity of root nodules was proportional to the nodule size up to 2.0−2.5 mm in diameter and was almost constant in nodules larger than 2.0 mm (Fig. 2A). The acetylene reduction activity per unit fresh weight of root nodule was highest in the medium-size group (1.5−2.0 mm size group); the nodules in 2.5−3.0 mm size group exhibited lower activities. At 113 DAS and 133 DAS, the acetylene-reduction activity of individual nodules were lower than those at 70 DAS (Fig. 2B and C).

The cross-section area of the nodules was closely related to the square of their diameter (R² = 0.975, Fig. 3A). The ratio of the rhizobium-infected area to the entire cross-section area of nodule was rather stable (about 0.65) irrespective of nodule size and plant growth stage (Fig. 3B). The acetylene-reduction activity of nodules in the <1.0, 1.0−1.5 and 1.5−2.0 mm size-groups were positively correlated with ca. 1.8 power of the rhizobium-infected area at 70 DAS (R² = 0.614, Fig. 4A, triangle plots). In contrast, nodules in the 2.0−2.5 and 2.5−3.0 mm size groups showed no such correlations (Fig. 4A, circle plots). At 113 and 133 DAS, the acetylene reduction activity of nodules in the <2.0 mm size groups were correlated with squared (R² = 0.342, Fig. 4B, square plots) and cubed (R² = 0.663, Fig. 4C, square plots) values of the rhizobium-infected area, respectively, though the relationships were not so clear at 70 DAS. The nodules of the <2.0 mm size groups at 113 and 133 DAS showed no such correlation at 70 DAS (Fig. 4B and C, circle plots).

The color of the rhizobium-infected areas in the cross-section of the root nodule varied among the size groups at 70 DAS (Fig. 1). Rhizobium-infected areas of nodules less than 1.0 mm in diameter were white, whereas those in larger size groups (1.0−1.5, 1.5−2.0 and 2.0−2.5 mm) were red, and those in the largest size group (2.5−3.0 mm) were greenish. The variation of the colors in the rhizobium-infected areas among the nodule sizes at 70 DAS was similar to them at 113 DAS and 133 DAS (data not shown).

Discussion
1. Nodule size as a possible index of nitrogen-fixing activity
So far the number of reports on the relationship between nodule diameter and nitrogen-fixing activity in legume crop is limited (King and Purcell, 2001;
Sato et al., 1999). Weisz and Sinclair (1988) reported that larger nodules (ca. 4 mm in diameter) had higher nitrogen-fixing activity than smaller ones (ca. 2 mm) in soybean. Peanut plants have smaller nodules than soybean and the anatomy of the peanut nodule differs from that of soybean. The non-infected peripheral area was much thinner in peanut than in soybean. Additionally, in central rhizobium-infected area, all the cells were infected in peanut nodule, whereas infected area was composed of infected and non-infected cells in soybean nodule (Tajima et al., 2004). These anatomical structures suggest that individual peanut nodules have high nitrogen-fixing activity in spite of their smaller size than soybean nodules.

In this study, reduction activity of root nodules is significantly varied with the nodule size and the plant growth stage. Such relationships suggest that the nitrogen-fixing activity in peanut increases with growth and development of nodules up to 2.0 mm in diameter and decreases rapidly thereafter.

Additionally, acetylene reduction activity is correlated with the rhizobium-infected area in the <2.0 mm size group, in particular at flowering stage (70 DAS, Fig. 4). The ratio of the infected area to the whole cross-section of the nodule is almost constant (Fig. 3B), and the cross-section area of the nodules is proportional to the square of their diameter (Fig. 3). Nitrogen accumulation during early reproductive stage is considered important for the yield of legume crops (George and Singleton, 1992). The present study indicates that the nitrogen-fixing activity of root nodules at flowering stage can be estimated from their diameter.

In this study, we classified root nodules by their size using a circle template. This procedure was quite easy and reliable for evaluating the size of numerous nodules in field-grown peanut (Table 1). This nodule size also reflects their nitrogen-fixing activities evaluated from acetylene reduction activity. The acetylene reduction activities varied among the size groups, independent of the plant growth stage, especially at 70 DAS. These results show that classification of nodules

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**Classification (mm in diameter)**

| Size Group (mm in diameter) | Acetylene Reduction activity per nodule (nmol h\(^{-1}\) nodule\(^{-1}\)) | Acetylene Reduction activity per fresh weight (nmol h\(^{-1}\) mg\(^{-1}\)) |
|----------------------------|-----------------------------------------------|--------------------------------------------------|
| <1.0                       | ![Graph A](image_url)                         | ![Graph B](image_url)                            |
| 1.0-1.5                    | ![Graph C](image_url)                         | ![Graph D](image_url)                            |
| 1.5-2.0                    | ![Graph E](image_url)                         | ![Graph F](image_url)                            |
| 2.0-2.5                    | ![Graph G](image_url)                         | ![Graph H](image_url)                            |
| 2.5-3.0                    | ![Graph I](image_url)                         | ![Graph J](image_url)                            |

Fig. 2. Acetylene reduction activity of the nodules classified into different size groups using the circle template. Columns indicate the activity for each nodule. Lines indicate the activity per unit of nodule fresh weight. Bars indicate the standard errors. A, 70 DAS; B, 113 DAS; C, 133 DAS. n \(\geq\) 5.
by their diameter is a rapid and effective way for estimating the amount of nitrogen fixed in the whole root system.

The acetylene-reduction activity of root nodule, however, was low at later growth stages. The nitrogen-fixing activity of the whole root system declines during the pod-filling stage in legume crops (McDermott and Graham, 1989; Vikman and Vessey, 1992) because of the competition for photosynthate between pods and root nodules (Sinclair, 2004). Such competition should occur at a later growth stage in this study.

Nodules of the 1.5−2.0 mm size group had the highest acetylene reduction activity at the flowering stage (70 DAS) and the activity declined rapidly at later growth stages (Fig. 2). Nodules belonging to this group occupied the major part of the whole root system (65−70% of nodules at flowering stage) in this cultivar (Tajima et al., 2006). It is estimated by the combination of the data about nodule number (Tajima et al., 2006) and acetylene-reduction activity per nodule (Fig. 2) that 70–80 % of nitrogen fixation in the whole root system of this cultivar at this stage (60–70 DAS) is attributed to the activity of the medium-sized nodules (1.0−2.0 mm). Therefore, nitrogen-fixing activity of this size group might determine nitrogen fixation of the whole plant in this cultivar during the pod-filling.

In a field experiment using 12 peanut cultivars, the number of nodules of this size group (1.5−2.0 mm) showed a higher correlation with leaf biomass than the other size groups (unpublished data), suggesting the functional importance of such medium-sized root nodules in peanut.

2. **Color of rhizobium-infected area as a possible index of nitrogen-fixing activity**

In soybean, larger nodules might have a larger ratio of rhizobium-infected area and this was suggested to be a reason why larger nodules exhibit markedly higher nitrogen-fixing activity (King and Purcell, 2001). The ratio of the rhizobium-infected area to the whole cross-section area of peanut nodule, however, was stable in this study, irrespective of nodule size and plant growth stage.

Moreover, the activity of rhizobium-infected area
seems to vary depending on nodule size, despite the stable ratio of the rhizobium-infected area to the cross-section of the root nodule. During early growth stages of nodules, the acetylene reduction activity per unit nodule fresh weight is proportional to nodule size, and the color of the rhizobium-infected area changed from white to dark red with the increase of the nodule diameter. Red color of nodule may be responsible for the high nitrogen-fixing activity because the color reflects the condition of leghemoglobin, an important enzyme for nitrogen fixation in rhizobia (Vikman and Vessey, 1993). In a later growth stages of nodules, the acetylene reduction activity per unit fresh weight of nodule decreased remarkably, and the rhizobium-infected area became greenish, which suggests senescence of the enzyme in the infected area. Root nodules in peanut are thereby classified into three growth phases based on nodule size and interior color: small nodules with white infected areas; medium-size nodules with red infected areas; and larger, older nodules with greenish infected areas. Among these, the medium-size nodules may have the highest nitrogen-fixing activity.

In summary, the nodule size might be a rapidly measurable effective index of the nitrogen-fixing activity of nodules, which can be monitored by the color of the rhizobium-infected area.

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