Note

Backhoe-assisted monolith method for plant root phenotyping under upland conditions

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Root system architecture (RSA) is one of the most important traits determining water and nutrient availability for plants. Modification of RSA is known to be a useful approach for improving root performance of crops. However, for conducting root phenotyping, there are few alternatives for the rapid collection of root samples from a constant soil volume. In this report, we propose a rapid root-sampling method, which uses a steel cylinder known as round monolith and backhoes to reduce the physical effort. The monolith was set on the ground surrounding individual rice plants and vertically driven back by a backhoe. Soil samples with 20 cm width and 25 cm depth were excavated by the monolith, from which root samples were then isolated. This backhoe-assisted monolith method requires at most five minutes to collect root samples from one plant. Using this method, we quantified the root traits of three rice lines, reported to form different types of root system such as shallow-, intermediate-, and deep-roots, using a root image analysis software. The data obtained through this method, which showed the same trend as previously reported, clearly demonstrated that this method is useful for quantitative evaluation of roots in the soil.

Key Words: monolith, rice, root morphology, root sampling, image analysis.

Introduction

Root system architecture (RSA) is one of the most important plant traits, as roots not only anchor plant body to soil but also determine its nutrient availability (Smith and De Smet 2012). Modifications of RSA would minimize the negative impact of abiotic stress on crop yield; for that reason, many genes and quantitative trait loci (QTLs) affecting RSA have been identified for crop improvement (de Dorlodot *et al.* 2007).

RSA is defined as the morphological and functional differences in root deployment in the soil (Lynch 1995). In cereals including rice (*Oryza sativa* L.), one radicle (primary root) firstly emerges after germination, followed by many crown roots (adventitious roots) that emerge from basal stem nodes (Hochholdinger *et al.* 2004, Itoh *et al.* 2005, Yoshida and Hasegawa 1982). Radicle and crown roots develop lateral roots, which play a key role in water and nutrient uptake (Atkinson *et al.* 2014). In other words, crown and lateral roots contribute to the rhizosphere area and the roots density in the soil, respectively. For example, the modifica-

tion of the RSA with the *DEEPER ROOTING 1 (DRO1)* gene increased rice yield under drought conditions by expanding the rhizosphere deeper (Uga *et al.* 2013). Another example of RSA relevance arises from simulation analyses carried out in maize, which revealed that phosphorous uptake is optimized by dense and short lateral roots, whereas nitrate uptake is optimized by sparse and long lateral roots (Postma *et al.* 2014).

Roots being underground organs, root phenotyping under field conditions is very laborious and time-consuming (Böhm 2012). Thus, root phenotyping systems in laboratories or artificially-controlled conditions have been widely used for the high-throughput of root characterization. For example, hydroponic culture is one of the cultivation systems that allow easy root sampling and phenotyping. Rhizobox, a kind of transparent culture box for root phenotyping, is also popular because root traits are easy to observe and measure. Both systems could deal with many samples at the same time, which is necessary for QTL mapping and genome-wide association studies (*e.g.* Courtois *et al.* 2013, Kitomi *et al.* 2018, Slovak *et al.* 2014, Song *et al.* 2016, Uga *et al.* 2018, Zhao *et al.* 2018). Since these methods involve artificial growth conditions, procedures allowing root phenotyping under real field conditions remain as a research need.

‘Shovelomics’ is a field root phenotyping approach based

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on root sampling by digging up the roots with shovels, followed by a visual scoring that allows root phenotyping (Trachsel *et al.* 2011). Due to its simplicity and high-throughput, shovelomics is one of the most popular methods for root phenotyping of field-grown plants. However, shovelomics is not suitable to the quantification of root traits because it uses shovels for digging up the roots, which may involve root losses or hurting, and employs a visual scoring method, which is semi-quantitative.

For collecting and measuring root samples, soil-core sampling and monolith methods are mainly used (Böhm 2012). Soil coring drives small-diameter cylinders into the ground vertically to collect soil samples, from which root samples are isolated and quantified (Wasson *et al.* 2014). The procedure is simple and easy, but the sampling area is limited. The monolith method drives relatively large boxes or cylinders with bottom side open into the ground vertically to collect soil block containing root samples (Wu and Guo 2014). The box and cylinder are conventionally known as ‘monolith’ and ‘round monolith’, respectively. Both soil coring and monolith methods require hammers or custom-built machines to drive the core samplers or monoliths into the ground, indicating that soil coring and monolith methods are more complicated and laborious more than shovelomics may.

Modern cultivation uses a lot of fertilizers to increase crop yields (Food and Agriculture Organization of the United Nations 2015), and the fertilizers are mainly distributed in the plow layer. Most of roots are also concentrated in the plow layer although the distribution of roots depends on subsoil tillage (Cai *et al.* 2014). Therefore, we considered that a rapid sampling method for roots growing in the plow layer, suitable for quantitative root phenotyping, would be helpful not only for the characterization of RSA of crops but also the breeding of new cultivars. In this regard, the monolith method is one of the most suitable ones for quantifying root traits in the plow layers. However, the monolith method is laborious and time-consuming compared with shovelomics.

In this study, we present a rapid sampling method for plant root phenotyping, based on the use of a round monolith and backhoes, to reduce physical effort. Using this method, we collected rice roots from a constant volume of the plow layer, taking the operation five minutes per sample. Furthermore, we quantified root traits of three cultivars or lines using a root image-analysis software and corroborated the difference in root characters related to RSA between *indica* and tropical *japonica* cultivars.

Materials and Methods

Plant materials

Three rice (*Oryza sativa*) cultivars or lines, ‘IR64’ (IRGC #66970), ‘Kinandang Patong’ (KP, IRGC #23364), and ‘Dro1-NIL’ (Uga *et al.* 2013), were used in this study. IR64 is a lowland *indica* cultivar and KP is an upland tropical *japonica* cultivar. It was previously reported that KP has

thicker, longer, and steeper roots than IR64 (Kondo *et al.* 2000, Uga *et al.* 2018), which is a typical character of upland rice. Dro1-NIL is a near-isogenic line of IR64 genetic background with the KP allele of *DRO1* gene, which has been identified as a gene controlling root growth angle by QTL cloning in a mapping population derived from a cross between IR64 and KP (Uga *et al.* 2013). IR64, KP, and Dro1-NIL show monomorphic shallow-, monomorphic deep-, and dimorphic-rooting patterns, respectively.

Field and experimental design

We cultivated rice plants during the summer season of 2018 at an upland field of the Institute of Crop Science (National Agriculture and Food Research Organization, Ibaraki, Japan; 36°02’89” N and 140°09’97” E). The soil at the experimental site is a volcanic ash soil of the Kanto loam type (Humic Andosol). The topsoil (0–30 cm) is a dark humic silty loam. The subsoil (below 30 cm) is a red-brown silty clay loam. There was a hardpan at a depth of about 20 to 25 cm. The field was fertilized with 5.2 g N m⁻², 15.4 g P₂O₅ m⁻², and 5.6 g K₂O m⁻² before rice planting. We employed a block design for evaluating rice traits. Three blocks were arranged as three replications. Each block included three plots for IR64, KP, and Dro1-NIL, and contained 15 hills spaced by 0.4 m × 0.4 m. Three hills were used for monolith sampling, while three other hills were used for the basket assay. Three seeds were sown in each hill on June 5. On July 3 and July 4, we adjusted seedling density to one seedling per hill by removing seedlings with growth defects and transplanting those with intermediate growth. Simultaneously, three plastic mesh baskets (15 cm diameter and 6 cm height) were filled with soil and buried at certain hills in each plot for basket assay. Water was supplied with a sprinkler before starting leaf rolling of rice seedlings.

Monolith design

The monolith was designed as a steel cylinder (20 cm diameter and 30 cm depth), tough enough to be driven from the ground by a backhoe. Although the plow layer thickness depends on several factors including crop rotations, climate, and schedule of planting, the depth of an upland rice plow layer is 25 cm at most (Gupta and O’Toole 1986). Thus, the monolith we developed is suitable for the evaluation of rice root properties in the plow layers. An external view of the round monolith and an unfolded scheme are shown in Fig. 1, respectively. The thickness of the monolith wall was 8.2 mm, and a steel disk was welded to the top side to improve its mechanical properties. The bottom side was sharpened to sever roots when monolith was driven into the ground. Four equidistant holes were drilled at 3 cm from the top side for craning the monolith from the soil.

Backhoe-assisted monolith sampling

The tiller number and height of the plants selected for monolith sampling were measured on July 25. Above-

Fig. 1. Round monolith used in this study. (A) Image of the monolith. (B) Unfolded scheme of the monolith. All diameters are shown as inside diameters. The thickness of the wall was 8.2 mm. A steel disk was welded to the top side of the monolith to improve its mechanical properties.

ground tissues from the three blocks were sampled on July 26, July 30, and August 1, in coincidence with the days of monolith sampling. The above-ground tissues were dried at 80°C for one week to measure their dry weight. Around late July and early August are the tillering stage for IR64, KP, and Dro1-NIL, the period during which rice plants increasing their tiller number and biomass. Since the tillering stage is one of the most important periods affecting rice yield (Fageria 2007), we collected shoot and root samples during these periods.

Rice roots were sampled with the monolith and backhoes. The monolith was set up on the ground with the plant base as the horizontal center and driven into the soil vertically by pushing the upper side of the monolith with the blade of the B27 backhoe (YANMAR, Japan) to a depth of 25 cm (Fig. 2A, 2B). The monolith containing soil and root samples was excavated by craning from the ground by the backhoe (Fig. 2C, 2D). Soil and root samples were detached from the round monolith with a RA400 loader (KUBOTA, Japan) by pushing the upper side of the sample (Fig. 2E, 2F). A more detailed procedure is shown in Supplemental Fig. 1.

Root samples were washed with water to remove soil and dust and stored in 70% ethanol at 10°C until use. The volume of each hole after excavating was 7,850 cm³ (20 cm diameter and 25 cm depth).

Basket assay

The ratio of deep rooting (RDR) was measured on July 24 with the basket assay, according to a previous study (Uga *et al.* 2009). All baskets were dug up and subjected to manual counting of the roots that penetrated the holes. The RDR was calculated by dividing the total number of roots penetrating the lower part of the mesh (53 to 90° to a horizontal axis) by the total number of roots penetrating the whole mesh.

Root image analysis

Total root length, total root surface area, total root vol-

Fig. 2. Rice root sampling with backhoes. (A) The monolith was set up on the stump. Large tree blocks were placed between the monolith and the backhoe as cushion. A small steel disk was set on the soil in the monolith, which was used for effective isolation of the soils. The disk can be confirmed in panel C. (B) The monolith was driven into the soil vertically with the blade of the backhoe. (C) Hook wires were connected with the monolith and backhoe. Four hooks were inserted into the holes of the monolith. (D) The monolith containing soil and root samples was excavated by craning. (E) The sample was detached from the round monolith by pushing the upper side of the sample with the loader and a large tree block. (F) Soils with root samples were collected in the box. Arrowheads indicate the monolith.

ume, and root diameter were measured with WinRHIZO™ Pro 2017a software (Regent Instruments, Canada). Crown roots were separated by cutting and rinsed with reverse-osmosis water, and all roots were spread out on the tray for scanning. The scanning resolution was 400 dpi. According to their diameters, roots were divided into two classes: less than 0.2 mm and over 0.2 mm. Since it is known that the diameter of lateral roots in rice is less than 0.2 mm (Henry *et al.* 2012), the former class includes lateral roots and the latter class includes radicle and crown roots. The average root diameter of each class was calculated as:

$$D = 2\sqrt{\frac{V}{\pi L}}$$

where D is root diameter (cm), V is total root volume (cm³), and L is total root length (cm). All data were processed with the R software (<https://www.r-project.org/>). After scanning, roots were dried at 80°C for three days to measure their dry weight.

Statistical analysis

Average within each block was calculated, these data were used for statistical analysis. Dunnett's test against IR64 was performed with 'multcomp' package (version 1.4.8) in R software. This study defined that comparisons with P values under 0.05 entail significant difference.

Results

Root phenotyping using the backhoe-assisted monolith method

Roots of IR64, Dro1-NIL, and KP were collected by the backhoe-assisted monolith method. The shapes of the whole roots including shoot bases are shown in **Fig. 3A–3C**. Whole roots in the plow layer were successfully collected. All crown roots were separated from each other by removing the shoot base and subjected to scanning (**Fig. 3D–3F**). Compared with IR64 and Dro1-NIL, KP had a larger diameter of crown roots and a lower number of crown and lateral roots, *i.e.*, shorter total root length. On the other hand, there were no apparent differences between IR64 and Dro1-NIL.

To compare root traits quantitatively, scan images were analyzed with the WinRHIZO™ software. Total lengths of both crown and lateral roots in KP were significantly lower than those in IR64 (**Fig. 4A, 4B**), indicating that root density of KP in the plow layer was sparse. Because crown roots in rice sprang out from nodes (Atkinson *et al.* 2014, Yoshida and Hasegawa 1982), one of the reasons for lower root density of KP was lower tiller number (**Supplemental Fig. 2A**). Total lengths of both crown and lateral roots in Dro1-NIL were also lower than those of IR64, though not significantly

Fig. 3. Representative scan images of whole root samples; (A) IR64, (B) Dro1-NIL, and (C) KP. Images of separated roots; (D) IR64, (E) Dro1-NIL, and (F) KP. Bars indicate 5 cm.

Fig. 4. Root phenotype measured with WinRHIZO™ software. Total length (A, B), total surface area (C, D), total volume (E, F), and average diameter (G, H) of crown and lateral roots were calculated from scan images of IR64, Dro1-NIL, and KP. P values calculated by Dunnett test against IR64 are shown on the figures. The number of samples of each rice line was three. Bars and error bars indicate average and standard deviation, respectively.

(Fig. 4A, 4B). A similar pattern was observed in the total surface area and the total volume of both crown and lateral roots (Fig. 4C–4F). Compared with those of IR64 and Dro1-NIL, crown and lateral roots diameters in KP cultivar were significantly higher (Fig. 4G, 4H). We observed no significant differences of root dry weight between IR64, Dro1-NIL, and KP (Supplemental Fig. 3A), which is explained by shorter but thicker roots of KP (Fig. 4A, 4B, 4G, 4H).

Phenotype of above-ground tissues and ratio of deep rooting

The basket assay revealed that the RDR of Dro1-NIL was significantly higher than that of IR64 (Supplemental Fig. 3B), confirming the effect of *DRO1* gene on deep rooting. Regarding phenotype of above-ground tissues, there were no significant differences of tiller number, plant height, and shoot dry weight between IR64 and Dro1-NIL (Supplemental Fig. 2A–2C). This result is consistent with that of a previous report demonstrating that *DRO1* gene in KP affects only root distribution in the soil (Uga *et al.* 2013). On the other hand, KP showed lower tiller number, higher plant height, and higher shoot dry weight. These findings corroborate the difference between *indica* and tropical *japonica* (Gupta and O'Toole 1986).

Discussion

A rapid root sampling method

We successfully demonstrated that the backhoe-assisted monolith method is a powerful technique for collecting root samples from the plow layer in the fields. Using this method, we collected root samples of three rice lines (IR64, Dro1-NIL, and KP) and found some quantitative differences on root traits. Notably, we estimated that the backhoe-assisted monolith method could collect root samples from individual plants within five minutes, which is equivalent or even quicker than the shovelomics process, which has been reported to consume about three to eight minutes per sample (Trachsel *et al.* 2011).

The number of persons required for the operation of this root sampling method is six: one who manipulates the backhoe, one who manipulates the loader, two who assist for handling the monolith near the backhoe and loader, and two who isolate root samples from the soil. Although at least six persons are required for efficient sampling, we conclude that the backhoe-assisted monolith method is a rapid root sampling procedure, suitable for rice root quantitative phenotyping in the fields. Furthermore, we consider that this method is also suitable for the root sampling of other crops whose cultivation involve a similar depth of the plow layer.

Root sampling from the plow layer

The depth of an upland rice plow layer is 25 cm at most (Gupta and O'Toole 1986) and the depth of the monolith presented in this study was 25 cm, meaning that the backhoe-

assisted monolith method is suitable for the evaluation of root properties in the plow layers. On the other hand, it is known that deep-rooting rice varieties have a high root density in soil layers deeper than 30 cm below the ground (Yoshida and Hasegawa 1982). Indeed, it was reported that KP elongated their roots to over 60 cm depth (Uga *et al.* 2013). Thus, the backhoe-assisted monolith method is not adequate to evaluate deep-rooting properties of rice varieties, for which other procedures such as the basket method (Oyanagi *et al.* 1993) or the trench method (Nemoto *et al.* 1998) would be suitable.

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