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# Colonization of Roots in Sago Palm Seedlings Associated with Commercial Mycorrhizal Inocula

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## Introduction

Sago palm (*Metroxylon sagu* Rottb.) extends across Southeast Asia and northwestern Melanesia (Papua New Guinea and the Solomon Islands). Among the Arecaceae genera, the trunk of the sago palm produces the largest amount of starch (over 200 kg dry starch per plant), and it is used variously for fresh food and processed food or floured for noodle making (Ehara et al., 2018). One of the oldest crops used by human beings, this species has been used as a food similar to bananas and taro (Barrau, 1959) since ancient times (Takamura, 1990).

Peat is an accumulation of partially decayed vegetation or organic matter. Peatland has low pH, and its parameters indicating natural soil fertilities are low per unit of soil volume. Approximately 30 million ha in Southeast Asia are estimated to be peatland (Chan, 2016). Sago palm is a useful plant resource that can adapt to tropical peat soil (Ehara et al., 2018). Even in peatland, this species will produce a comparatively large quantity of starch, an estimated 164 –180 kg per plant on a dry weight basis, according to Yamamoto et al. (2003). Although the yield of sago palm in peatland is lower than that in mineral soil (200 to 300 kg per plant [Ehara et al., 2018]), sago palm can be considered one of the most valuable peatland crops. Anugoolprasert et al. (2012a, b) reported from their laboratory level experiment and field survey in southern Thailand that sago palm can maintain the uptake of macronutrients, which may be a major reason why sago palm can adapt to growing in strongly acidic soil in a natural habitat.

Arbuscular mycorrhizas are mycorrhizas whose hyphae penetrate plant cells, producing a structure of arbuscules as a means of nutrient exchange. Mycorrhizal associations exert great influence on diverse agroecological and ecosystemic processes (Silvana et al. 2018). Inoculation with arbuscular mycorrhizal fungi (AMF) is an interesting strategy for improving crop yields because arbuscular mycorrhizal symbiosis benefits crops in many ways (Gianinazzi et al., 2010), including enabling the efficient use of fertilizers and soil nutrients (Javaid, 2009), protection against drought stress (Porcel et al., 2007, Porcel and Ruiz-Lozano, 2004) and diseases (Liu et al., 2007), increased N-fixation in legumes (Barea and Azcon-

Aguilar, 1983, Haselwandter and Bowen, 1996), and improved soil physical properties (Hallett et al., 2009). Most plant species form mycorrhizal symbioses, and, therefore, many crops could potentially benefit from inoculation with the correct AMF inoculum; however, the degree to which plants benefit varies greatly (Phosri et al., 2010), ranging from very little benefit to strong mycorrhizal dependence (Janos, 2007). Arbuscular mycorrhizas are key components of sustainable plant–soil ecosystems because they play an essential role in the nutrient acquisition, plant diversity, and nutrient cycling of plants (Jeffries and Barea, 2000).

Phosri et al. (2010) investigated the application of AMF technology for oil palm cultivation and reported that AMF has the potential to increase conventional agricultural productivity and is crucial for the functioning of agricultural ecosystems through more sustainable management and the practical use of AMF. Chan et al. (2002) detected that an endotrophic mycorrhiza or AMF association existed in sago roots with mycorrhiza identified as belonging to Glomales of the Zygomycetes. According to Chan et al. (2010), AMF inoculation should form an integral component of the micropropagation process of sago palm plantlets. Keys to the practical use of AMF are culture technology and diagnosis technology for effects; in order to offer it at low cost, a single culture technique should be prepared. It is also important to clarify the environmental conditions in which AMF works effectively. Further integrated studies are needed to utilize specific AMF for promoting sago palm growth. In this study, we tried to use commercial mycorrhizal inoculums, including *Rhizophagus irregularis*, which is a well-known AMF, and their associated colonization in the roots of sago palm seedlings was examined. Here, as a part of improving the cultivation method and commercialization of sago palm with interdisciplinary approaches, we investigated the possibility of utilizing the existing AMF technology for the cultivation of sago palm seedlings.

## Materials and Methods

### Plant materials

The experiment was conducted in an air-conditioned green house (phytotron) under natural sunlight at Nagoya University in Japan. In the phytotron, the air temperature and relative humidity were set at 28 °C and 60 %, respectively.

Sago palm seedlings germinated from seeds produced by a spiny mother palm in West Papua, Indonesia, were grown to the 8th leaf stage and used for the experiment. During the nursery period, seedlings were cultivated individually in 1/10000a Wagner pots filled with vermiculite and Kimura B culture solution containing (μM) 36.5 $(NH_4)_2SO_4$, 9.1 $K_2SO_4$, 54.7 $MgSO_4$, 18.3 $KNO_3$, 36.5 $Ca(NO_3)_2$, 18.2 $KH_2PO_4$, and 3.9 $FeO_3$ (Baba and Takahashi 1958). At the 8th leaf stage, seedlings were transplanted individually to 7 L plastic pots (20 cm depth, 12 cm diameter) filled with commercial black soil (Protoleaf, Japan) on August 27, 2018. When the seedlings were transplanted, lateral roots of less than 1 mm diameter were sampled and used for microscopic observation to confirm whether mycorrhizal colonization existed. After no mycorrhizal colonization was confirmed, transplanted seedings were inoculated individually with two commercial mycorrhizal inocula, considering the application rate recommended by the providers: (1) 200 μL (per plant) Mycogel including *Rhizophagus irregularis* (HYPONex Japan, Japan) (corresponding to 10000 spores) was mixed with 1 L tap water and applied to a plant; (2) 14 g (per plant) of Yukimotogoenotakumi including unpublished mycorrhizal materials (Protoleaf, Japan) was mixed with soil in a pot when the seedling was transplanted. Besides, Yukimotogoenotakumi was a spore-form inoculum. The amount of each inoculum applied was determined in consideration of the instructions from each provider. The pots, which were placed individually onto a plastic tray filled with tap water at a depth of 3.5 cm, were placed in the phytotron. Water was supplied to the tray daily to counter evapotranspiration. Any additional nutrients were not

applied for two months following transplanting. On October 24, 2018, one seedling each inoculated with Mycogel or Yukimotogoenotakumi was sampled for collecting lateral roots less than 1 mm diameter. The laterals roots were collected from approximately 5, 10, or 15 cm deep from the soil surface and were observed microscopically. When the seedlings were harvested at the end of the experiment, 20 root samples approximately 5 cm in length were taken at three different depth layers from soil surface as follows: depth of 5 cm: root approximately 5 cm in length distributed in the soil layer 3 to 7 cm deep (5 ± 2 cm) from the soil surface; depth of 10 cm: root approximately 5 cm in length distributed in the soil layer 8 to 12 cm deep (10 ± 2 cm) from the soil surface; depth of 15 cm: root approximately 5 cm in length distributed in the soil layer 13 to 17 cm deep (15 ± 2 cm) from the soil surface.

**Analysis of soil and root samples**

The soil sample that was used for the cultivation of transplanted plants was air-dried at room temperature and prepared for analyzing the soil' s chemical properties by sieving through a 2 mm mesh. Soil pH was measured at a soil:water or 1 N KCl ratio of 1:2.5 (w $v^{-1}$) by a pH meter. The total nitrogen and carbon concentrations were measured by a C-N coder (JM1000CN, J-SCIENCE LAB, Japan). The amount of available phosphorus was determined by the Bray II method (Bray and Kurtz, 1945). The exchangeable $K^+$, $Ca^{2+}$, and $Mg^{2+}$ were measured by the ammonium acetate saturation method (1 N ammonium acetate ($NH_4OAc$) solution (pH 7.0)) (Schollenberger and Dreibelbis 1930). All exchangeable cations were determined by atomic absorption spectrophotometer (170-30 AA, Hitachi, Japan). The cation exchange capacity was calculated from the amounts of $K^+$, $Ca^{2+}$, and $Mg^{2+}$ measured in the sample.

The lateral roots were sampled twice, before the inoculation and at the end of experiment. The root samples were treated as follows: 1) cleaning with tap water, 2) soaking and incubation in 10 % KOH (w/v) at 80 °C for 10 minutes, 3) rinsing with tap water, 4) soaking in 1 M HCl for 1 minute (until the root was completely bleached), 5) staining and incubating in a solution containing acid fuchsin:glycerol:lactic acid:distilled water (1. 5 g:125 ml:1750 ml:125 ml) at 80 °C for 10 minutes, 6) destaining in a solution containing glycerol:lactic acid:distilled water (125 ml:1750 ml:125 ml) for 3 hours at room temperature. Stained roots were observed under a compound microscope to determine the presence or absence of mycorrhiza. A camera attached to the microscope recorded photomicrographs at high magnification.

**Results and Discussion**

Daytime air temperature in the phytotron ranged from 29 to 31 °C during the experiment, and the maximum irradiance flux density inside the phytotron was around 1000 μmol $m^{-2}$ $s^{-1}$. The properties of the soil used are shown in Table 1. The nutrient contents of the soil were as follows: total nitrogen (4.4 g $kg^{-1}$), phosphorus (231.7 mg $P_2O_5$ $kg^{-1}$), and exchangeable potassium (207 mg $K_2O$ $kg^{-1}$). The stained root samples were examined under a compound light microscope, and the presence of AMF was assessed for each sample. AMF were observed in the root samples of sago palm seedlings by the presence of their vesicular structures and the intracellular and intercellular hyphae in the roots (Fig. 1). The 20 sampled roots taken from each depth were cut into 1 cm lengths and stained. Fifty samples were selected as well-stained ones among one hundred samples and observed under the microscope. In the seedlings inoculated with Mycogel, hyphae and arbuscules were observed in the samples taken from 10

**Table 1.** Properties of soil used

| pH ($H_2O$) | pH (KCl) | Total C | Total N | $P_2O_5$ (Bray II) | $K_2O$ | $K^*$ | $Mg^*$ | $Ca^*$ | $CEC^{*2}$ |
|---|---|---|---|---|---|---|---|---|---|
| | | ------ g $kg^{-1}$ ------ | | ----- mg $kg^{-1}$ ----- | | ---------- $cmol_c$ $kg^{-1}$ ---------- | | | |
| 5.68 | 5.02 | 71.3 | 4.4 | 231.7 | 207.4 | 0.44 | 0.37 | 5.40 | 40.91 |

*: exchangeable cation, *2: cation exchange capacity

cm and 15 cm below the soil surface. On the other hand, in the seedling inoculated with Yukimotogoenotakumi, hyphae were observed in the samples from 5 cm, 10 cm, and 15 cm, and arbuscules and vesicles were observed in the samples from 10 cm and 15 cm below the soil surface (Table 2, Fig. 1).

Many studies have focused on the use of AMF in sustainable agriculture as a mechanism for maintaining commercial crops efficiently (Andrews et al., 2012; Pellegrino et al., 2011). There have also been various studies on the use of AMF for tropical and subtropical crops (e.g., coffee, cacao, avocado,

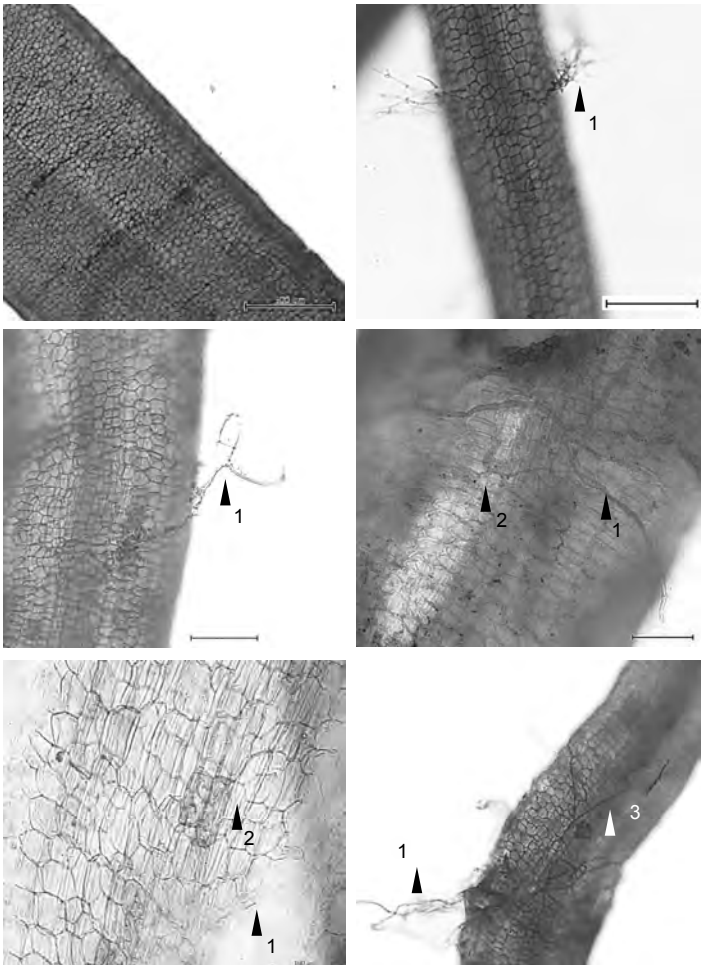


**Fig 1.** Photomicrograph of root samples of sago palm seedlings inoculated with Mycogel (left) and Yukimotogoenotakumi (right)
Left top: root from 5 cm below the soil surface, bar = 500 µm; left middle: root from 10 cm below the soil surface, bar = 200 µm; left bottom: 15 cm below the soil surface, bar = 100 µm; right top: root from 5 cm below the soil surface, bar = 200 µm; right middle: root from 10 cm below the soil surface, bar = 100 µm; right bottom: 15 cm below the soil surface, bar = 200 µm; arrow 1: hypha, arrow 2: arbuscule, arrow 3: vesicle

**Table 2.** Presence or absence of hyphae, arbuscule, and vesicle in lateral roots taken from soils of different depth from the soil surface

| Inoculum | Depth | Hyphae | Arbuscule | Vesicle |
|---|---|---|---|---|
| Mycogel | 5 cm | – | – | – |
| | 10 cm | + | + | – |
| | 15 cm | + | + | – |
| Yukimotogoenotakumi | 5 cm | + | – | – |
| | 10 cm | + | + | + |
| | 15 cm | + | + | + |

+: presence, –: absence

banana, pineapple, and citrus) and forest trees, which showed the positive effects of mycorrhization on their biometric characteristics (Adriano-Anaya et al., 2011, Aguirre-Medina et al., 2011, Colozzi-Filho and Siqueira, 1986; da Silveira et al., 2003; de Oliveira and de Olivera, 2005). Sago palm seedlings inoculated with both Mycogel and Yukimotogoenotakumi were also confirmed to be colonized by AMF as their vesicular structures and their intracellular and intercellular hyphae were observed in their roots in this experiment. As mentioned earlier, a practical application at low cost is absolutely essential for the utilization of AMF. To put useful microorganisms into practice, the isolation and screening of target(s), its single culture, and the verification of its influence are needed. When the AMF density in the soil is naturally high, the positive effect of AMF inoculation on the growth of crops will not be expected. In such a case, it is necessary to actively utilize indigenous AMF for crop production with cultivation management and/or some other practices; the application of powdered coal is considered effective in Japan (https://www.naro.affrc.go.jp/training/files/2005_14-04.pdf). The current result indicated the possibility that an AMF symbiotic association could exist in the roots of sago palm seedlings when commercial inocula were used.

Understanding the environmental conditions that will promote the efficient influence of AMF is also a subject for further study. The percentage of the mycorrhiza colonization of roots in sago palm seedlings was estimated to be a few percent in seedlings inoculated with Mycogel or Yukimotogoenotakumi in this experiment (data are not shown). Wood (1992) took a more cautious view and suggested 5 ppm Olsen P (5 mg $kg^{-1}$ available $P_2O_5$) as a general minimum for plants to benefit from mycorrhizal effects; he showed that the mycorrhizal colonization of roots decreased rapidly as P levels rose from 5 to 10 ppm (5 to 10 available $P_2O_5$ mg $kg^{-1}$). However, dose–response curves presented by Stribley et al. (1980) suggest that the mycorrhizal effect in the highly mycorrhizal-dependent leek plant was not negated until around 200 ppm Olsen P in the soil solution. The available $P_2O_5$ in the soil used was about 231.7 mg $kg^{-1}$ in this experiment, which might be comparatively high for associating with mycorrhizal inocula, considering the former publications of Wood (1992) and Stribley et al. (1980). Chan et al. (2010) reported that mycorrhizal colonization was observed two months after transplanting without phosphate application in the plantlets inoculated with mycorrhiza isolated from sago palm roots (in peat soil collected in Sarawak, Malaysia; no property data available). In the former publication of Chan (2016), there was no significant difference in spore abundance among the four different sites, where the available phosphorus content in the soil was significantly different (1033 to 1577 mg $L^{-1}$); however, the mean value of spore abundance looked to be high in soil that included lower phosphorus among the four sites. Chan (2016) reported the available phosphorus by concentration in dried soil; therefore, their data cannot be compared with that of the other publications referred to above. In any case, the mycorrhizal effect will be related to the phosphorus content in the soil. Phosri et al. (2019) stated that there are certainly doubts as to whether mycorrhizal inocula have real potential to improve oil palm productivity in established plantations, especially if growers continue high P input. A sustainable management had better be considered carefully for maintaining the undisturbed soil microbiota in the case of sago palm growth as well.

As described above, it is possible that AMF's symbiotic association existed in the roots of sago palm seedlings when commercial inocula were used. Verification of the effect of commercial inocula on the growth of sago palm seedlings and environmental conditions in which AMF works effectively associated with sago palm are future subjects that must be studied to contribute to environmentally friendly cultivation in intensive production.

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