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Histological Observation of Primary and Secondary Aerenchyma Formation in Adventitious Roots of *Syzygium kunstleri* (King) Bahadur and R.C.Gaur Grown in Hypoxic Medium

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Abstract: Trees growing in wetlands develop adventitious roots from the trunk during the rainy season and adapt to the flooded environment by forming primary (schizogenous or lysigenous) and secondary aerenchyma in the roots. Therefore, it is necessary to clarify the formation process of each type of aerenchyma in these adventitious roots. In this study, saplings of *Syzygium kunstleri* (King) Bahadur and R.C.Gaur were grown under four different treatments, and a total of 12 adventitious roots generated from trunks were used to clarify the distribution of each aerenchyma type in the roots using light or epi-florescence microscopy. Schizogenous aerenchyma was observed in the root tips where the root color was white or light brown, whereas lysigenous aerenchyma was found at some distance from the root tip where the root color gradually changed from light to dark brown. The secondary aerenchyma and periderm were observed in dark brown parts near the root base. None or only one layer of phellem cells was detected in the white roots near the root tip, but dark brown roots near the root base had at least three layers of phellem cells. Considering these results, oxygen transportation may occur between primary and secondary aerenchyma at the point where two or more layers of phellem cells are formed.

Keywords: flooding tolerance; oxygen deficiency; oxygen transport; primary aerenchyma; secondary aerenchyma; waterlogging

1. Introduction

Periodic or permanent flooding is a dominant environmental stress that critically impedes the growth, yield, and distribution of plants [1,2]. This reduction in growth and yield is caused mainly by gas diffusion being approximately 10,000-fold slower in water than in air [3,4]. Wetland plants have, therefore, adapted to hypoxic conditions through metabolic and morphological methods [5]. However, switching from aerobic respiration to the anaerobic glycolysis of ATP induces a severe reduction in energy available for maintenance, growth, and nutrient uptake [6]. Thus, in continuously hypoxic conditions, morphological and anatomical adaptation is a more efficient response for plants. Specifically, the stress caused by low O_2 concentrations results in the development of aerenchyma. Aerenchyma can be found in the shoots and roots of flood-tolerant species and has been shown to improve the internal diffusion of atmospheric O_2 from the aerial parts of plants to the flooded roots so as to prevent severe damage to the root system [7–11]. Thus, the development of aerenchyma is thought to be one of the most important anatomical adaptations to hypoxic conditions.

There are two kinds of aerenchyma, namely, primary and secondary [12]. Most aerenchyma develops in the primary tissues, especially in the root cortex, and is called the 'primary aerenchyma'. The primary aerenchyma is classified based on the different ways it is formed. The first type is schizogenous aerenchyma, which forms from cells separating during tissue formation and is developmentally controlled and separate from any external stimuli [13,14]. The second type is lysigenous aerenchyma, which is formed by the death and disintegration of cells in the cortex. Here, cell death appears in a predictable pattern and is controlled by a hormone (ethylene); this is an example of programmed cell death [15]. Previous studies have shown that many important crop species, such as maize [16–18], rice [19], barley [20], and wheat [21,22], develop lysigenous aerenchyma, whereas schizogenous aerenchyma is formed in some wetland herb species such as *Rumex* species [23–25]. In addition, only a few species, such as *Sagittaria lancifolia* L., form both lysigenous and schizogenous aerenchyma, which develop in different tissues [26].

In contrast, secondary aerenchyma is a tissue of secondary origin and differentiates from the phellogen (cork cambium), cambium, or pericycle, which can produce either a porous secondary cortex or an aerenchymatous phellem in stems, hypocotyls, and roots [12,24,27–29]. In some plants, secondary aerenchyma develops radially outward from phellogen [12,30,31]; this sort of secondary aerenchyma is homologous to phellem, and thus it is referred to as aerenchymatous phellem [10,30,31]. In *Lythrum salicaria* L., the aerenchymatous phellem consists of alternating tissues of radially elongated cells and small isodiametric cells [12]. Past studies have shown that some flood-tolerant species such as *Glycine max* (L.) Merr. [27,28], *Sesbania aculeata* (Schreb.) Poir. [32], *Se. cannabina* (Retz.) Pers. [33], *Se. rostrata* Bremek. and Oberm. [34], *Neptunia oleracea* Lour. [35], and *Viminaria juncea* (Schrad.) Hoffmanns. [36] develop aerenchyma in the phellogen region of the stems and roots under flooded conditions. Secondary aerenchyma is anatomically different from primary aerenchyma. Although secondary aerenchyma is also thought to play an important role in supplying O_2 to flooded roots [27,37], information on the anatomical process by which secondary aerenchyma develops in woody plants with secondary growth is still limited [28].

Some tree species growing in wetlands develop adventitious roots from the trunk during the rainy season as an adaptation to the flooded environment [38]. Therefore, in order to understand how trees adapt to growing in such an environment, it is necessary to clarify the formation of aerenchyma in the adventitious roots, which show growth in diameter and lignification. Although there have been some reports on the induction of primary aerenchyma in a flooded environment using current-year tree seedlings [11,28,33,39], few studies have been conducted using saplings.

Since only primary aerenchyma occurs at the root tip without secondary aerenchyma development [40], oxygen taken up by aboveground organs needs to be transported to the primary aerenchyma of the root tip through aerenchyma in the plant body. However, it is known that primary aerenchyma is absent near the root base [28], and oxygen to the root tip needs to be transported through secondary aerenchyma formed in the root. Several studies have been conducted on the development of secondary aerenchyma in roots [28,33,40–43]. When adventitious roots are formed, primary aerenchyma is formed anatomically at the start, and then secondary aerenchyma develops with root growth [40]. It is unknown at which stage the primary and secondary aerenchyma are connected to each other to transport oxygen. Thus, it has been hypothesized that young portions of roots may have schizogenous and lysigenous aerenchyma that connect with secondary aerenchyma at the point in the root where two or more layers of phellem cells are formed.

In this study, we aimed to examine how primary aerenchyma connects with secondary aerenchyma in adventitious roots using *Syzygium kunstleri* (King) Bahadur and R.C.Gaur, which is a woody plant distributed throughout Southeast Asia, especially in the peatlands of Thailand, peninsular Malaysia, and Borneo. For that purpose, we clarified how the distribution of primary and secondary aerenchyma changed in adventitious roots generated from trunks using light or epi-florescence microscopy.

2. Materials and Methods

2.1. Plant Material and Treatment

Seeds of *S. kunstleri* were collected from floodplains in Narathiwat Province, Thailand, in 2011, and brought to Japan. These seeds were sown and grown for two years in a phytotron at the University of Tokyo, Japan, under a regime of 12 h of daylight at 30 °C (06:00–18:00), 12 h dark at 25 °C (18:00–06:00), and relative humidity of 60–80%.

In 2013, two-year-old *S. kunstleri* seedlings were cut at 15 cm intervals; the diameter of each cutting ranged from 0.2 to 0.9 cm (average 0.6 cm). The cuttings were grown in a plastic container (60 × 26 cm, 27 cm in depth, Beramiso 60T, Daiwa, Japan) containing Akadama soil (granular loamy soil) in the same controlled-environment phytotron under well-watered conditions for five months. During the first five months, to protect the cuttings from drying, the top of the pot was wrapped with vinyl sheet. Then, the cuttings were rooted in lightproof plastic pots (7 cm in diameter, 20 cm in height, self-made) filled with quartz sand for approximately one year.

After confirming that the cuttings began to form roots, flooding treatment was started. Treatment was performed in lightproof plastic containers (55 × 40 cm, 30 cm in depth, s-54II, Sekisui, Japan) containing the media, which were covered with styrene foam (A-XPS-B-1b, Kaneka, Japan), and wrapped with aluminum foil. In total, six containers (i.e., three containers containing hydroponic media and three containers containing agar media) were prepared. Each container included two to more rooted cuttings. Both media in the containers were hypoxic and included the following nutrients; 4 mM NH_4NO_3, 0.6 mM NaH_2PO_4, 0.6 mM KCl, 0.35 mM $CaCl_2$, 0.25 mM $MgSO_4$, 10 μM $FeSO_4$, 20 μM H_3BO_3, 2 μM $MnCl_2$, 2 μM $ZnSO_4$, 2 μM $CuSO_4$, and 2 μM Na_2MoO_4 [44]. The agar medium contained 0.1% agar in order to prevent convection and thus to reduce introduction of atmospheric oxygen into the medium. Lightproof plastic plates (white) covered the surface of the media to prevent them from drying, shrinking, or being contaminated. During the experimental period, the media were refreshed every two weeks to reduce the effects of changes in composition of dissolved O_2, ethylene, and CO_2.

2.2. Preparation of Samples

We prepared four treatments (treatments A to D) as explained below.

Treatment A (n (number of adventitious roots) = 3): three-month flooding with hydroponic medium.

Treatment B (n = 3): three-month flooding with agar medium.

Treatment C (n = 3): six-month flooding with hydroponic medium.

Treatment D (n = 3): six-month flooding with agar medium.

At Treatments A and C, which the same three containers containing hydroponic medium was used in, one cutting was sampled from each of the three containers after three-month flooding (Treatment A) and six-month flooding (Treatment C), respectively. One adventitious root newly extending directly from the stem was selected from each of the cuttings. Similarly, at Treatments B and D, which the same three containers containing agar medium was used in, each adventitious root was selected from the cuttings in each of the three containers after three-month flooding (Treatment B) and six-month flooding (Treatment D), respectively. The lengths over which the roots were differently colored, i.e., the white parts at the root tip, the light brown parts immediately below the root tip, and the dark brown parts near the root base, were measured using a ruler.

The selected adventitious roots were gently washed in tap water, cut into 0.5 cm pieces, from the tip to the root base, using a razor blade. The specimens from each root cross-section were fixed in formalin/acetic acid/alcohol (FAA) (in the ratio, formalin:acetic acid: 99.5% ethanol:H_2O = 1:1:9:9) and were vacuumed until the tissues had all sunk to the bottom. Dehydration was carried out by treating the desiccated specimens sequentially with 50%, 70%, 90%, and 99.5% ethanol for 2 h at each concentration and finally with 100% ethanol for 24 h. The specimens were then infiltrated and embedded in Technovit 7100 resin (Kulzer and Co. GmbH, Wehrheim, Germany). The polymerized

resin blocks were glued onto wooden blocks with epoxy resin adhesive and sectioned into 7 μm-thick blocks using a rotary microtome (RM2235, Leica, Wetzlar, Germany).

The sections were mounted on glass slides, heated to 50 °C, then stained for 3 min in 0.05% toluidine blue O and washed with water for 10 min. To confirm the presence of phellem cells, root sections were stained for 1 h with a solution of fluorol yellow 088 (0.01%, in lactic acid) [45,46]. The aliphatic components of the phellem cells in cell walls can be identified by their yellow/green fluorescence under UV light. The observations of the stained sections were performed by light microscopy (BX51, Olympus, Tokyo, Japan) and fluorescence microscopy (BX51, Olympus, Tokyo, Japan; excitation filter 330–385 nm; dichroic mirror, DM 440 nm; barrier filter, BA 420 nm) and photographed with a microscope digital camera (Visualix Pro2Metrics, Visualix, Kobe, Japan).

2.3. Observation of Aerenchyma Formation

The distributions of primary and secondary aerenchyma were observed in all the digitized images of the root cross sections taken by the microscope digital camera. In addition, the thickness of periderm (radial length) and the number of layers of phellem cells were measured. When we counted the layers of phellem cells, if the innermost layer was partly connected with the second layer and it was difficult to separate from one another, we counted it as 0.5. Moreover, the proportions of cortex, periderm, and stele along the roots were also calculated from the root cross sections in Treatment C and D, according to the following procedure.

The digitized images of root cross sections were subjected to cross-section analysis [47] with ImageJ software (Ver. 1.48u). ImageJ contains built-in functionality for measuring lengths and areas using area selection tools. The outlines of cortex, periderm, and stele in digitized images of root cross sections were traced with‘ a cursor (freehand selection tool), and their areas were quantified. The ratio of pixels to scale bar length was established during image analysis and the measurements of the selected areas were converted from pixel counts to units of μm and μm^2.

Applying the method in the program RootScan [48], the cross section of the root was divided into cortex, periderm, and stele. Where no periderm was observed, such as near the root tip, the stele was the central part of the root; the endodermis was the outermost boundary of the stele next to the cortex. The outer boundary of the cortex was the epidermis, and root hairs were excluded. If periderm was present between the cortex and stele, range between the outermost and innermost layers of phellem was defined as periderm area.

Thus, total root cross-sectional area was defined as follows:

$$\text{(total root cross-sectional area)} = \text{(cortex area)} + \text{(periderm area)} + \text{(stele area)}, \quad (1)$$

When we measured the thickness of periderm, the layers of phellem cells, and the respective areas in each root cross-section, the measurement was done on five serial sections in each part of root and then average values were used in the following analysis. To test the difference in those values among root colors (i.e., white, light brown, and dark brown) and media, Bonferroni corrected Mann–Whitney pairwise comparison was carried out following Kruskal–Wallis test ($p < 0.05$) using SPSS Statistics Version 25 (IBM Software, New York, NY, USA). In this study, the difference among containers was not considered.

3. Results

3.1. Distribution of Aerenchyma in Syzygium kunstleri Adventitious Root

3.1.1. Primary Aerenchyma in Cortex

Schizogenous aerenchyma was observed in and near the root tips (Figure 1), and these roots were white and light brown. Lysigenous aerenchyma was detected throughout the roots except near the root tip in the three-month flooding treatment roots; these roots were light brown and dark brown

(Figure 1). In the roots grown in the six-month flooding treatment, cortical cell death and degradation were observed throughout the roots. Also, cortex collapse was observed near the root base and the periderm was exposed (Figure 1).

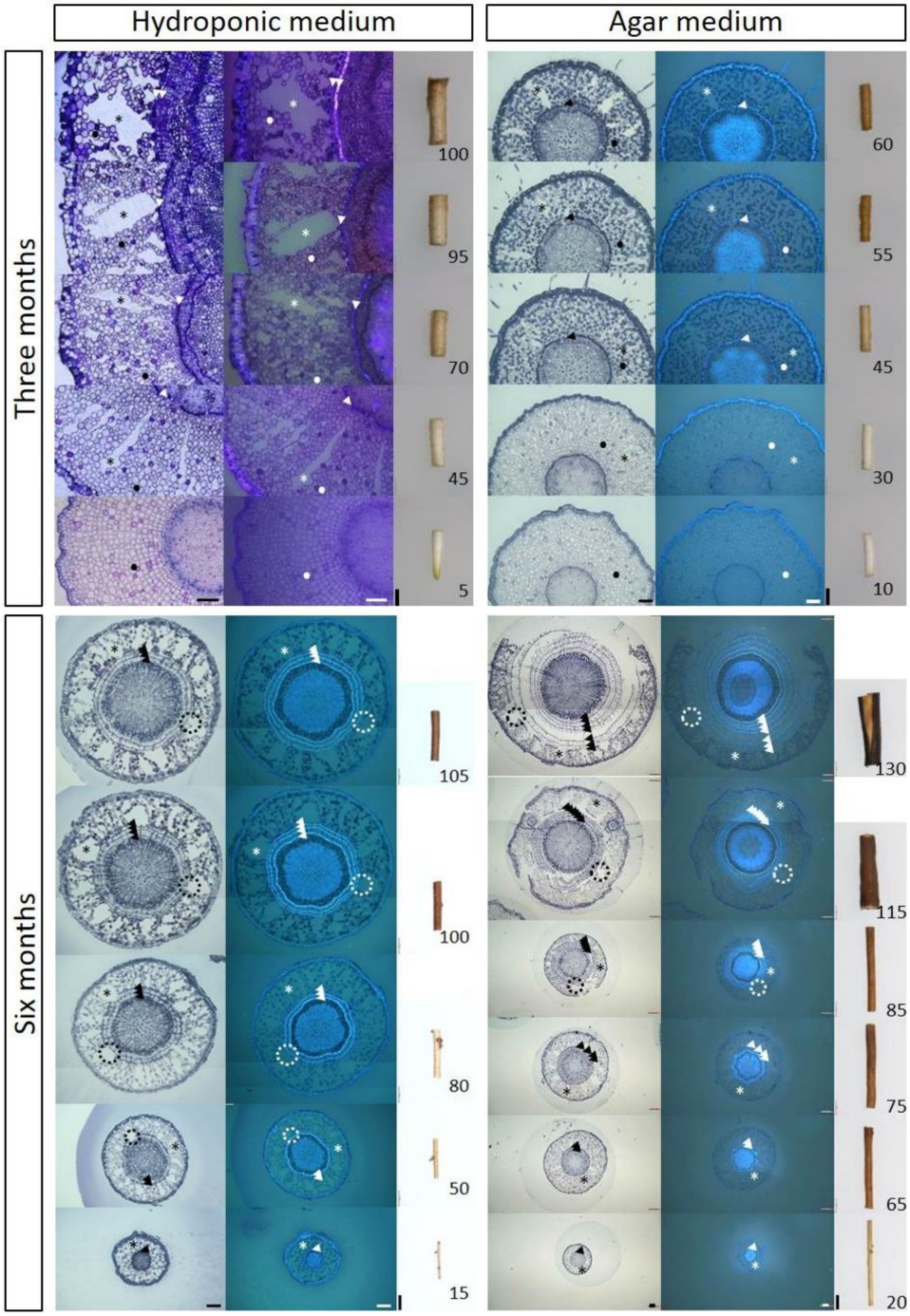

Figure 1. Serial transections of adventitious roots of *Syzygium kunstleri* (King) Bahadur and R.C.Gaur grown for three or six months on hydroponic and agar media. (**Left**) Light microphotograph; (**Middle**) fluorescence microphotograph; (**Right**) stereo microphotograph. Arrowheads (▲): layers of phellem cells; asterisks (*): lysigenous aerenchyma (cortical intercellular space); dashed circle (): broken outermost layers of phellem cells, connected to primary aerenchyma (See Figure 3A,B); filled circle (●): schizogenous aerenchyma. Number indicates distance behind the root tip (mm). Scale bar: left, middle = 100 μm; right = 2 mm.

3.1.2. Development of Periderm and Secondary Aerenchyma

Colored roots (light and dark brown) had at least one layer of phellem cells, and notably the strongly colored roots (dark brown) had at least three layers (Figure 2). Interestingly, when the roots were white, no or only one layer of phellem cells was detected (Figure 2). Dark brown parts were not observed in roots grown for three months in flooding treatments, whereas observed in root base of roots which grown for six months in flooding treatments. The root was darkest in the root base and faded in the direction of the root tip. The thickness of periderm was large in root base and small towards root tip, which was similar to the trend of color change. The number of layers of phellem cells also tended to decrease towards root tips (Figure 2). In roots grown for six months in hydroponic medium, the average thicknesses of periderm in the light and dark brown were 18.4 µm and 95 µm, respectively. On the other hand, in roots grown for six months in agar medium, the average thicknesses in the light and dark brown were 47.4 µm and 281.7 µm, respectively. Those values were significantly larger in roots grown for six months in agar medium. In addition, the average numbers of layers of phellem cells were 1.7 (light brown) and 3.4 (dark brown) in roots grown for six months in hydroponic medium, and 2.3 (light brown) and 5.7 (dark brown) in roots grown for six months in agar medium. Both the numbers in roots grown for six months in agar medium were also significantly larger than those in roots grown for six months in hydroponic medium. Near the root base, destruction of the outermost layer of phellem cells was observed in several root cross sections (Figure 1).

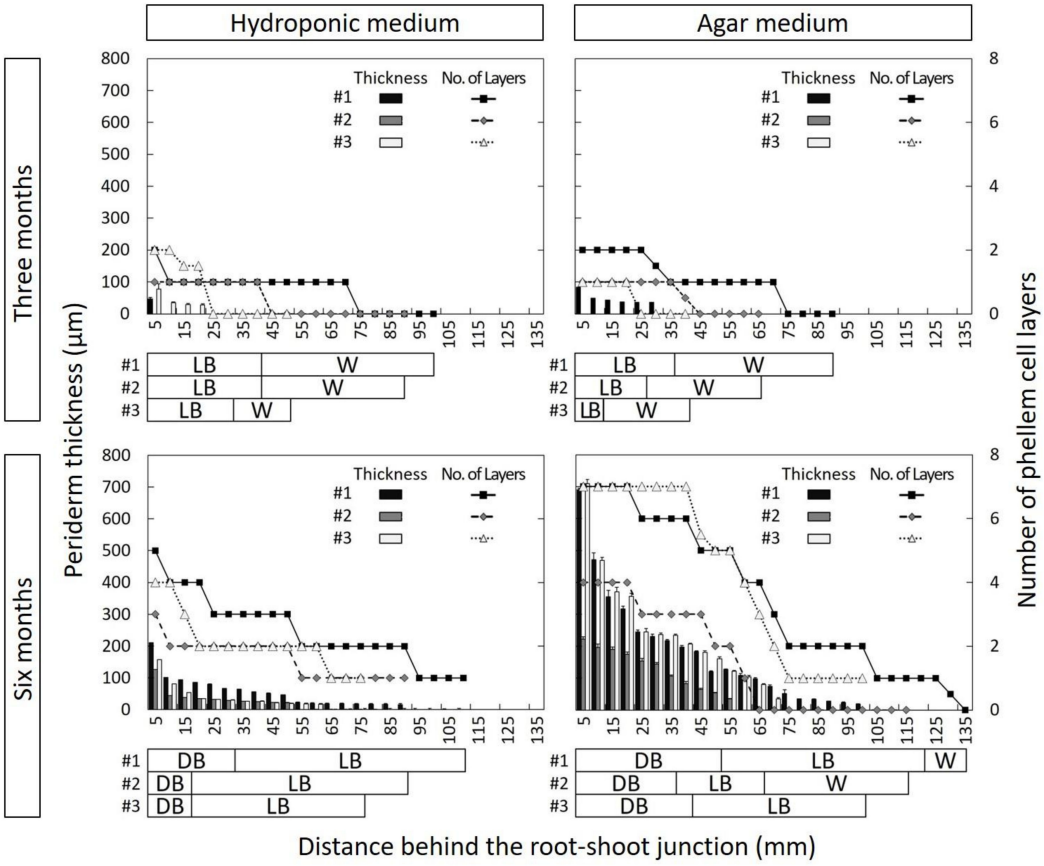

Figure 2. Distribution of periderm development along adventitious roots of *Syzygium kunstleri* grown for three or six months on hydroponic and agar media. Thickness of periderm (radial length) was measured and number of layers of phellem cells was counted. Bars under the graph indicate the color of root surface along the each root. Each part of roots was measured by the average of five serial sections. W: White, LB: light brown, DB: dark brown.

The early formed layer of phellem cells detached from the next layer and the elongated cells were observed between the layers of phellem cells (Figure 3). Thus, the secondary aerenchyma was observed in the periderm (Figure 3).

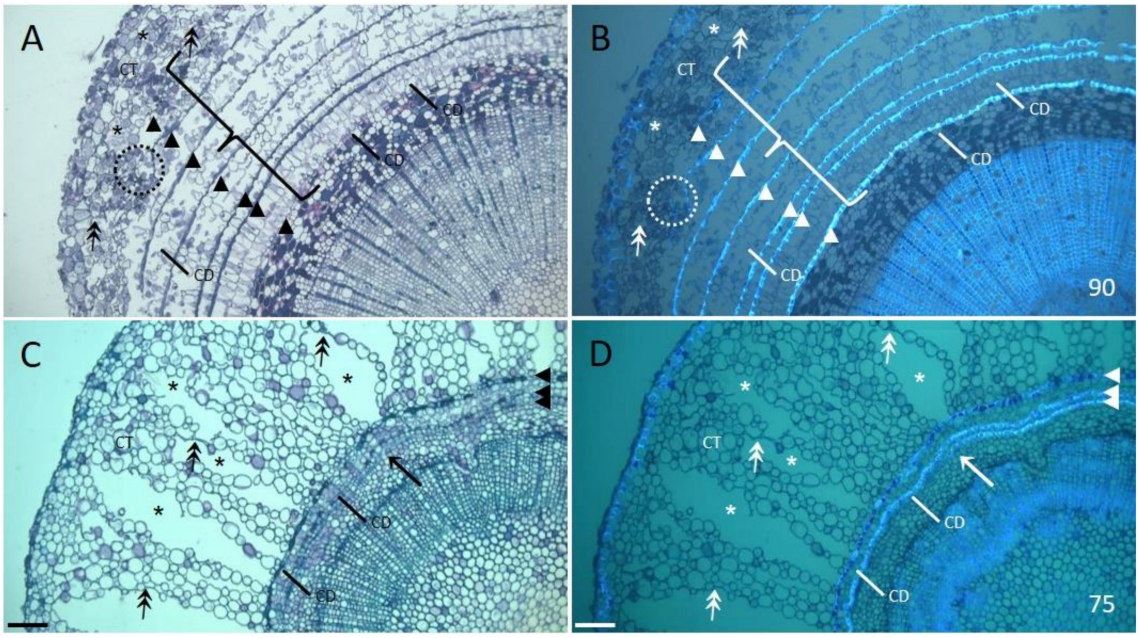

Figure 3. Transverse sections showing periderm and secondary aerenchyma formation in the adventitious roots of *Syzygium kunstleri* grown in agar medium for six months. (**A**,**B**) 90 millimeters behind the root tip, Figure 2 agar medium in six months #3; (**C**,**D**) 75 mm behind the root tip, Figure 2 agar medium in six months #2. (**A**,**C**) Light microphotographs; (**B**,**D**) Fluorescence microphotographs. Arrows (→): newly formed phellem cells; arrowheads (▲): phellem cells; asterisks (*): cortical intercellular space (lysigenous aerenchyma); bracket ({): periderm with intercellular space (secondary aerenchyma); dashed circle (): broken outermost layers of phellem cells, connected to primary aerenchyma; two headed arrows (): cortical cell degradation and death. CD: cell division occurs; CT: cortex. Scale bar = 100 μm.

3.2. Proportion of Cortex, Periderm, and Stele in Each Adventitious Root Cross Section

By the observation, we came to conceive that the thickening of periderm under the experimental conditions is made by elongation of some cells resulting in intercellular spaces. Then we thought that the area of periderm could be used as a parameter available for the comparison of aerenchymas. Since primary and secondary aerenchyma are developed in the cortex and periderm, respectively [12,13,24,30,31], the proportion of cortex and periderm implies the development of primary and secondary aerenchyma, respectively. Thus, the proportions of cortex, periderm, and stele along the roots were also calculated from the root cross sections in roots grown for six months in flooding treatments (Figure 4). Cortex area comprised more than 48% of the white and light brown parts of the roots grown for six months in hydroponic and agar medium (Figure 4). The highest proportion of the periderm area was about 22% and 15% in the dark brown parts of the roots grown for six months in agar medium and hydroponic medium, respectively (Figure 4).

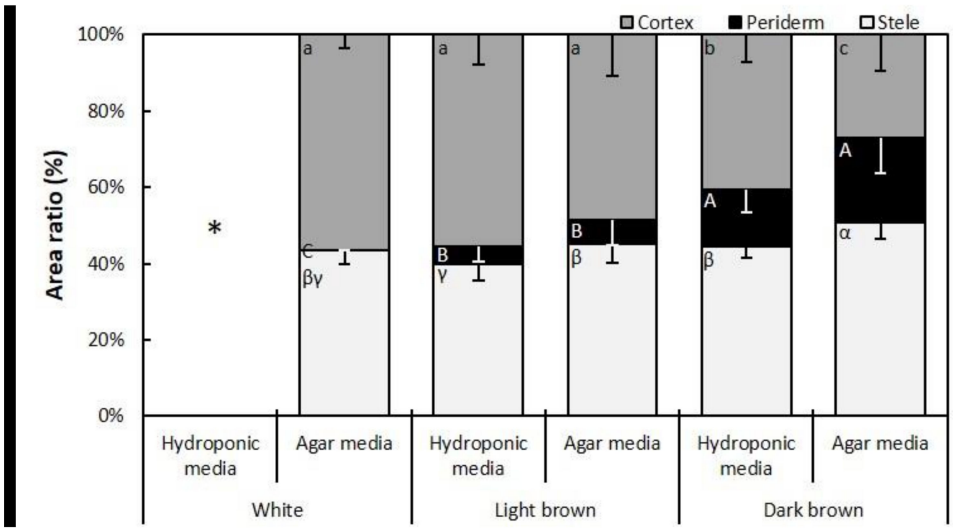

Figure 4. Changes in the proportions of cortex, periderm, and stele for different root colors of *Syzygium kunstleri* grown for six months in hydroponic (n = 3) and agar media (n = 3). Root cross-sectional area = (cortex area) + (periderm area) + (stele area). Error bar; standard deviation, White, Light brown, and Dark brown; root colors, Symbols; the same letter means no significant difference within each tissue (p <0.05, Bonferroni corrected Mann–Whitney pairwise comparisons), *: Not observed.

3.3. Comparison between Different Treatments

The presence of both primary and secondary aerenchyma were confirmed irrespective of the medium used (Figure 1) and the color of the roots was found to correspond with the respective aerenchyma types (Figure 2). When flooding treatment was applied for six months, the proportion of the periderm in the dark brown part of the roots was not significantly different between the roots grown in the hydroponic and agar medium, but the cortex was significantly smaller in the agar medium (Figure 4).

4. Discussion

4.1. Development and Spatial Patterns of Primary Aerenchyma in Cortex

The order of occurrence of aerenchyma types in *S. kunstleri* was similar to that in soybean roots exposed to flooding [41]. Schizogenous and lysigenous aerenchyma appeared first in the cortex, and then secondary aerenchyma developed in the periderm.

In this study, the section of the adventitious roots in which the color changed from white to brown almost coincided with the section in which lysigenous aerenchyma started growing (Figure 1). Investigators usually use a combination of indicators when assessing the aging of roots, such as color changes, the loss of cortex, and the disappearance of fine roots [49]. Thus, because the advance of cell degradation in the cortex differs depending on the distance from the root tip (Figure 4), the cortical intercellular space varied depending on the section of root (Figure 1). The difference of development in cortical intercellular space along the roots would enable longitudinal diffusion of O_2 from the shoots to the root tips.

Considering the observed cortical collapse near the root base, the independent development of lysigenous aerenchyma in roots cannot be seen as an optimal root form for hypoxic conditions [50]. The development of lysigenous aerenchyma is caused by cell death and degradation [13], but continued hypoxic conditions lead to cortical collapse that in turn leads to a decrease in the function of primary aerenchyma. Additionally, since the cortex is destroyed by secondary thickening, in some species cortical aerenchyma formation may be less important for survival under conditions of low O_2 [24,50,51].

Thus, in *S. kunstleri*, tissues that replace the function of primary aerenchyma as an internal O_2 pathway can be considered as secondary aerenchyma.

4.2. Development and Spatial Pattern of Secondary Aerenchyma in Periderm

Secondary aerenchyma in soybean roots was seen to arise from cell divisions in the pericycle, and the entire secondary aerenchyma was found to consist only of cells resulting from that process [41]. Although the secondary aerenchyma of *S. kunstleri* also developed by cell division, in contrast to soybean, layers of phellem cells were observed, forming several layers in *S. kunstleri* (Figures 1 and 3). The repeated structure formed by the layers of phellem cells and the division of elongated cells between the layers of phellem cells seem to be a distinctive process in *S. kunstleri* for the formation of secondary aerenchyma. The secondary aerenchyma is part of the periderm, and occupies a large portion in the vicinity of the root base (Figures 1–3). Therefore, it seemed that the development of secondary aerenchyma was related with that of periderm.

The longitudinal distribution of periderm and secondary aerenchyma in a single adventitious root indicates that the development of periderm was an indicator of secondary aerenchyma development (Figures 1 and 3). As a result of observing the development of periderm, secondary aerenchyma seemed to be most developed in the dark brown sections of the roots (Figure 4). In addition, radially elongated cells positioned between the layers of phellem cells also appeared in multiple layers as a result of cell division, and intercellular space was observed (Figures 1 and 3).

4.3. Function as Oxygen Transportation Pathway in Primary and Secondary Aerenchyma

As the secondary growth of the roots progresses, cortex including primary aerenchyma collapses and peels off (Figure 1). Unless the collapse occurs, the primary aerenchyma is presumed to function as a pathway for oxygen [40]. As such a loss was not observed where the roots were white or light brown (Figure 1), the primary aerenchyma was considered to be functional in these parts of the root.

In order to function as a pathway for oxygen transport, it is important that there are enough spaces for secondary aerenchyma between layers of phellem cells [40]. In this study, only one layer of phellem cells was confirmed where the light brown roots were near the white parts or in the white parts themselves (Figures 1 and 2). This means that a pathway for oxygen was absent or low in these regions. On the other hand, in the light brown root, slightly away from the white parts, the layers of phellem cells were observed as two or more layers, with thick periderm (Figure 2), possibly functioning as a pathway for oxygen transport.

The primary aerenchyma was also predicted to be functional in this root region. Therefore, oxygen diffusion might occur through the primary and secondary aerenchyma types in this root region. Moreover, secondary aerenchyma also developed from the light brown region toward the root base (Figures 1 and 2), suggesting possible oxygen transport between the primary and secondary aerenchymas in this part of the root.

4.4. Comparison among the Different Treatments

Comparing the results of flooding treatment was applied for six months, proportion of the cortex in dark brown part of roots was significantly smaller in the agar medium than in the hydroponic medium (Figure 4). This result implies that the lower oxygen concentration induces cell degradation and cell death in the cortex [18]. Based on Figure 2, the thickness of periderm and number of layers of phellem cells were most developed in dark brown parts near the root base. Especially, the dark brown part of roots that grown for six months on agar media was the most developed. The development of secondary aerenchyma in the dark brown parts near the root base, where cortical cells degradation and secondary thickening were performed, was possibly believed to enhance adaptation to the hypoxic conditions.

5. Conclusions

In *S. kunstleri*, which is a known flood-tolerant species, both primary and secondary aerenchyma were observed in adventitious roots grown in hypoxic conditions. In order to transport atmospheric oxygen to the root tip, the primary and secondary aerenchyma must be connected to one another, because primary aerenchyma is developed along the entire length of the roots, whereas secondary aerenchyma is not. Oxygen transportation occurs between primary and secondary aerenchyma from the point where two or more layers of phellem cells are formed, and the outermost layer of phellem cells is directly connected to the cortical intercellular space, allowing oxygen transfer between the two tissues to occur. That is, secondary aerenchyma in roots grown under hypoxic conditions can complement the oxygen transport function of primary aerenchyma. This study would provide the basis for understanding the morphological and anatomical adaptive mechanism to flooding condition of the tolerant woody plant species, which is expected to contribute to selection of woody tree species as planting stocks for afforestation of degraded peatlands.

Author Contributions: H.-D.S. and M.M. conceived and designed the experiments; H.-D.S. performed the experiments and analyzed the data; H.-D.S. and H.K. wrote the manuscript; T.T. supervised the research.

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