



TITLE:

Insights into the mechanism of diurnal variations in methane emission from the stem surfaces of *Alnus japonica*

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1 **Insights into the mechanism of diurnal variations in methane emission**

2 **from the stem surfaces of *Alnus japonica***

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- 31 **Brief heading (< 93 characters)**
- 32 Diurnal amplitudes in stem CH₄ emissions are season-dependent.
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35 Summary

- 36 ● Recent studies have suggested that in certain environments, tree stems emit methane (CH_4). This
- 37 study explored the mechanism of CH_4 emission from the stem surfaces of *Alnus japonica* in a
- 38 riparian wetland. Stem CH_4 emission rates and sap flux were monitored year-round, and fine-root
- 39 anatomy was investigated.
- 40 ● CH_4 emission rates were estimated using a closed-chamber method. Sap flux was measured using
- 41 Granier-type thermal dissipation probes. Root anatomy was studied using both optical and cryo-
- 42 scanning electron microscopy.
- 43 ● CH_4 emissions during the leafy season exhibited a diurnally changing component superimposed
- 44 upon an underlying continuum in which the diurnal variation was in phase with sap flux. We
- 45 propose a model in which stem CH_4 emission involves at least two processes: a sap flux–
- 46 dependent component responsible for the diurnal changes, and a sap flux–independent component
- 47 responsible for the background continuum. The contribution ratios of the two processes are
- 48 season–dependent.
- 49 ● The background continuum possibly resulted from the diffusive transport of gaseous CH_4 from
- 50 the roots to the upper trunk. Root anatomy analysis indicated that the intercellular space of the
- 51 cortex and empty xylem cells in fine roots could serve as a passageway for transport of gaseous
- 52 CH_4 .

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54 **Key words:** methane flux, stem, diurnal variations, sap flux, *Alnus japonica*, fine-root, anatomy,
55 optical and cryo-SEM images

56

57 Introduction

58 Atmospheric methane (CH_4) is an important greenhouse gas; therefore, the identification of its
59 source and its quantification are crucial issues in addressing climate change. The global CH_4 budget
60 remains highly uncertain because of the diversity of biogenic and abiotic CH_4 sources and emission

61 processes, reduction by chemical reaction with the short-lived hydroxyl radical, and lack of
62 observations of sources and sinks (Saunois et al., 2020). Wetland CH₄ emissions are recognized as the
63 largest natural source in the global CH₄ budget, contributing to roughly one third of total natural and
64 anthropogenic emissions. However the estimated CH₄ emission strengths are still highly uncertain
65 (Kirschke et al., 2013), clearly indicating that there is a need for improved estimates.

66 In recent years, emission of CH₄ from the stems of living and dead trees has drawn considerable
67 attention as a potentially important new source of atmospheric CH₄ (Carmichael et al., 2014, 2018,
68 Barba et al., 2019a; Covey and Megonigal, 2019). Findings of experimental studies have increasingly
69 suggested that tree-mediated processes can contribute significantly as a pathway of CH₄ emission in
70 wetland ecosystems (Terazawa et al., 2007, Gauci et al., 2010; Pangala et al., 2015, Pangala et al, 2017,
71 Terazawa et al., 2021). Even in upland forest ecosystems in which surface soils under aerobic
72 conditions are generally considered to act as CH₄ sinks, emission of CH₄ from tree stems may diminish
73 the capacity of surface soils to take up CH₄ (Pitz and Megonigal, 2017, Pitz et al., 2018). However,
74 the understanding of the mechanisms by which CH₄ is emitted from the stems of living trees remains
75 ambiguous. For example, it is unclear whether trees function as passive “pipes” through which CH₄ is
76 transported diffusively from the rhizosphere to the atmosphere, whether they serve as pipes in which
77 xylem flow transports CH₄ in the dissolved state, or whether living trees produce CH₄ in the heartwood
78 (Covey et al., 2012, Barba et al., 2019a). A unique application of ²²²Rn gas in a recent study by
79 Megonigal et al. (2020) enabled them to investigate the mechanism for gas transport through trees of
80 three species of *Fagus grandifolia* Ehrh., *Liriodendron tulipifera* L., and *Quercus rubra* L. Their
81 suggested mechanism is based on the fact that transpiration lowers stem water content. Transpiration
82 draws water out from water-filled intercellular spaces in the xylem, reducing the tortuosity of diffusion
83 pathways, and thereby increasing methane diffusion rates. Aside from soil and heartwood production
84 of CH₄, methane-oxidizing bacteria have recently been identified within tree bark (*Melaleuca*
85 *quinquenervia*) and sapwood (*Populus* sp.), which may be able to affect CH₄ emissions from trees
86 (Jeffrey et al., 2021a; Feng et al. 2022). In addition, under conditions where tree stems are waterlogged,

transport of gaseous CH₄ through the bark has been reported for *M. quinquenervia* that features a distinctive bark that is thick, layered, spongy, paper-like, shaggy and peeling bark (Jeffrey et al., 2020).

The aim of the present study was to elucidate the mechanisms of CH₄ transport inside wetland trees. Our group has been investigating the process by which CH₄ is emitted from the stems of *Alnus japonica* (Thunb.) Steud. trees growing in a riparian wetland with a monsoon climate. We recently reported CH₄ emission rates and seasonal variations dependent on individual trees based on year-round measurements using a closed-chamber method coupled with near-infrared laser spectrometry for *in situ* CH₄ detection (Sakabe et al., 2021). The rates of CH₄ emission from the stems of *A. japonica* trees exhibited maxima in summer and minima in winter, and this pattern was closely related to the methanogenic activity in the soil, as demonstrated by path analysis. In addition, our results indicated that intensive rainfall could modulate the rates of CH₄ emission, as transient rainfall-associated changes in the soil environment, such as fluctuations in groundwater level, could affect the rates of CH₄ emission. In the present study, hourly based measurements conducted throughout the year enabled us to examine the diurnal properties of CH₄ emission rates in detail. We discuss the possible processes giving rise to diurnal variation in the rates of CH₄ emission from the stem surfaces of *A. japonica*, especially in terms of the relation between the CH₄ emission rates and sap flux. A diurnal feature of stem CH₄ flux was clearly observed in *L. tulipifera* L in an upland forest (Pitz and Megonigal, 2017), but their data were limited to only three days. They briefly discussed the need to study the relationship between diurnal variations in CH₄ flux and sap flow. Thereafter, sap flux has been shown to associate with temporal CH₄ emissions at diurnal and seasonal scales (Barba et al., 2019b; Jeffrey et al., 2020). Sap flux occurs when water in the soil is absorbed by the roots and ascends via the transpiration of leaves; this system connects the underground to the aboveground parts through the inside of trees. Attention has also been focused on the relation between the day-night variations in CH₄ flux and air temperature (Barba et al., 2019b; Jeffrey et al., 2020). By contrast, other studies have found no clear evidence of diurnal variations in CH₄ emissions (Pangala et al., 2014; Terazawa et al., 2015; Schindler et al., 2021). In the present study, we conducted a detailed examination of the relationship between the

properties of diurnal CH₄ emission and the results of sap flux measurements.

In addition, in order to explore the mechanistic insights into stem CH₄ emissions in the present study, samples of fine roots were collected and observed using optical and cryo-scanning electron microscopy (cryo-SEM). Previous optical microscopy studies have indicated that the pathway for gas exchange between the roots and aboveground environment is composed of the aerenchyma, a type of tissue comprising a relatively high proportion of spaces or lacunae (Evert 2006; Takahashi et al., 2014). The function and formation of lysigenous aerenchyma have been well studied, particularly in roots of rice and maize. These structures, which are formed by cell death and subsequent lysis, provide oxygen from shoots to root, enabling the plant to tolerate anaerobic conditions (Drew et al., 2000; Yamauchi et al., 2013). Distinct root aerenchymas have also been observed in mature mangrove trees, the proportion of which expands from the root apex (Purnobasuki & Suzuki, 2004). The other tissue, called secondary aerenchyma, is a white sponge tissue formed in the stem, hypocotyl, tap root, adventitious roots, and root nodules of some leguminous, herbaceous, and woody plants, which plays a role in flood tolerance (Stevens et al., 2002; Verboven et al., 2012; Yamauchi et al., 2013). In addition to the characteristics of these specific tissues that can be examined using optical microscopy, cryo-SEM can provide unique information regarding the distribution of water in the observed tissue at a given point in time (e.g., Webb et al., 1986; Azuma et al., 2016). Using these tools to examine the root tissues of *A. japonica* could help elucidate the mechanisms by which CH₄ is transported in fine roots, either in the gaseous form or dissolved in sap flow. Here, we report the identification of intercellular spaces that could function as conduits for transporting CH₄ molecules in the gaseous state.

Materials and methods

Measurements of stem CH₄ emission and sap flux

The experimental design and the target wetland for *in situ* measurements of CH₄ flux from the stem surfaces of *A. japonica* was the same as that described in our recent paper (Sakabe et al., 2021); thus, only a brief description will be given here. Measurements were conducted in a temperate

coniferous forest in the Kiryu Experimental Watershed (KEW, 34°58'N, 136°00'E) in Shiga Prefecture, which is located in central Japan. The detailed site description including a topographic map has been provided elsewhere (Kogusi et al., 2007; Itoh et al., 2007; Sakabe et al., 2016, 2021). The elevation range of the study site is 190 – 255 m. The forest comprises 60-year-old Japanese cypress trees (*Chamaecyparis obtusa* Sieb. et Zucc.) that were planted in 1959. Some riparian wetlands were located upstream of check dams that were constructed across the main stream of the watershed approximately 100 years ago.. The dominant tree species in the wetland is *A. japonica*. Three mature *A. japonica* trees growing naturally in the riparian wetland were selected for this study (hereafter: Trees 1, 2, and 3). The height and diameter at breast height of Trees 1, 2, and 3 were 11.3 m and 12.7 cm, 8.3 m and 8.9 cm, and 12.2 m and 10.8 cm, respectively. The trunks of *A. japonica* trees were not waterlogged at all. Surface soil around the trees may be saturated with water for a few hours during and after extreme heavy rainfall..

Stem CH₄ flux was measured using a dynamic closed-chamber system, in which a laser-based instrument (FGGA907-0010, Los Gatos Research, CA, USA) was employed for interference-free, real-time monitoring of gaseous CH₄ concentrations at atmospheric levels. The span of the laser spectrometer was calibrated against standard gas containing atmospheric levels of CH₄ diluted in synthetic air (Koatsu Gas Kogyo Co., Ltd., Osaka, Japan). A chamber that enclosed the whole stem circumference was attached to each tree. Each chamber consisted of a custom-made cylinder made of clear acrylic resin (25 cm outer diameter × 30 cm high) and was equipped with an inlet and outlet connected to the laser-based analyzer via PFA tubes (4.25 mm inner diameter). The chambers were attached to the stem surface at a specific height to cover all directions, and remained in place throughout the observation period. The height with respect to the bottom of the chamber ranged from 30 to 50 cm above the ground surface. The chambers were usually ventilated using ambient air. The target chamber system was temporally closed for 10 min during measurements, and the air from the chamber was circulated using a diaphragm pump. The sample air flow was circulated at a constant flow rate of 1.5 L min⁻¹, which was regulated using a mass flow controller (MPC0005BBRN010000,

azbil, Tokyo, Japan). Sequential switching among the chambers was regulated using solenoid valves (CKD-CD16AC, Campbell Scientific, UT, USA). The sampled air was pre-dried using a gas dryer (PD-50T-24MPS, Perma Pure Inc., NJ, USA). A portion of the circulating sample air was introduced into the laser spectrometer to measure the concentrations of CH₄ and residual water vapor. The CH₄ concentrations were corrected for water vapor dilution. The stem CH₄ flux per stem surface area (nmol m⁻² s⁻¹) was deduced from the temporal changes in gas concentration using the following equation:

$$Flux = \frac{dc}{dt} \times \frac{V}{S} \times \rho$$

where dc/dt represents the slope of the linear regression of the temporal change in gas concentration *c* (ppm) at time *t* (s) during chamber closure, *V* represents the volume of the chamber headspace, *S* represents the surface area of the stem, and *ρ* represents the air mole density (mol m⁻³). In our study, *S* was determined based on geometric measurements, and the bark of *A. japonica* characteristically exhibits small, shallow cracks. The flux data from each of the three chambers were obtained hourly. All flux values determined in this study were positive, which means that net emission of CH₄ occurred throughout the year. The CH₄ fluxes were accepted with a condition that the determination coefficient of linear regression was larger than 0.90. The flux data discarded were 0.3, 0.5, and 0.3 % of the raw data of measurements for Tree 1, 2, and 3, respectively. Based on the root mean square error of the linear regression within the measurements, the minimum detection limits for CH₄ flux were 0.04, 0.07, and 0.04 nmol m⁻² s⁻¹ for Trees 1, 2, and 3, respectively, for the 6-min chamber deployment period.

Sap flux was measured for each tree every 30 min using the thermal dissipation method and Granier-type sensors (Granier, 1987). Sap flux was measured using the same method reported by Tateishi et al. (2008). Each sensor consisted of a pair of thermocouple probes (a heater probe and a reference probe) 20 mm in length and 2 mm in diameter. The probes were inserted vertically into the sapwood (150-mm apart, 0-20 mm depth). All sensors were installed on the north-facing side of the trunk and covered to avoid exposure to direct sunlight. The upper heater probe was continually supplied with a power of 0.2 W. The heat generated by the heater probe dissipated into the sapwood

and the vertical sap flow surrounding the probe. The temperature difference between the heater and reference probes (ΔT) was converted to the sap flux per cross-sectional area of the sapwood at the heater probe ($\text{cm}^3 \text{ m}^{-2} \text{ s}^{-1}$) as reported by Granier (1987). ΔT values were measured every second, and all values averaged over 30 min were recorded using a CR-1000 data logger (Campbell Scientific, USA). The highest temperature difference was defined as the zero-flux condition for each day.

Environmental parameters including air temperature, rainfall, and short-wave radiation in the KEW were measured as described in previous studies (Kosugi et al., 2007; Sakabe et al. 2016, 2021). The average annual air temperature was 13.7°C , and the average annual rainfall between 2010 and 2019 was 1,799 mm. The amount of rainfall was measured using a tipping bucket rain gauge (RT-5, Ikeda Keiki, Japan). This paper analyses the observation data of CH_4 flux, sap flux and environmental parameters between September in 2017 and August in 2018.

Optical microscopy and cryo-SEM

To elucidate the pathway of CH_4 transport in plant tissue from the soil, fine roots ($n = 6$) were carefully collected from approximately 5-cm depth of soil close to the study trees and then flash frozen *in situ* in liquid nitrogen. The frozen fine roots were then cut from the individual trees, sealed in plastic tubes, transported to the laboratory under a large amount of dry ice, and stored at -80°C until analysis. To observe the anatomic characteristics of the fine roots without dehydrating the tissues, which would alter the water status at the time of collection, transverse sections were observed using cryo-SEM (SU8230, Hitachi, Japan). Secondary electron images were obtained at an accelerating voltage of 3 kV with shallow sublimation. To examine the fine root anatomy in detail, some fine roots were sealed in plastic bags before flash freezing and transported to the laboratory. Transverse sections ($26 \mu\text{m}$ thickness) of these fine roots were prepared and double stained with safranin–fast green. The sections were then observed under an optical microscope (Eclipse 80i, Nikon, Tokyo) and photographed using a digital camera (E-620, Olympus, Tokyo). Fine root samples examined by cryo-SEM were also sectioned transversely and photographed under the optical microscope, as described above.

The potential for sublimation of moisture from frozen fine root tissues during transport from the field to the laboratory or during storage in a freezer raises concerns regarding artifacts that may appear on cryo-SEM images. To determine whether any such artifacts were present in the above observations, the samples were compared with those prepared in an experiment using an *A. japonica* sapling grown in the laboratory under well-watered conditions (tree height = 83 cm). In this experiment, fine roots of the sapling ($n = 3$) were observed under cryo-SEM using the same procedure described above, with the exceptions of sample transport from the field to the laboratory and storage in a freezer. That is, in the sapling experiment, cryo-SEM was conducted immediately after the samples were flash frozen, as this condition is unlikely to introduce artifacts. Comparison of the cryo-SEM images of the sapling experiments (Fig. S1) confirmed that no artifacts were present in the images of the samples collected at the study site.

Results

Diurnal properties of stem CH₄ flux and model of the emission mechanism

Figure 1 shows the stem CH₄ fluxes from three *A. japonica* trees in different seasons, along with the air temperature, rainfall, downward short-wave radiation, and sap flux. Flux measurements with a high time resolution of one hour revealed the existence of diurnal changes that were superimposed over the background continuum. Stem CH₄ emissions peaked **about an hour after noon** in the afternoon in all three trees. In addition, the daytime increase in CH₄ emission was almost synchronous with the increase in sap flow. Intriguingly, declines in sap flux due to rainfall or cloudy weather were related to a decline in the magnitude of the daytime increase in CH₄ emissions (Fig. 1A), suggesting that the diurnal amplitude in CH₄ emissions depends on sap flux. We note that measurements of the stem CH₄ fluxes and sap flux were conducted between September in 2017 and August in 2018, of which such diurnal features were evident only during leafy season. Another feature to be addressed here is that the *total* CH₄ emission rates exhibited overall decreasing and increasing trends in autumn and summer, respectively. This is due to the seasonal variation in CH₄ emissions, in

which they differed by up to about two orders of magnitude between the minima in February-March during defoliation and the maxima in August of leafy season (Sakabe et al., 2021). Similarly, as reported previously, relative differences in sap flux between autumn and summer were due to its seasonality in which the sap fluxes kept quite low during defoliation and began to increase with the spread of leaves in April, and decreased gradually after the summertime maxima as the season progressed.

Figure 2 shows the monthly average stem CH_4 and sap fluxes for the three study trees over the periods September to November 2017 and May to July 2018. The CH_4 emission peaks were almost synchronous with the sap flux peaks. In calculating the monthly averages, data for periods when the rainfall amount over the previous 24 h exceeded 10 mm were excluded. Intense rainfall events were associated with transient increases in stem CH_4 emissions, and these increases were typically triggered within a few hours of the start of the intense rainfall event and then gradually faded over the course of several days (Fig. 1A). This phenomenon will be associated with the dynamic response to rainfall of the vertical distributions of gaseous and dissolved CH_4 concentrations in subsurface soil layers, as discussed in our recent paper (Sakabe et al., 2021). The data in Figure 2 show that the diurnal amplitude of CH_4 emissions declined with the progression from autumn to winter, along with a seasonal decrease in sap flux, whereas the diurnal amplitude increased with the progression from spring to summer. It should be noted that no diurnal component of CH_4 emissions could be detected from late autumn to early spring, although emission of CH_4 could be clearly detected even in winter, as we recently reported (Sakabe et al., 2021). At the study site, *A. japonica* trees drop their leaves in late November, and begin to leaf in April. In addition, a careful look at Fig. 2 shows that, in Tree 3, the diurnal peaks in stem CH_4 emission and sap flux appeared about an hour later than those in Tree 1 and 2. This may be due to the fact that the study trees grow in a shallow valley, which causes individual differences in the time of sunlight exposure. Sap flow is linked to leaf transpiration, and leaf transpiration is linked to sunlight exposure.

We make an attempt to separate the CH_4 flux data into two components, namely, diurnally

varying and unvarying components, based on their comparison with the sap flux data. The diurnal patterns of stem CH₄ and sap fluxes were found to be almost in phase with each other, which allows us to infer that there could be a process associated with sap flow. Accordingly, we will henceforth refer to the diurnally varying component as the “sap flux-dependent” component, whereas the diurnally unvarying component as the “sap flux-independent” component. We note that, however, using the terms of “sap flux-dependent” and “sap flux-independent” does not negate any CH₄ transporting process driven by other than sap flow. Possible CH₄ transport mechanisms regulating the stem CH₄ emissions from the stem surface of *A. japonica* trees will be discussed later. Here, we propose a model enabling estimation of the relative contribution of the sap flux–dependent and –independent components to the rate of stem CH₄ emission. Figure 3 shows scatter plots of the stem CH₄ and sap flow fluxes in September 2017 for each of the study trees. Linear regression analysis was used to determine the y-intercept corresponding to the stem CH₄ flux at which the sap flux becomes virtually zero. Assuming that the estimated y-axis intercept value is constant over the entire sap flux of interest, we calculated the monthly–averaged ratio of the relative contribution of the sap flux–dependent to –independent components in the stem CH₄ flux for each tree. The estimated relative contribution ratios over a 7-month period were found to be sample tree–dependent, with a maximum relative contribution of the sap flux-dependent component occurring around July with no more than about 11 % (Table 1). To our knowledge, no such estimate has been reported previously.

Anatomy of fine roots

The vascular cylinder (stele) was surrounded by a thick cortex in the primary growth root (Fig. 4A), and secondary growth gradually proceeded (Fig. 4B). The outermost layer of roots in primary growth was surrounded by ectomycorrhizal hyphae (Fig. 4A). Analysis of longitudinal sections of roots during secondary growth revealed intercellular spaces extending vertically in the cortex (Fig. 4C). We expected that such lacunae function as pathways for the diffusion of CH₄ in the gaseous form through the root. Cryo-SEM was used to determine whether the fine-root tissues were filled with water

(Fig. 5A and B). In the cortex, a number of large, empty (i.e., no water) intercellular spaces were observed (Fig. 5C and D). By contrast, in the endodermis, the intercellular spaces were small and filled with water (Fig. 5E and F). All parenchyma cells were filled with water and cytoplasm, whereas some xylem cells were empty in the vascular cylinder (Fig. 5G and H). Similar results were observed in all six fine-root samples analyzed (Fig. S2).

Discussion

Year-round measurements of the CH₄ emission rates from the stem surfaces of *A. japonica* trees revealed the presence of a diurnal component in the emission rates, and the daytime increase in emission was found to be almost synchronous with a daytime increase in sap flux. We propose here a simple model in which the total CH₄ emission rate consists of “sap flux–dependent” and “sap flux–independent” components. A possible mechanism of CH₄ transport for the sap flux–dependent component could involve absorption of soil water containing dissolved CH₄ by the roots, with subsequent transport of the dissolved CH₄ through the conduits; volatilized CH₄ could then be detected with the daytime increase in the stem CH₄ emission rates. However, we note that the observed synchrony between CH₄ and sap fluxes does not exclude the possibility that the diurnal variation cannot be explained solely by sap flow. The mechanisms regulating stem CH₄ emission in plants remain controversial, although most previous studies have supported the primary importance of CH₄ transport in the gaseous form within the tree (Barba et al., 2019a). As mentioned in the introduction section, a variety of the CH₄ transport mechanism inside the trees have recently been suggested, for example, plant transport of soil gases is controlled by plant hydraulics as reported by Megonigal et al. (2020). Some studies have suggested that methanogen living in the heartwood could also be a source of CH₄ (Wang et al., 2016, 2017; Yip et al., 2018). Aside from CH₄ production in soil and heartwood, microbial oxidation within trees may weaken stem emissions (Jeffrey et al., 2021a; Feng et al. 2022). Clearly further investigation is needed for understanding the **sap flux-dependent and -independent CH₄ emission** mechanisms functioning in *A. japonica* trees. Our recent study (Sakabe et al., 2021)

suggested that, as supported by most studies for different wood species, the transport of gaseous CH₄ produced by methanogenic archaea in rhizospheric soil is primarily responsible for stem CH₄ emission from *A. japonica* throughout the year. This conclusion was based on the following evidence: (i) stem emission was observed even in winter; and (ii) stem emission was observed even during the night, when the sap flow rate was nearing zero (see also Fig. 1 of this paper).

The ratio of the relative contribution of the sap flux–dependent and sap flux–independent components, estimated based on the model proposed above, were found to vary seasonally (Table 1). The sap flux–dependent component reached a maximum in summer with no more than about 11 % (Table 1). It will be intriguing to consider whether the contribution ratio depends on the height of the stem chamber within a given *A. japonica* tree. Measurements of the vertical profile of stem CH₄ emission from some species provided clear evidence that the stem CH₄ emission rates vary widely, with a general trend toward decreasing CH₄ emission with increasing stem height for flux measurements (Wang et al., 2016; Pitz & Megonigal, 2017; Barba et al., 2019b; Jeffrey et al., 2020). This observation was explained by the gradual flow of gaseous CH₄ out of the tree and into the atmosphere during transport from the roots to the upper trunk. Our preliminary measurements indicated that the rate of CH₄ emission from *A. japonica* stems decreased with increasing height of the stem flux monitoring chambers, consistent with the results of previous studies. However, the sap flux–dependent component also likely depends on stem height, as CH₄ taken up by the roots and dissolved in the sap may gradually volatilize as it moves through the tree. In addition, there is no information on whether stem-height dependent microbial CH₄ oxidation within trees, which was evident for *M. quinquenervia* and *C. glauca* (Jeffrey et al., 2021), are occurring in *A. japonica*. Accordingly, the possibility that the contribution ratio estimated for each tree does not remain constant throughout an individual tree cannot be ruled out.

Besides sap flow, another possible factor associated with the diurnal feature of stem CH₄ flux is the molecular diffusivity and its temperature dependency. The coefficient of molecular diffusivity of CH₄ in air, for example at 30 °C, is 6% larger than that at 20 °C, according to Massman (1998). The

rate of increase in diffusion coefficient seems to be comparable to the estimated contributions of diurnally varying component (Table 1). Although the gas diffusivity through wood (Soriz and Hietz, 2006) as well as stem height dependence of the relative contribution ratios need to be quantified, it would be worthwhile to examine this physical chemistry mechanisms in the future. Possible effects of temperature on diffusive transport of CH₄ in *Alnus glutinosa* were briefly discussed by Pangala et al (2014). Relation between air temperature or stem temperature and stem CH₄ flux was tested to discuss the day-night variations in CH₄ flux (Barba et al., 2019b; Jeffrey et al., 2020).

From the viewpoint of elucidating the CH₄ transport mechanism leading to the sap-dependent component, we would like to mention one more point. A closer look at the data in Figure 2 shows, there appeared to be a time lag between the daily peaks of stem CH₄ emission and sap flux, particularly in Tree 3 in Sep.-Oct. 2017. This observation is qualitatively similar to the results of Pitz and Megonigal (2017), in which a diurnal feature in stem CH₄ emission from *L. tulipifera* peaked in the late afternoon, while the sap flux reached its maximum around noon. In order to achieve a better understanding of the time lag, if existed, in *A. japonica*, CH₄ flux (and sap flux) with its stem height dependence should be measured at a higher temporal resolution. Furthermore, effective gas diffusivity in wood, both in axial and radial direction, and sunlight exposure conditions should also be investigated.

Our proposed model for the process of stem CH₄ emission in *A. japonica* suggests the primary importance of gaseous CH₄ transport, which can be detected as sap flow-independent emission from the stem surfaces. Therefore, in this study, we analyzed fine roots using optical and cryo-SEM to explore plant tissues that may facilitate the diffusion and transfer of gaseous CH₄. Tree roots take up and transport water and nutrients via their direct contact with the soil, and apical fine roots are particularly active in this process (Strock & Lynch, 2020). In this study, no lysigenous aerenchyma or secondary aerenchyma were observed in the cortex of the fine roots of *A. japonica*. However, the presence of scattered longitudinal intercellular spaces (Figs. 4 and 5) suggests that CH₄ in the gaseous form can be diffused from the soil to the tree stem via the roots. In addition, some xylem cells, such as vessels and fibers, were empty in the vascular cylinder of the fine roots, thereby providing a possible

pathway for gas diffusion. Although other researchers have speculated that anatomical features such as intercellular spaces or air-filled elements in the secondary xylem could function as gas pathways in waterlogging tolerance (Philipson & Coutts, 1980), our present study is the first to demonstrate this phenomenon by *in situ* field observations in adult *A. japonica* trees. However, optical microscopy analyses of roots of cultivated seedlings of *A. japonica* and *Salix martiana* Leyb. under experimental flooding conditions suggested the formation of aerenchyma (Yamamoto et al., 1995; De Simone et al., 2002). As the results of the present study were based on roots collected from the surface soil layer, it will be necessary to examine the anatomic features of roots in deeper soils as well as in a variety of species. Lenticels are another potential plant gas transport pathway, as they permit the entry of air though the periderm (Groh et al. 2002). As many lenticels were observed in visual observations of the fine-root surfaces in the present study, these structures may also play a role in gas transport. Our anatomical analyses suggest that the intercellular spaces in the cortex and the empty xylem cells in the fine roots of *A. japonica* could serve as passageways for the diffusive transport of gas molecules.

Our long-term, hourly based measurements of CH₄ emission from the stem surfaces of *A. japonica* growing in a riparian wetland revealed an interesting phenomenon of diurnal variation with a season-dependent amplitude. Our results provide clear evidence that a component of the rate of stem CH₄ emission is proportional to the sap flux, allowing us to infer a possible mechanism in which CH₄ molecules dissolved in the sap are volatilized and emitted from the trunk of *A. japonica* trees. However, as discussed above, there could be another mechanism which accounts for diurnally varying component in stem CH₄ flux, and therefore observed clear association between the diurnal properties in CH₄ flux and sap flux merits further investigation. A large portion of stem CH₄ emission was found to be independent of sap flow, indicating that gaseous CH₄ molecules are also transported diffusively from the rhizosphere to the tree stem. Analysis of fine roots of *A. japonica* using optical microscopy and cryo-SEM suggested that intercellular spaces in the cortex and empty xylem cells could serve as passageways for the diffusive transport of gas molecules.

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406 Author Contributions

407 K.T., A.S., and W.A. are equally contributing first authors who designed and led the research. K.T.,
408 A.S., and M.I. conducted flux observations. W.A. and M.T. conducted the sap flux measurements.
409 W.A., T.I. and Y.M. conducted microscopic analyses of fine-roots. Y.K. measured
410 micrometeorological parameters. K.T, A.S. and W.A. drafted the manuscript. All authors contributed
411 to revision of the manuscript.

413 Data availability

414 The data that support the findings of this study are available from the corresponding author upon
415 reasonable request.

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538
539

540 **Supporting Information**

541 **Fig. S1** Cryo-SEM images of fine roots of *A. japonica* sapling grown in the laboratory under well-
542 watered conditions.

543 **Fig. S2** Optical and cryo-SEM images of fine roots of *A. japonica* trees.

544

545 **Table 1:** Ratios of the relative contribution of sap flux–dependent to sap flux–independent components
546 in the rates of CH₄ emission from the stem surfaces of *Alnus japonica* trees: sap flux–dependent
547 component / sap flux–independent component. Values in parentheses indicate the coefficient of
548 determination for regression ($p < 0.01$), as typically shown in Figure 3 (see main text for details).

549

Observed	Tree 1	Tree 2	Tree 3
Month/year			
Sept 2017	2.7% (0.90)	3.8% (0.92)	8.7% (0.78)
Oct 2017	1.5% (0.26)	1.7% (0.36)	4.2% (0.41)
Nov 2017	2.0% (0.41)	1.0% (0.29)	2.2% (0.44)
May 2018	3.1% (0.75)	3.7% (0.77)	N/A*
June 2018	3.4% (0.84)	5.6% (0.86)	10.2% (0.87)
July 2018	4.1% (0.89)	N/A*	12.2% (0.62)
Aug 2018	3.3% (0.87)	2.8% (0.33)	9.7% (0.75)

550 *N/A: not estimated due to lack of sap flux data.

551

552

553 **Figure captions**

554 **Figure 1:** CH₄ flux from the stem surfaces of three *Alnus japonica* trees in (A) September 9-16, 2017,
555 and (B) July 11-18, 2018, together with sap flux, downward short-wave radiation (denoted as S↓), air
556 temperature, and rainfall (bar graph). Both periods in figure 1a and 1b are leafy season. Measurements
557 of CH₄ flux and sap flux were conducted with 1-h resolution (see main text for details). Sap flux data
558 for Tree 2 in (B) were not available due to unavoidable circumstances. Red, Tree 1; blue, Tree 2; green,
559 Tree 3. No rainfall was observed in July 11-18, 2018.

560

561 **Figure 2:** Monthly average stem CH₄ flux and sap flux for three *Alnus japonica* trees for September
562 to November 2017 and May to July 2018; diurnal data with 1-h resolution were averaged over each
563 month. Shaded ranges represent 95% CI. Data were excluded when the cumulative rainfall in the
564 previous 24 h exceeded 10 mm. Color codes are the same as indicated in the legend for Figure 1. The
565 sap flux data for Tree 1 in May 2018 and for Tree 3 in July 2018 were not available due to instrument
566 malfunction.

567

568 **Figure 3:** Plots of correlation between CH₄ flux and sap flux for three *Alnus japonica* trees in
569 September 2017, using the data shown in Figure 2. Each graph has 24 data points, each of which is an
570 hourly one-month average corresponding to the data shown in Figure 2 (mean and 95% CI). The
571 determination coefficients for regression were 0.89, 0.93, and 0.84 for Tree 1, Tree 2, and Tree 3,
572 respectively ($p < 0.01$). The y-axis intercepts correspond to the stem CH₄ fluxes at which the sap fluxes
573 become virtually zero and were assumed to indicate the contribution of the diurnally unvarying
574 component of CH₄ emission from the stem surface. The estimated y-axis intercept value was subtracted
575 from the observed CH₄ flux values, whose output was integrated over the range of the observed sap
576 flux, thereby the contribution of the diurnally varying component could be calculated (See main text
577 for details).

578

579

580 **Figure 4:** Optical microscopy images of the fine-root anatomy of *A. japonica* trees. Transverse
581 sections were double stained with safranin-fast green to indicate roots in primary growth (A) and
582 secondary growth (B). Intercellular spaces (indicated by arrows) extended vertically in the cortex of a
583 longitudinal section double stained with safranin-fast green of a secondary growth root (C).

584

585 **Figure 5:** Optical and cryo-SEM images of fine roots of Japanese *A. japonica* trees. Transverse surface
586 of a root in primary growth observed by optical microscope (A) and cryo-SEM (B). Panel C–H are
587 magnified images corresponding to the rectangle-denoted areas in panel B. Large, empty (i.e., no
588 water) intercellular spaces were observed in the cortex (C, D). Intercellular spaces (arrows) were small
589 and filled with water (frozen) in the endodermis (E, F). All parenchyma cells were filled with water,
590 whereas some xylem cells were empty (i.e., no water) in the vascular cylinder (G, H).

591

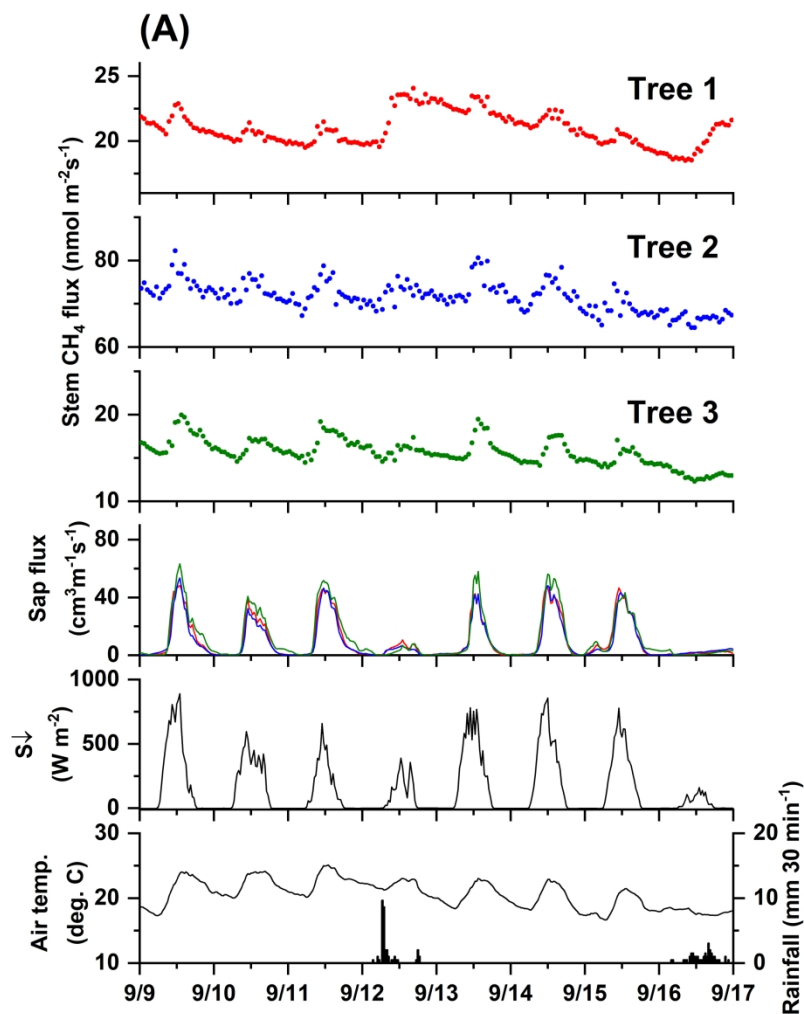


Fig. 1A

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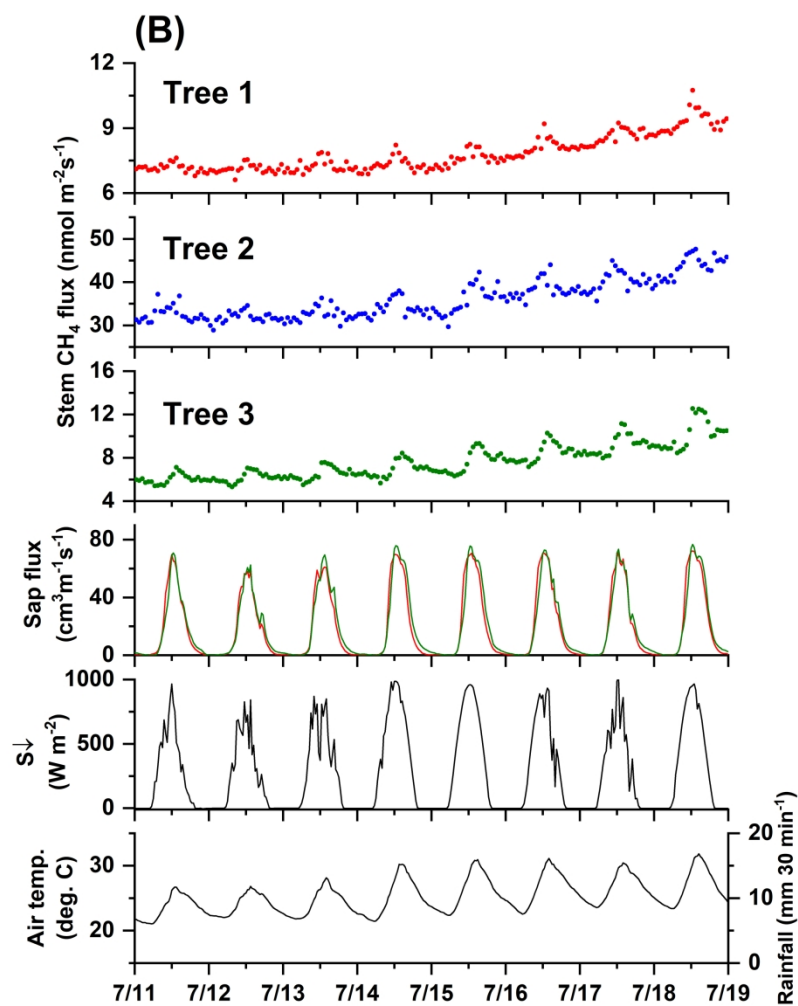


Fig. 1B

209x296mm (300 x 300 DPI)

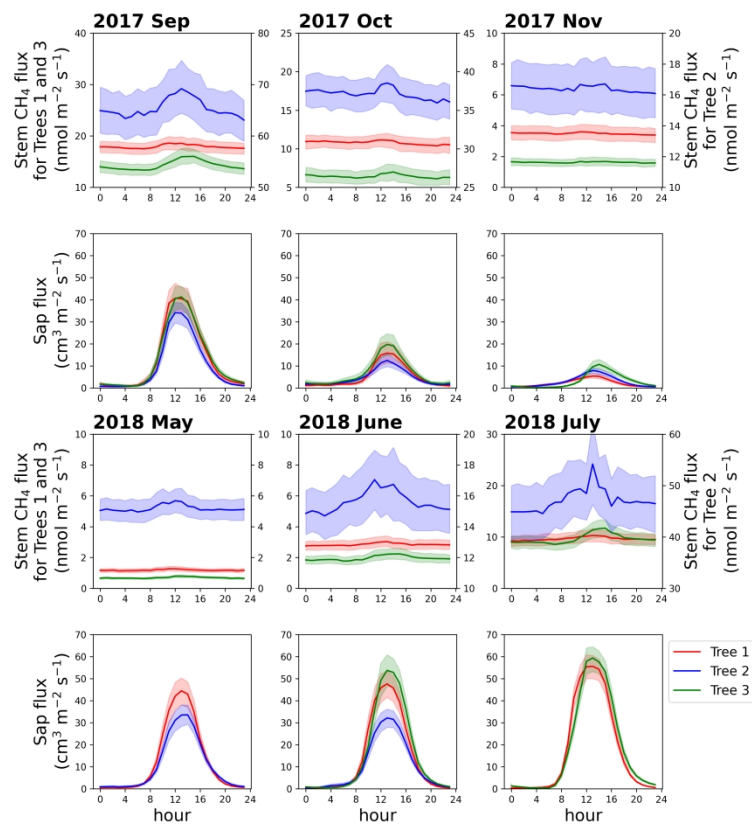


Fig. 2

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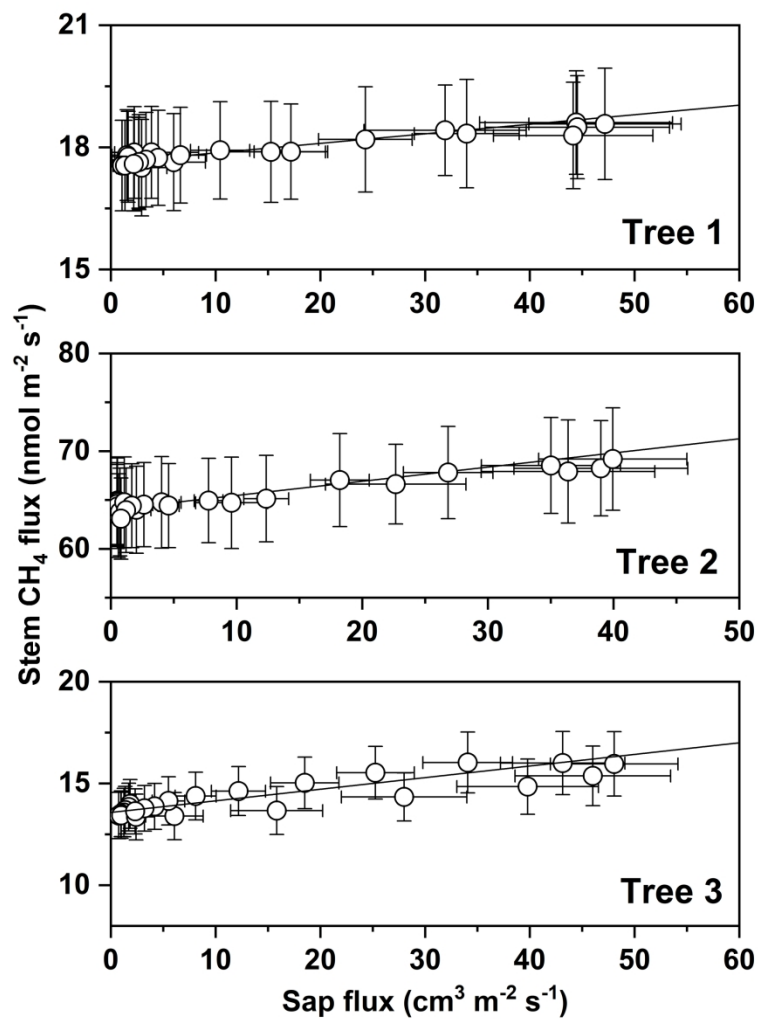


Fig 3

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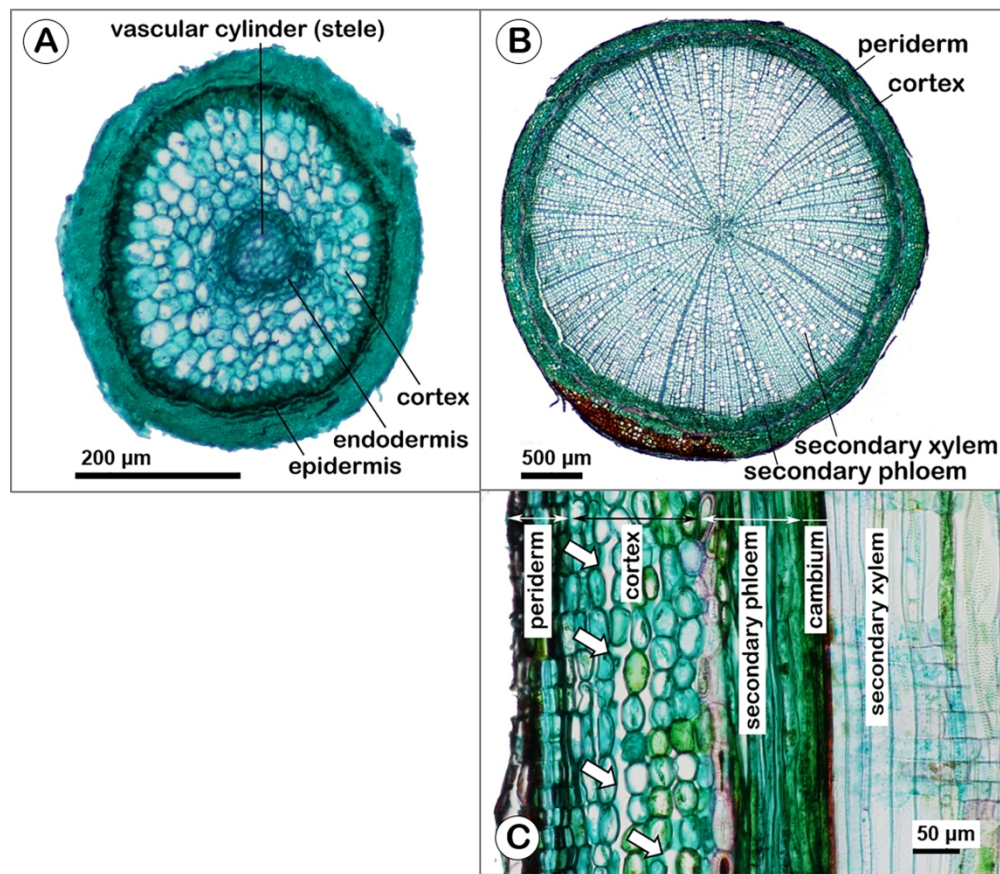


Fig. 4

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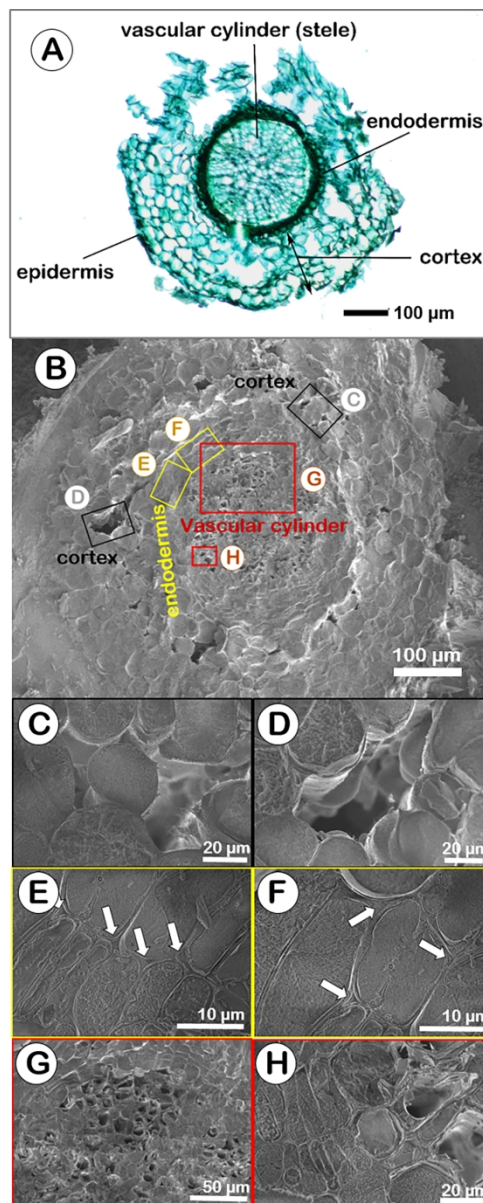


Fig. 5

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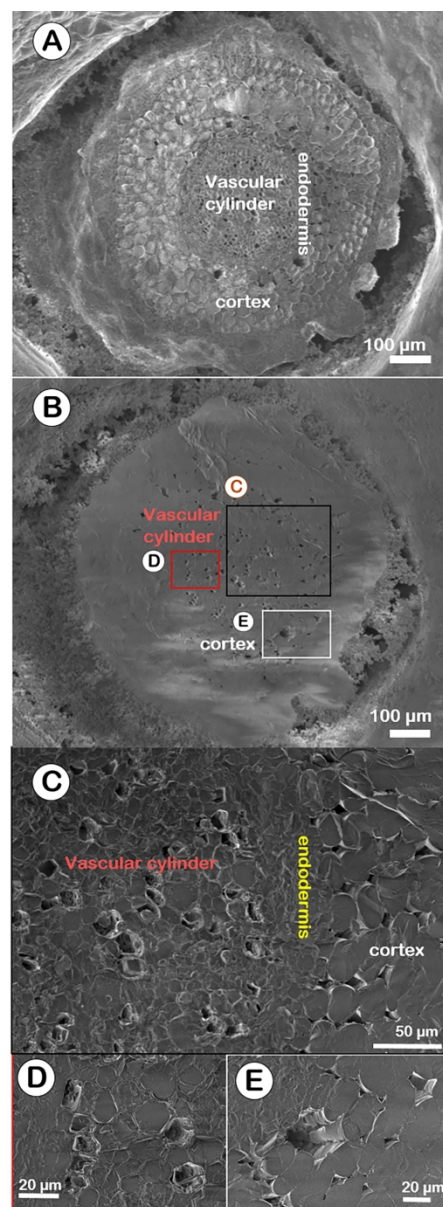


Fig. S1

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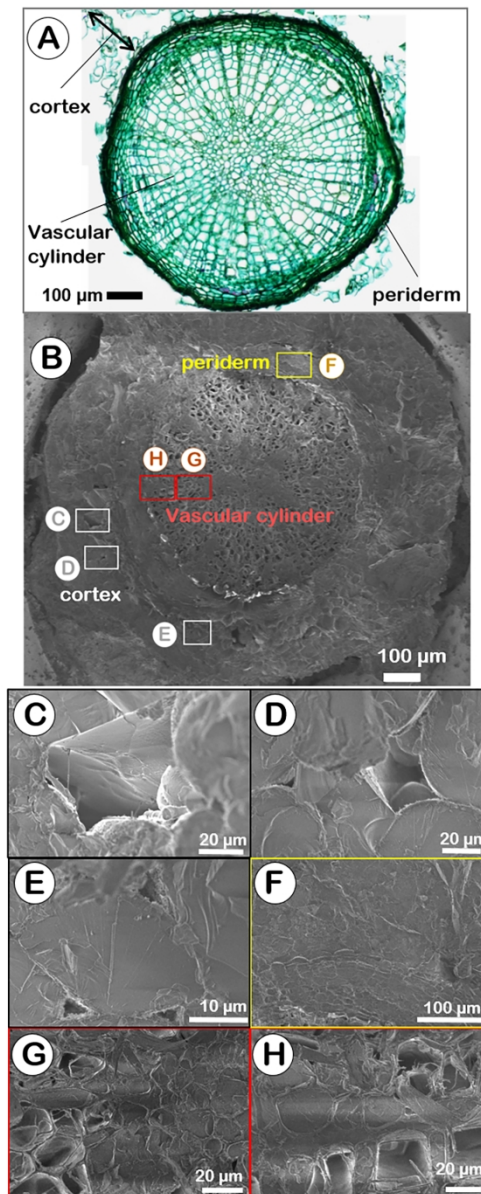


Fig. S2

98x234mm (300 x 300 DPI)