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Title: Chemiluminescence-based quantification of the colonization rates of *Lotus japonicus* roots by arbuscular mycorrhizal fungi

Running title: Chemiluminescent detection of mycorrhiza

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Abstract

Simple evaluation of the levels of root colonization by arbuscular mycorrhizal (AM) fungi is challenging for conventional histochemical staining because most roots show an endogenous background signal. Herein, we report a chemiluminescence-based novel method for the quantification of the relative colonization rates of AM fungi. *Lotus japonicus* seedlings were grown in two types of soils with different inoculum potential [i.e. the former vegetation was maize (host) or buckwheat (non-host)]. Indigenous AM fungi colonizing roots were labelled with wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP), which specifically targets the *N*-acetylglucosamine polymers of fungal cell walls. After the roots were removed from the soil and rinsed with water, all procedures were conducted in 24-well plates. AM fungi were detected by chemiluminescence in the presence of enhanced chemiluminescence (ECL) reagents, and the signal strengths were measured by image analyses. Post-staining of AM fungal structures in the presence of 3,3'-diaminobenzidine (DAB) and H_2O_2 (DAB staining) revealed that even small colonization events (e.g. hyphopodia) could be detected, whereas the background signals observed in DAB staining were eliminated in the chemiluminescence method. Chemiluminescence thus provides both high sensitivity and high specificity. The chemiluminescence-based colonization levels of roots grown in maize soil were significantly higher than those of roots grown in buckwheat soil, demonstrating the feasibility for high-throughput quantification of the relative

colonization rates of *L. japonicus* seedlings by AM fungi.

Keywords

arbuscular mycorrhizas; 3,3'-Diaminobenzidine (DAB); enhanced chemiluminescence (ECL); horse radish peroxidase (HRP); WGA (wheat germ agglutinin)

1 Introduction

Arbuscular mycorrhizal (AM) fungi have been regarded as a natural bio-fertilizer that helps crops to take up phosphate, nitrogen, and other nutrients from soils. Cultivation of AM host crops increased the amount of propagules of indigenous AM fungi (e.g. spores, colonized roots, or hyphal networks) in soils, which may enhance the uptake of nutrients by succeeding crops and permit a decrease in the use of fertilizers (Karasawa 2004; Oka *et al.* 2010). However, the cultivation of non-host crops (e.g. Brassicaceae or Amaranthaceae) or conventional field management methods, including bare fallow, fumigation, excess fertilization, or intense tillage, may decrease the biomass of AM fungi in soils (Smith and Read 2008). Moreover, the productivity of AM propagules fluctuates depending on the vegetation type, light intensity, geography and the physical, chemical and biotic properties of the soil (Oehl *et al.* 2010; Jansa *et al.* 2014). Thus, evaluating the level of AM fungal propagules (i.e. the inoculation potential of AM fungi) in soils before cultivation is important to maximize the benefit of this symbiosis for low input and sustainable agriculture.

Considering that strict host specificity has not been observed in AM symbiosis, the inoculum potential of field soils can be assessed by analyzing the colonization level of 'bait' plants via pot culture. In such analyses, the complexity as well as time and labour

expenses are the main bottlenecks hindering high-throughput evaluation. For example, conventional staining methods for the evaluation of colonization levels of roots using trypan blue or ink often produce high background staining of cortical cells or parts of the stele (Dodd and Jeffries 1986). Fluorescence-labelled wheat germ agglutinin (WGA) specifically targets fungal cell walls and provides excellent high-contrast images; however, a stele often has an autofluorescence that is not easily distinguishable from AM fungal colonization under low magnification. Therefore, in general, these methods require the preparation of specimens and observation under high magnification (McGonigle *et al*. 1990). We previously reported that the application of WGA-conjugated horseradish peroxidase (HRP) with 3,3'-diaminobenzidine (DAB) (WGA-HRP-DAB staining) successfully excludes the background signals of cortex and steles (Kobae and Ohtomo 2015). This method enables the identification of AM fungal structures in roots with extremely high contrast and simple bright-field imaging that can be performed with bright-field stereomicroscopy at only 15× magnification (Kobae and Ohtomo 2015). However, rhizosphere materials with brownish color often adhere to roots removed from some soils and are difficult to distinguish from DAB staining under low magnification.

Here, we describe a chemiluminescence-based method for detecting the colonization of AM fungi in the roots of *Lotus japonicus* grown in a small-scale cultivation system. Roots were cleared and labelled with WGA-HRP in 24-well plates, and the luminescence of oxidized enhanced chemiluminescence (ECL) reagents was detected by a cooled charge-coupled device (CCD) optical imaging system. This method enables a simple, high-sensitivity, high-throughput evaluation of the AM-inoculum potential of field soils.

2 Materials and Methods

Plant materials and sample preparation

To avoid the secondary effects of nodulation on the level of AM colonization, AM+/nod– *nfr1* mutant of the Gifu ecotype of *L. japonicus* (Kawaguchi *et al.* 2002; Sandal *et al.* 2006) was used as bait plant. Seeds were scarified and sterilized with a sodium hypochlorite solution, rinsed with sterile water, and allowed to germinate under sterile conditions. Three-day-old seedlings were grown singly in 12-mL polypropylene tube. The soil consists of 7-mL (bottom layer) of Ezo sand (small pumice), and sample soil (to the brim). Two types of soils with different inoculum potential [i.e. former vegetation was maize (host) or buckwheat (non-host)] from the farm fields (43º00′30″N, 141º24′25″E) of Hokkaido Agricultural Research Center (Japan). The bottom of the tubes had cross slits in which filter paper was attached (**Supplementary Figure 1A**). Tubes were placed in test tube rack, which was placed in a flat-bottom tray, and arranged randomly in a growth cabinet under 15-h-light/9-h-dark photoperiods (25°C/23°C) (**Supplementary Figure 1B**). No nutrients were added, and water was supplied from the bottom.

Chemiluminescent detection of fungal cell wall using WGA- HRP

Plants were harvested at 14 day post plantation (dpp). Roots were severed from plants, washed with water to remove soil. Following procedures were performed in 24-well plates. Solutions were changed with gentle pipetting. Severed roots were immediately immersed in 50% (v/v) ethanol and placed at room temperature for 3 h. Roots were cleared with 20% (w/v) KOH for 2 days at room temperature and then rinsed five times with water and once with phosphate-buffered saline (PBS) (pH 7.5). Roots

were then immersed in 1-mL PBS containing 1% (w/v) bovine serum albumin (Wako, Osaka, Japan) and 0.4 μg/ml WGA-HRP (Vector, Burlingame, CA, USA). Roots were kept in this solution for more than 16 h at room temperature before being rinsed twice with PBS and then immersed in 1-mL Amersham ECL reagent (GE, Chicago, IL, USA) for 10 min at room temperature. Before imaging, ECL reagent was removed to settle the roots at the bottom. Chemiluminescence was detected using an ImageQuant LAS4000mini (GE, Chicago, IL, USA). Images were saved as 8-bit grayscale TIFF format with standard resolution. For post staining with DAB, roots were gently rinsed one with PBS and then immersed in 1-mL PBS containing 0.2 mg mL^{-1} DAB (Nakarai Tesque, Kyoto, Japan) and 0.1 μL mL^{-1} 30% H_2O_2. The roots were kept in the DAB solution for 1 h at room temperature and then soaked in Tris–ethylene diamine tetra acetic acid (EDTA) (TE) buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8.0) to stop the HRP reaction. Images were obtained using a Nikon stereomicroscope (SMZ800) equipped with a CCD camera. The lengths of colonized regions and the signal strengths were measured using ImageJ (http://imagej.nih.gov/ij/). Chemiluminescence signal strengths in wells were determined within selected area without thresholding option.

3 Results and Discussion

We previously established a WGA-HRP-DAB staining method for the bright-field imaging of AM fungal mycelia in roots of diverse plant species (Kobae and Ohtomo 2015). This staining method is high contrast and low background and allows the recognition of symbiotic structures of AM fungi under low microscopic magnification. Despite the low background signals in roots, rhizosphere soils or adhering matter are often observed on the root surface as unwanted background signal. These sticky adhesions are

145 difficult to remove by sonication and were distinguished from AM fungal colonization
146 only by observation under high magnification. Thus, in this study, the detection method
147 was improved. ECL reagents have been well used as the substrate of HRP in Western
148 blotting analysis, and many enhanced versions of similar products are commercially
149 available. WGA-HRP specifically targets the GlcNAc polymer of the fungal cell wall *in*
150 *situ* (Kobae and Ohtomo 2015). HRP then oxidizes ECL to emit strong luminescence
151 (**Figure 1**).

152 In a small-scale cultivation system of *L. japonicus* (**supplementary Figure 1**),
153 DAB staining of mycorrhizal roots indicated that initial colonization occurred at 8 dpp
154 and the number of colonized regions increased at least up to 16 dpp in roots grown in
155 maize soil (**Supplementary Figure 2A**), while the maximum expansion of colonized
156 regions was observed at 12–14 dpp (**Supplementary Figure 2B**). The roots at 14 dpp
157 were used for chemiluminescence-based evaluation. At this time point, the luminescent
158 signal strength of roots grown in maize soils was significantly higher than that in
159 buckwheat soil (**Figures 2A, 2B, and 2E**), suggesting that this method could quantify the
160 relative mycorrhization rates. To check whether chemiluminescence accurately reflected
161 the presence of AM fungal hyphae, ECL detection was followed by DAB staining. Double
162 detection revealed that the chemiluminescence-based method was able to identify
163 extremely small colonization events (e.g. hyphopodia) as well as heavily colonized
164 regions as a strong luminescence (**Figures 2A, 2B, 2C, and 2D**), even at low resolution
165 (**Supplementary Figure 3**). No AM fungal colonization was observed in roots of
166 buckwheat plants. However, it should be noted that the bases of hypocotyls were often
167 heavily infected by non-AM fungi, which were also detected as strong luminescent
168 signals (**Figures 2A, 2B; Supplementary Figure 3**). Therefore, removal of the hypocotyl

169 is strongly recommended for accurate evaluation of the colonization rate. Unfortunately,
170 this method is not able to distinguish the signals of non-AM fungi from those of AM fungi.
171 Although healthy *L. japonicus* roots were rarely infected by fungi except AM fungi at
172 least up to 3 weeks in this cultivation system, we recommend that the presence of
173 intraradical AM structures (e.g. arbuscules) is confirmed by ECL/DAB double staining to
174 avoid the potential risk of the overestimation of AM colonization levels.

175 **Figure 3A** shows that DAB staining could not exclude the background signals
176 of rhizosphere soils, and furthermore, the background signal was much higher than the
177 true signal from AM fungal colonization. Although DAB staining had low background
178 inside roots; this external background could lead to the overestimation of colonization
179 level under low magnification in a high-throughput analysis. In contrast,
180 chemiluminescence-based detection successfully eliminated the background signals from
181 rhizosphere soils (**Figure 3B**), suggesting that this method is more suitable for high-
182 throughput analysis than DAB staining.

183 In this report, we described a novel method for a simple, high-specificity, high-
184 throughput evaluation of the AM inoculum potential of soils. This method provides the
185 relative colonization rates of roots grown in field soils that have different levels of AM
186 inoculum potentials (e.g. presence or absence of host plant in previous cropping), but are
187 not able to provide the absolute quantification of colonization levels. For example,
188 overlapped roots in a well may lead to the underestimates of colonization levels. Thus,
189 this method is useful for the comparative analysis among multiple soil samples that have
190 different inoculum potentials. In the field, the inoculum potential of AM fungi in soils
191 fluctuates depending not only on vegetation type but also by year and by field
192 management (Karasawa 2004). In addition, remote sensing technologies have revealed

heterogeneity in fertility and crop yield within a single field and suggested the importance of diagnostic information for the potential productivity of soils (Mulla 2013). Thus, accurate evaluation of the inoculum potential of AM fungi requires an analysis of numerous soil samples within a single field. The novel method presented in this study will be useful for the high-throughput evaluation of AM inoculum potential.

Acknowledgments

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Disclosure statement

Conflicts of interest: No conflicts of interest declared.

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Figure legends

Figure 1 Schematic drawing of chemiluminescent detection of fungal cell wall

WGA, wheat germ agglutinin; HRP, horseradish peroxidase; ECL, Enhanced chemiluminescence; GlcNAc, *N*-acetylglucosamine

Figure 2 Quantification of relative colonization rates of *Lotus japonicus* roots by indigenous AM fungi

(A) Chemiluminescent images of *L. japonicus* mycorrhizal roots. Plants were grown in soils of which the former vegetation was maize (left) or buckwheat (right). (B) ImageJ thresholded images of (A). Arrows indicate the unwanted infection by non-AM fungi. The signal strength within black-dotted area were measured by imageJ. (C) Image of WGA-HRP-DAB staining. Magnified image of the white-dotted area in (A). (D) Magnified image of the black-dotted area in (C). (E) Mean signal strength of chemiluminescent in (B). Data are given as means±standard deviation. ** $P < 0.01$, Welch's *t* test (maize versus buckwheat). a.u., arbitrary unit. Bar = 1 cm (A and B).

Figure 3 Background signals from rhizosphere materials were eliminated in chemiluminescent detection system

(A) Inverted grayscale image of DAB staining (bright-field image) of *L. japonicus* roots colonized by indigenous AM fungi. Signal intensity profile was quantified along a transect including a colonized region and rhizosphere materials (RM), indicated by white dotted lines. (B) Chemiluminescent image of (A) in the presence of ECL reagent. Plot profiles for dotted line in (A) and (B) are shown in (C) and (D), respectively. a.u., arbitrary unit. Bar = 2 mm (A and B).

Supporting information legends

Supplementary Figure 1 Small scale cultivation system of *L. japonicus*

(A) Cross slits were put at the bottom of polypropylene tubes, in which filter paper was inserted. (B) Twelve-mL tube cultivation system.

Supplementary Figure 2 Development of AM colonization in small tube cultivation system of *L. japonicus*

(A) Number of colonized region per plant. (B) Mean length of colonized region (n=2–18).

Supplementary Figure 3 Chemiluminescent detection of AM colonization in *L. japonicus* roots

(A) Chemiluminescent image of *L. japonicus* mycorrhizal root. Zoom images of DAB staining of boxed area 1 and 2 in (A) were shown in (B) and (C), respectively. Arrowhead in (B) indicate the position of small colonization (hyphopodium). Arrow in (C) indicate the infection of non-AM fungi.

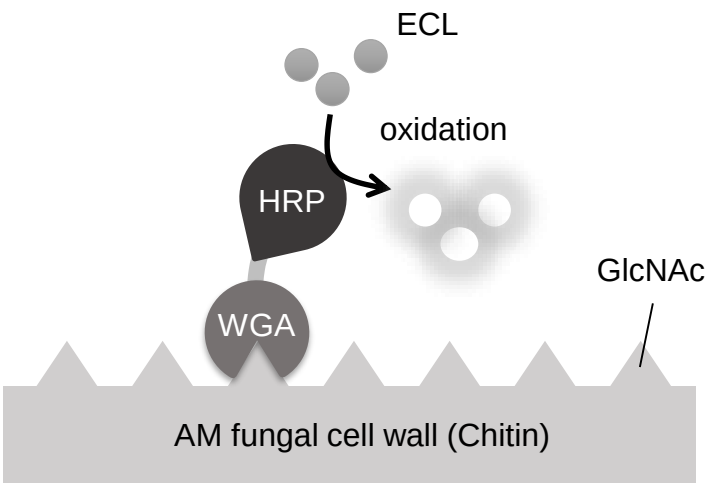

Figure 1
Schematic drawing of chemiluminescent detection of fungal cell wall
WGA, wheat germ agglutinin; HRP, horseradish peroxidase; ECL, Enhanced chemiluminescence; GlcNAc, *N*-acetylglucosamine

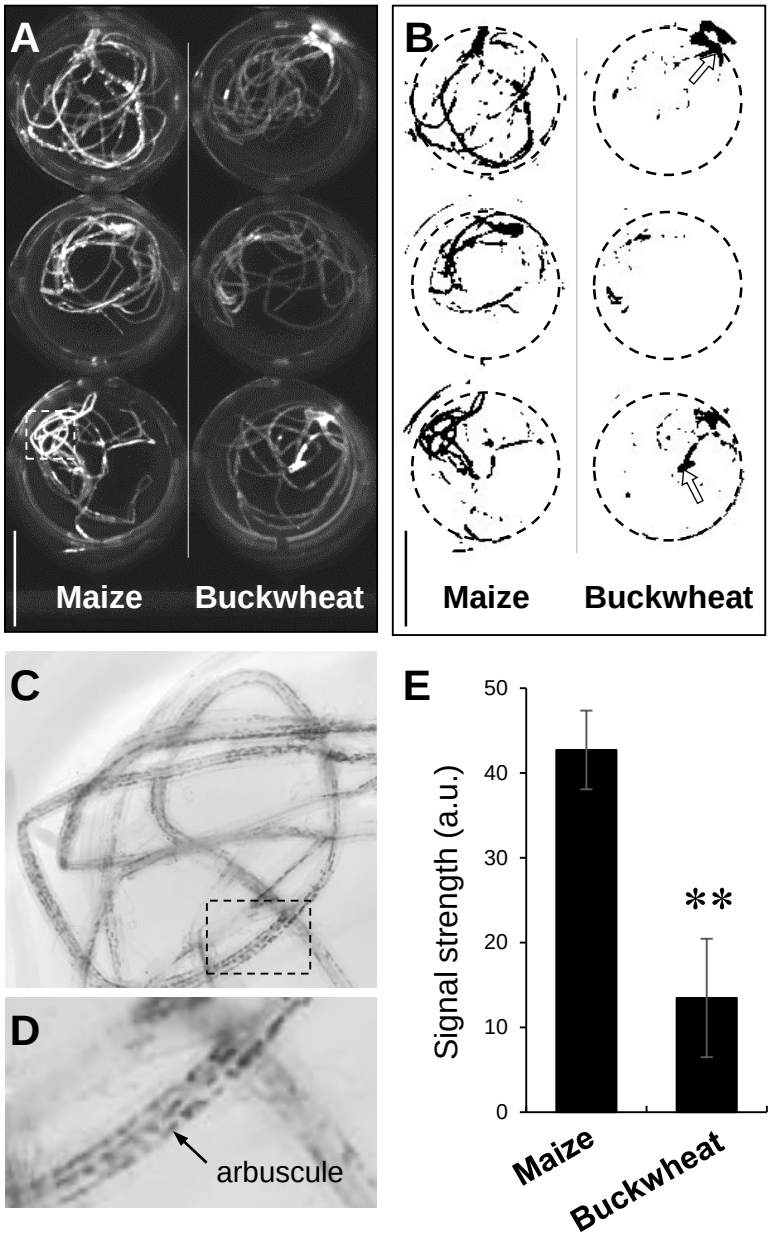

Figure 2
Quantification of relative colonization rates of *Lotus japonicus* roots by indigenous AM fungi
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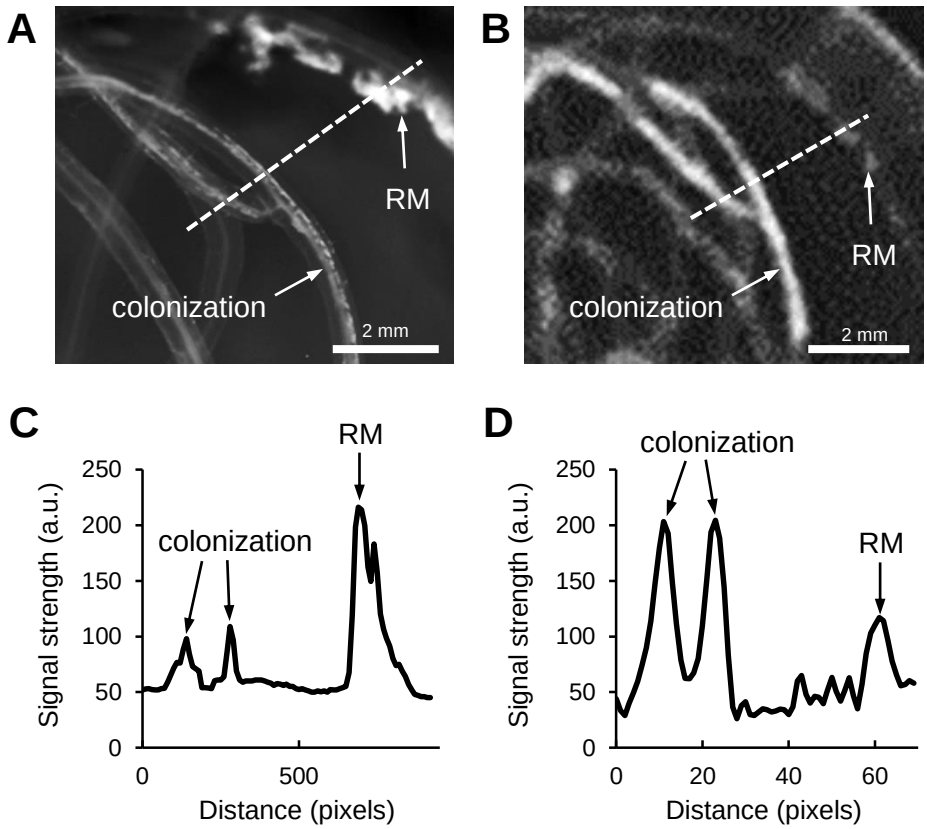

Figure 3

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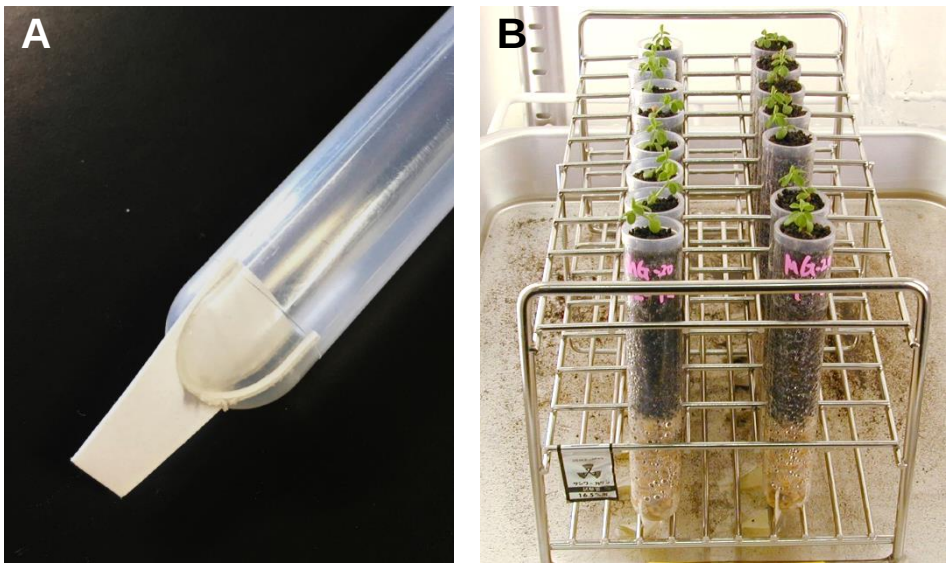

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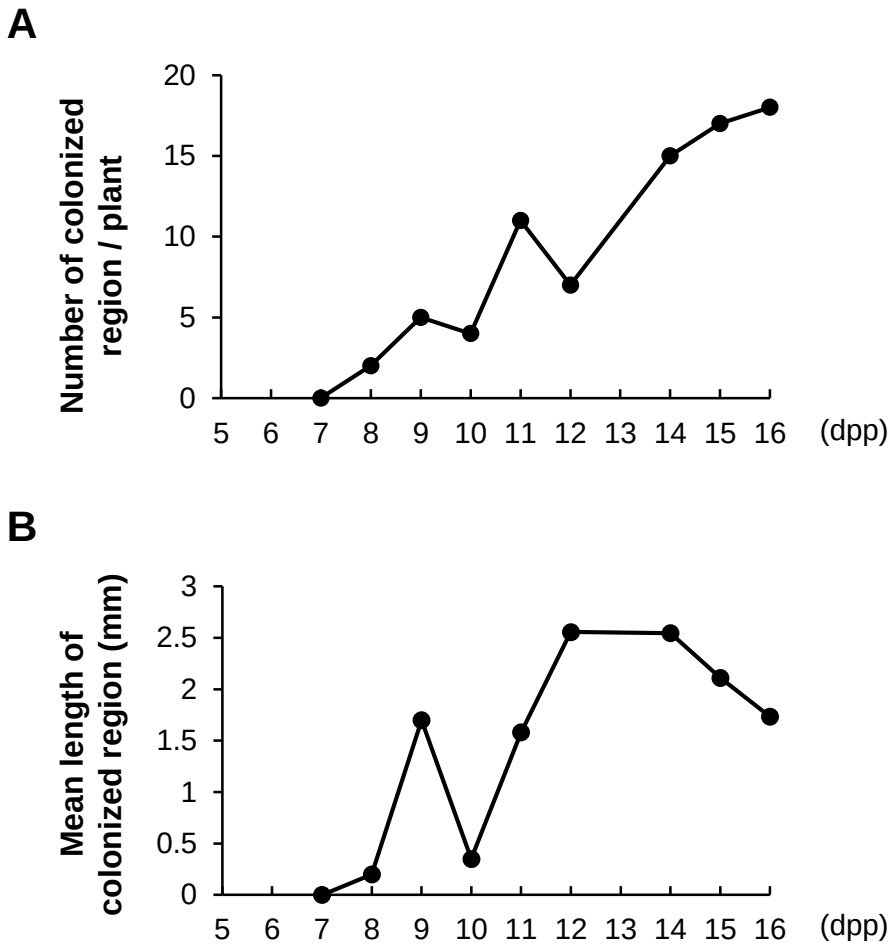

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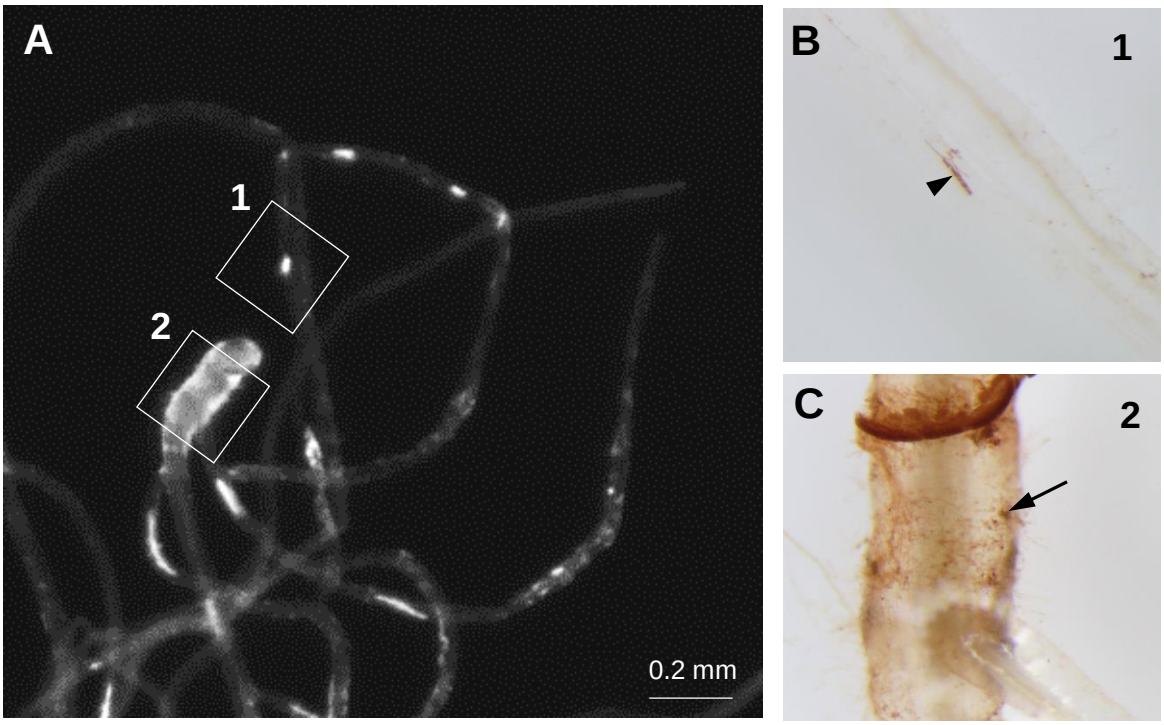

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