Microbial Diversity in the Edible Gall on White Bamboo Formed by the Interaction between *Ustilago esculenta* and *Zizania latifolia*

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Abstract

An edible gall is formed between the third and fourth nodes beneath the apical meristem near the base of *Zizania latifolia* shoots. This gall is harbored by and interacts with the smut fungus *Ustilago esculenta*. The gall is also a valuable vegetable called "white bamboo", "*jiaobai*" or "*gausun*" in China and "*makomotake*" in Japan. Five samples of the galls harvested at different stages of swelling were used to isolate microorganisms by culturing. Isolated fungal and bacterial colonies were identified by DNA sequencing and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS), respectively. Several strains of *U. esculenta* as well as 6 other species of fungi and 10 species of bacteria were isolated. The microbiome was also evaluated by simple and outlined DNA profiling with automated rRNA intergenic spacer analysis (ARISA), and the amount of DNA of *U. esculenta* was determined by qPCR. At least 16 species of fungi and 40 species of bacteria were confirmed by ARISA of the overall sample. Interestingly, the greatest bacterial diversity, i.e., 18 species, was observed in the most mature sample, whereas the fungal diversity observed in this sample, i.e., 4 species, was rather poor. Based on qPCR, *U. esculenta* occurred in samples from all stages; however, the abundance of *U. esculenta* exhibited unique U-shaped relationships with growth. These results may explain why the interaction between *U. esculenta* and *Z. latifolia* also influences the unique microbial diversity observed throughout the growth stages of the swollen shoot, although the limited sample size does not allow conclusive findings.

Keywords: endophyte, microbiome, ARISA, qPCR

Introduction

Various plant-fungal interactions are known, ranging from symbiosis to parasitism [8, 9]. The edible gall of *Zizania latifolia*, traditionally and widely used as a vegetable in the Orient, is induced by *Ustilago esculenta* harbored in the plant stem. Morphological and histological observations have shown that the hyphae of *U. esculenta* are mostly confined to the parenchyma and distributed systemically throughout the stem tissues, whereas no hyphae were found in leaf and root tissues [1, 13, 16]. The hyphae are especially abundant in the apical meristem of young stems, and they intra- and intercellularly colonize most vegetative tissues except for the leaves and roots [16]. In other studies, fungal hyphae were found mainly in the upper swollen parts, the nodal regions of mature culms and old rhizomes and buds or shoots, but they were rare in the internodes of mature culms and at a low abundance in the internodes of old rhizomes [7, 19]. A rapid increase in hyphal aggregations occurs before culm swelling, and sori and teliospores form subsequently in the swelling tissues [20]. Different strains of *U. esculenta*, namely, sporidial (T) and mycelial (M-T) strains, were isolated from sporulating and nonsporulating galls, respectively, and showed distinctive morphological and molecular features [18, 21].

The nutritional requirements of the fungus [3] and distribution of the plant growth regulator indole-3-acetic acid were also investigated throughout the growth stages of the edible gall [2]. Recent studies have focused primarily on the detailed mechanism of edible gall formation caused by the plant-fungus interaction with whole-genome [5, 16] or RNA expression [14] analysis. These studies identified a number of fungal and host candidate genes with DNA sequence losses/mutations or RNA expression changes. However, research has rarely focused on microbial diversity except for *U. esculenta* with or without a causal relationship throughout the process of the gall formation of *Z.*

latifolia. In addition, there are many studies on the amounts of *U. esculenta* based on semiquantitative analyses during the enlargement process [1, 12, 15]; however, a precise quantitative evaluation has not yet been reported. Therefore, we focused on microbial diversity and variation in *U. esculenta* abundance throughout the growth stages of the edible gall based on a culture and DNA analysis approach. The DNA-based microbiome was evaluated by automated rRNA intergenic spacer analysis (ARISA) [10, 11], and the amount of *U. esculenta* DNA was determined by qPCR.

Materials and Methods

Samples

Fresh edible galls were harvested at Ito Noen, Komono-cho, Mie Prefecture, in September 2014 and 2015 (Fig. 1). Samples collected during different stages of swelling from smaller (Nos. 1 and 2; tender) to larger (No. 5; aged) shoots were used, in which Nos. 3 and 4 (mature) were the optimal harvest times (Fig. 2). The approximate length of each sample was 15 cm for No. 1, 20 cm for No. 2, 25 cm for No. 3 and 30 cm for Nos. 4 to 5. The *Z. latifolia* host was single-season crop that was harvested once a year [4]. The gall is formed between the third and fourth nodes beneath the apical meristem near the base of the *Zizania latifolia* shoot [2].

Sample treatment

Edible parts of the five samples at different degrees of swelling from which the sheath had been removed were cut into pieces and homogenized using an Ika T-10 Basic Ultra homogenizer (Cole-Parmer, IL, USA) sanitized with 70% ethanol (Fig. 2A). The sample of No. 5 (aged) was blackened since *U. esculenta* had initiated the formation of

many black spores (Fig. 2B). A total of 10 g of the homogenate was placed in a sterile plastic bag, combined with 90 mL of sterile saline, and then mixed by a shaker (Seward Medical, London, England). One milliliter of the original mixed solution was diluted by mixing 9 mL of sterile saline serially until final 1×10^5 dilution and summitted to culture isolation for microbes. DNA was extracted from the centrifugal residue (10,000 r/min for 10 min at 25° C) of 40 mL of the mixed solution for molecular analysis of microbial diversity.

Additionally, an edible gall individual at the optimal harvest stage was divided into 3 parts: top (apical side), middle (internode) and bottom (node) (Fig. 1B). The parts were blown dry with hot air and subsequently powdered by a pulverizer (Iwatani, Tokyo, Japan) to maintain efficiency and homogeneity of DNA extraction from the samples.

Microbe culture

The mixed-sample solutions were combined with saline, serially diluted and added to the following media for microbe culture.

Potato dextrose agar (PDA) medium

The fungi were cultured on PDA medium. A total of 39 g of PDA (E-MF21, Eiken Chemical, Tokyo, Japan) and 100 mg of chloramphenicol (Nacalai Tesque, Kyoto, Japan) were added to 1 L of distilled water, and the mixture was autoclaved at 121° C for 20 min to generate the medium plate (10 cmØ, 15-20 mL). After 0.2 mL of the diluted solution was spread per plate, the medium was cultured at 25° C for one week.

Standard agar plate (SAP) medium

The bacteria were cultured on SAP medium. A total of 24.5 g of agar (Eiken Chemical) was added to 1 L of distilled water, and the mixture was autoclaved at 121° C

for 20 min to generate the medium plate (10 cmØ, 15-20 mL). After 0.2 mL of the diluted solution was spread per plate, the medium was cultured at 37° C for one day.

Identification of fungal species by DNA analysis

DNA extraction

Colonies on the PDA plate were collected into bead-beating tubes from an ISOIL bead-beating kit (Nippon Gene, Atsugi, Japan). DNA was extracted according to the instructions of the provider. The details were as follows: 950 μL of lysis solution BB and 50 μL of lysis solution 20 s were added to the tube and pulverized by a cell crusher (Central Scientific Commerce, Tokyo, Japan) at 2,000 r/min for 3 min. After centrifugation at 15,000 r/min for 5 min at 25° C, 600 μL of the supernatant was transferred to a new tube and mixed with 400 μL of purification solution. Then, 600 μL of chloroform was added to the tubes, followed by vortexing for 15 sec and centrifugation at 15,000 r/min for 15 min at 25° C. After 800 μL of the supernatant was transferred to a new tube, 800 μL of precipitation solution was added and mixed in, and the tubes were centrifuged at 15,000 r/min for 15 min at 4° C. The supernatant was removed, and 1 mL of wash solution was added to the precipitate; after inversion, the mixture was centrifuged at 15,000 r/min for 10 min at 4° C. After the supernatant was removed, 1 mL of 70% ethanol and 2 μL of Ethachinmate were added into each tube. After mixing, the tubes were recentrifuged at 15,000 r/min for 5 min at 4° C. After the supernatant was removed, 100 μL of TE buffer (pH of 8.0) was added, the precipitate was dissolved, and the DNA solution was finally obtained.

PCR amplification

The DNA solution was used as a template for PCR amplification with primer pairs for fungal identification (Table 1). The 20 μL PCR solution was composed of injectable

water (14.1 μL), 10× Ex Taq buffer (2 μL), 2.5 mM dNTPs (1.6 μL), Ex Taq (0.1 μL) (Takara Bio, Kusatsu, Japan), primer mix (0.2 μL of 50 μmol/L forward and reverse primers) (Fasmac, Atsugi, Japan) and DNA template solution (2 μL). The amplification reaction included initial thermal denaturation at 94° C for 10 min, followed by 30 cycles of heat denaturation at 94° C for 30 sec, annealing at 55° C for 30 sec, and extension at 72° C for 30 sec (Bio-Rad, Hercules, USA).

Agarose gel electrophoresis

PCR products were confirmed for amplified fragment length polymorphisms by agarose gel electrophoresis. Two percent (w/v) agarose in 30 mL of 1× Tris acetate EDTA (TAE) buffer (pH of 7.8) was heated to melting, and a gel was prepared by adding 6 μL of 2.5 mg/mL ethidium bromide. The electrophoresis solution was composed of the same concentrations of TAE buffer and ethidium bromide as the agarose gel. The loading buffer contained 15 g of glycerin, 15 mg of bromophenol blue and 3 mL of 0.5 mol/L EDTA, all of which were dissolved in distilled water at a total volume of 50 mL. A total of 10 μL of PCR product was mixed with 2 μL of loading buffer, and 10 μL of each mixture was injected into a well of the gel in the electrophoresis solution. Ten microliters of size marker (50 μg/mL 100 bp DNA ladder) was also added. Electrophoresis was performed at 100 V for 20 min, and bands were confirmed by a UV gel imaging apparatus (Toyobo, Osaka, Japan).

PCR product clean-up

The PCR products were purified using a PCR clean-up kit (Takara Bio) according to the manufacturer's instructions. For the step in which NE3 buffer was added, the volume was changed from 700 μL to 650 μL to avoid overflow from the spin column. The details were as follows: 10 μL of PCR product was diluted 10-fold with injectable water, and 200 μL of NT1 buffer was added. Then, the mixed solution was transferred

to a spin column and centrifuged at 11,000 r/min for 1 min at 25° C, the liquid permeated through the column was removed, and 650 μL of NT3 buffer was added to the spin column. The column was centrifuged (11,000 r/min for 1 min at 25° C) again, and the permeated liquid was removed. This operation was repeated once more to completely remove the solution in the spin column. Then, a new tube was placed in the spin column, 30 μL of NE buffer was added to the column and allowed to stand for 1 min, and the tube was subsequently centrifuged (11,000 r/min for 1 min at 25° C). The resulting eluate was purified PCR product.

Nucleotide sequencing analysis

Forward or reverse PCR primers of Fun-5 (Table 1) were diluted to 1.6 pmol/L by TE buffer (pH of 8.0). Purified PCR products were diluted 10- or 20-fold with injectable water in accordance with the amount of targeted PCR product confirmed by agarose gel electrophoresis. The mixture totaling 14 μL, including the purified-diluted PCR products (10 μL) and primer (4 μL), was submitted for DNA sequencing (Fasmac). The sequenced data were applied in a BLAST homology search. The criterion for a species match was a concordance rate of 97% or greater.

Identification of bacterial species by MALDI-TOF MS

Sample preparation

Bacterial test standard (BTS) (Bruker Daltonics, Billerica, USA) and matrix solution (α-cyano-4-hydroxycinnamic acid, HCCA) (Bruker Daltonics)) were used as recommended by the provider. The colonies from the SAP culture were collected and smeared thinly, evenly and in duplicate on a polished steel target plate (Bruker Daltonics), and a 1-μL spot of BTS was added as the calibration standard. The plate was then air dried at room temperature. For each spot, 1 μL of HCCA was spotted

over the smears and air dried again.

Species identification

The dried spots were measured by MALDI-TOF MS with Flex Control software, and the obtained spectra were matched in parallel by MALDI Biotyper Real Time Classification (RTC) software (Bruker Daltonics) to those in the database. The highest score between every pair of duplicate spots was chosen. According to the manufacturer's instructions, matching scores of 2.30-3.00 were classified as "highly probable species", those of 2.00-2.29 were "secure genus, probable species", those of 1.70-1.99 were "probable genus identification", and those of 0.00-1.69 were "unreliable identification". In practice, scores of 2.00 or more are considered to indicate candidate species [12, 15].

ARISA of microbiota

Microbial analysis

The extracted DNA solutions were submitted to PCR amplification with a universal primer pair for fungi (Fun-1 labeled with HEX fluorescence) and a universal primer pair for bacteria (Bac-2 labeled with FAM fluorescence) in a separate tube (Table 1). The water-diluted primer concentration was 25 μmol/L in each tube. The number of PCR cycles was 35. After PCR amplification was confirmed by agarose gel electrophoresis, 10 μL of each PCR product was combined with Fun-1 and Bac-2. The 20 μL mixture was submitted to fragment analysis (Fasmac).

Species-specific fragments

The DNA solutions extracted from fungal and bacterial colonies for which the species had been identified were subjected to PCR amplification for ARISA under the same conditions as described above. The species-specific ARISA fragments obtained were

compared to those of the samples. The species were assumed to be the same if the size difference between the corresponding peaks was within 0.1% [12].

Quantitative analysis of *U. esculenta* by qPCR

The *U. esculenta*-specific primer pair, namely, qUED, targeting the D1/D2 region of 26S rDNA, was used for quantitative analysis by qPCR (Table 1). The DNA solutions were extracted from 10 mg of dried samples of edible gall with different degrees of swelling (Nos. 1-5) and from three different parts of edible gall (top, middle and bottom) (Figs. 1, 2). The calibration curve was obtained by using a dilution series of DNA extracted from 3×10^7 cells of *U. esculenta*. The cell number was determined by the counting method and McFarland turbidimetry. The qPCR reagent used was LightCycler FastStart DNA Master^{Plus} SYBR Green I (Roche, Basel, Switzerland). The total mixture was composed of 13.9 μL of H_2O, 4 μL of 5× Master Mix, 0.1 μL of each 25 μmol/L primer mix (Table 1) and 2 μL of DNA solution diluted 20-fold with injectable water. The PCR conditions were as follows: initial thermal denaturation at 95° C for 10 min, followed by 45 cycles of heat denaturation at 95° C for 10 sec, annealing at 55° C for 10 sec and extension at 72° C for 10 sec. The fluorescence reflecting the PCR process was measured in real time by a LightCycler 1.5 (Roche).

Data analysis

All experimental data were obtained by single measurement except for MALDI-TOF MS identification.

Results

Microbe culture

A large number of white or light yellow colonies were growing on the SAP medium after 24 hours of incubation at 37° C. Several colonies with different appearances in each plate were selected for species identification by MALDI-TOF MS. On the PDA medium at 25° C, some colonies with hyphae grew rapidly and covered the entire plate within 5 days. Conversely, other colonies grew slowly and remained very small even after 1 week. Fungal colonies with different appearances were also collected. These species were identified by DNA sequencing analysis.

Microbe identification

Seven species of fungi and 10 species of bacteria were isolated, including several strains of *U. esculenta* (Table 2). According to the sequencing results for fungi, *Sarocladium strictum* and *Cladosporium* sp. were identified from all five samples throughout the different stages of swelling. *Cryptococcus flavescens* and *Arthrinium phaeospermum* were found at the smallest stage (No. 1); *Arthrinium arundinis* in Nos. 2 and 3; *Microdochium* sp. in No. 4; and *U. esculenta* in Nos. 3 and 5. Concerning bacteria, *Arthrobacter woluwensis* and *Pantoea ananatis* were frequently isolated from 4 of the 5 samples. *Serratia marcescens*, *Xanthomonas* sp. and *Microbacterium testaceum* were identified only in No. 1; *Bacillus* sp. in No. 3; and *Acinetobacter nosocomialis* in No. 4. *Acidovorax* sp. was detected in Nos. 2 and 4; *Acinetobacter baumannii* in Nos. 3 and 4; and *Microbacterium* sp. in Nos. 2 and 5.

Species-specific fragment size in the ARISA

Different stages of swelling and sampled parts

The distribution of the sizes of fragments, which were amplified ITS regions of microbial DNA extracted from samples of different stages of swelling and parts, was

measured by ARISA (Figs. 3, 4). Blue bacterial peaks (FAM label) were detected in all of the samples; however, the numbers of bacterial peaks in larger samples and their signal intensities were distinctly higher than those in smaller samples. In contrast, the green fungal peaks (HEX label) were much less abundant and weaker, except for the largest peak corresponding to 757.72 bp (±0.1%) for Fun-1.

Isolated microbes

The isolated and identified microbes were submitted to ARISA, and each species-specific peak was obtained as a single major peak, except for *Serratia marcescens* (Table 3). The specific fragment size of *U. esculenta* was 757.72 bp for Fun-1, which was consistent with those observed in all samples with an average of 757.72±0.8 bp (Fig. 4). In addition, the fungi *Cladosporium* sp. in No. 1 and *Microdochium* sp. in No. 4 (Table 4) and the bacteria *P. ananatis*, *Xanthomonas* sp. and *Acidovorax* sp. in Nos. 4 and 5 were also identified based on their specific fragment sizes (Table 5).

Quantitative analysis of *U. esculenta*

The qPCR results also revealed a high abundance of *U. esculenta* in the swelling stems of *Z. latifolia* ranging from 10^5 to 10^8 cells/g of dried matter. The abundance of *U. esculenta* was much greater in smaller or larger samples among the different stages of swelling and greater in the top and bottom parts than in the middle part (Fig. 5). Thus, the abundance of *U. esculenta* presented unique U-shaped relationships with the stages of swelling and with the parts of the swollen shoot. The cell numbers in calibration curves were based on the spore count.

Discussion

U. esculenta interacts with *Z. latifolia* to form the well-known edible gall known as white bamboo in China. This study confirmed the high abundance of *U. esculenta* in the swollen shoots of *Z. latifolia* by quantitative analysis of the DNA of this fungus. The limited isolation of *U. esculenta* in only 2 samples may have been due to the small sample size and the number of fungal colonies in the culture. In addition, the greatest possible variety of colonies with different appearances were selected since the major purpose of this study was to estimate microbial diversity rather to evaluate the abundance of specific species such as *U. esculenta*, in which case fungi species with more rapid growth than that of *U. esculenta* would have been selected predominantly (Table 2). To confirm such complex interactions, it will be necessary to perform replicate measurements using both intra- and intersampling. Nevertheless, we also isolated 6 other species of fungi and 10 species of bacteria (Table 2), suggesting that the interactive microbes were diverse. ARISA identified many other microbes, especially bacteria, in the aged and spored gall of No. 5 samples (Fig. 3, Tables 4, 5). In addition, the larger gall samples Nos. 4 and 5 were found to contain more bacteria species and abundant *U. esculenta* (Figs. 3, 5). In other words, smaller gall samples Nos. 1 and 2 are protected from infection by or growth of microbes other than *U. esculenta*. However, whether the isolated fungi and bacteria are symbiotic microbes or not as well as whether they interact with each other require further verification. Here, we used a *Z. latifolia* cultivar used as a single-harvest crop in the fall. If another type of cultivar that can be harvested twice in the fall and once the following summer had been used, the microbial diversity might have been varied to some extent.

Quantitative analysis of *U. esculenta* revealed a U-shaped relationship with the stages of swelling and the different parts (top, middle and bottom) of the edible gall. *U. esculenta* accumulates substantially in young plant tissues such as the apical meristem

[1, 16, 19]. Subsequently, under the influence of *U. esculenta*, secreted indole-3-acetic acid stimulates the duplication of plant cells, and the plant stem expands rapidly. One hypothesis is that *U. esculenta* may continue to proliferate until the nutritional status of the plant becomes exhausted, and at this point, the fungus begins to produce large numbers of teliospores throughout the gall, as in the spored sample of No. 5. Otherwise, a plant-defense system may cause the formation of teliospores at the time of maturity, including the flowering stage.

In the different parts of the edible gall, *U. esculenta* accumulated considerably in the apical meristem (top), similar to observations in previous studies [1, 16, 19]. During the interaction between *Z. latifolia* and *U. esculenta*, indole-3-acetic acid is secreted and transmitted to the basal tissue of a preswollen shoot in the bottom part of the stem, leading to the greatest enlargement of the gall in the middle part. This secretion and movement of indole-3-acetic acid may be why the middle part of the shoot is larger than the bottom but contains fewer individuals of *U. esculenta*.

Conclusion

The results of this study obtained by microbial culture and DNA analyses revealed a very close interaction between *U. esculenta* and *Z. latifolia* growth and enlargement. However, whether the presence of the other microbes is beneficial or harmful to the growth of edible gall remains unknown. Furthermore, to improve the poor detection rate of *U. esculenta* in the culture method than in DNA-based ARISA, the culture conditions should be modified and focused on the specific detection of *U. esculenta* rather than reflecting a wider fungal spectrum.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflicts of interest.

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Table 1 PCR primers for ARISA and DNA sequencing analysis

Primer pair	Target	Forward Reverse	Fluorescence label	Literature
Fun-1	Fungal ITS	2234C: 5'-GTTTCCGTAGGTGAACCTGC 3126T: 5'-ATATGCTTAAGTTCAGCGGGT	- HEX	[8]
Fun-5	Fungal D1/D2	NL1: 5'-GCATATCAATAAGCGGAGGAAAAG NL4: 5'-GGTCCGTGTTTCAAGACGG	- -	[5]
Bac-2	Bacterial ITS	ITSF: 5'-GTCGTAACAAGGTAGCCGTA ITSReub: 5'-GCCAAGGCATCCACC	- FAM	[7]
qUED	Fungal D1/D2	UED-F: 5'-AATCCCAGGCCGCATCTCT UED-R: 5'-GACCGATAGCGAACAAGTACA	- -	This study

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Table 2 Microbial species isolated from different stages of swelling of the edible gall

	Species	Stages of swelling				
		1	2	3	4	5
Fungi	*Arthrinium arundinis*	-	Isolated	Isolated	-	-
	Arthrinium phaeospermum	Isolated	-	-	-	-
	Cladosporium sp.	Isolated	Isolated	Isolated	Isolated	Isolated
	Cryptococcus flavescens	Isolated	-	-	-	-
	Microdochium sp.	-	-	-	Isolated	-
	Sarocladium strictum	Isolated	Isolated	Isolated	Isolated	Isolated
	Ustilago esculenta	-	-	Isolated	-	Isolated
Bacteria	*Acidovorax* sp.	-	Isolated	-	Isolated	-
	Acinetobacter baumannii	-	-	Isolated	Isolated	-
	Acinetobacter nosocomialis	-	-	-	Isolated	-
	Arthrobacter woluwensis	Isolated	Isolated	Isolated	-	Isolated
	Bacillus sp.	-	-	Isolated	-	-
	Microbacterium sp.	-	Isolated	-	-	Isolated
	Microbacterium testaceum	Isolated	-	-	-	-
	Pantoea ananatis	-	Isolated	Isolated	Isolated	Isolated
	Serratia marcescens	Isolated	-	-	-	-
	Xanthomonas sp.	Isolated	-	-	-	-

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Table 3 Species-specific fragment size in the ARISA of isolated microbes

	Species	Species-specific fragment size (bp)	
		Fun-1	Bac-2
Fungi	*Arthrinium arundinis*	562.23	-
	Arthrinium phaeospermum	591.98	-
	Cladosporium sp.	530.63	-
	Cryptococcus flavescens	512.84	-
	Microdochium sp.	537.75	-
	Sarocladium strictum	560.32	-
	Ustilago esculenta	757.72	-
Bacteria	*Acidovorax* sp.	-	711.03
	Acinetobacter baumannii	-	700.39
	Acinetobacter nosocomialis	-	700.33
	Arthrobacter woluwensis	-	603.56
	Bacillus sp.	-	306.78
	Microbacterium sp.	-	495.17
	Microbacterium testaceum	-	491.71
	Pantoea ananatis	-	457.66
	Serratia marcescens	-	-
	Xanthomonas sp.	-	556.22

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Table 4 Fungal fragment sizes amplified with the primer pair Fun-1 in the ARISA of samples of edible galls at different stages of swelling

Fragment size (bp) detected at different stages of swelling					
1	2	3	4	5	Species identified
130.51	-	130.58	-	-	-
-	-	-	-	164.51	-
-	-	218.46	-	-	-
-	260.08	260	-	-	-
345.57	-	345.43	-	-	-
-	-	367.2	-	-	-
379.22	379.23	379.3	-	379.31	-
390.42	-	-	-	-	-
-	-	-	-	391.2	-
457.62	-	-	-	-	-
514.38	-	-	-	-	-
530.15	-	-	-	-	*Cladosporium* sp.
-	-	-	537.68	-	*Microdochium* sp.
-	-	673.25	-	-	-
756.75	756.7	756.73	756.73	-	-
757.52	757.54	757.59	757.50	757.49	*Ustilago esculenta*

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Table 5 Bacterial fragment sizes amplified with the primer pair Bac-2 in the ARISA of samples of edible galls at different stages of swelling

Fragment size (bp) detected at different stages of swelling					
1	2	3	4	5	Species identified
-	-	-	-	138.21	-
-	205.92	-	-	-	-
-	-	226.78	-	-	-
-	-	287.53	-	-	-
292.41	-	-	-	-	-
-	-	-	-	310.51	-
-	-	-	-	330.67	-
345.73	-	-	-	-	-
-	-	356.69	-	-	-
380.86	-	-	-	-	-
-	-	-	-	393.38	-
-	-	-	457.67	457.52	*Pantoea ananatis*
-	-	-	458.84	-	-
-	-	-	-	465.1	-
-	-	-	-	483.1	-
-	-	-	-	485.19	-
-	-	-	486.35	-	-
-	542.53	-	-	-	-
-	-	-	556.56	556.71	*Xanthomonas* sp.
559.69	-	-	-	-	-
-	569.52	-	-	-	-
-	-	-	-	591.36	-
-	-	644.25	-	-	-
-	-	-	-	650.21	-
-	-	-	-	678.19	-
-	679.27	-	679.43	-	-
-	-	-	-	680.66	-
-	-	-	693.64	-	-
-	-	700.73	-	-	-
702.1	-	-	-	-	-
707.13	-	-	-	-	-
-	-	-	711.34	711.43	*Acidovorax* sp.
-	-	-	-	755.93	-
-	-	-	776.52	-	-
-	-	-	799.47	-	-
-	-	-	850	-	-
992.89	-	-	-	-	-
-	-	-	-	1131.41	-
-	-	-	-	1149.6	-
-	-	-	-	1190.96	-

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Figure captions

Fig. 1 Mature edible gall samples harvested from farmland, Ito Noen (A), and divided into 3 parts (top, middle and bottom) after removal of the sheath (B).

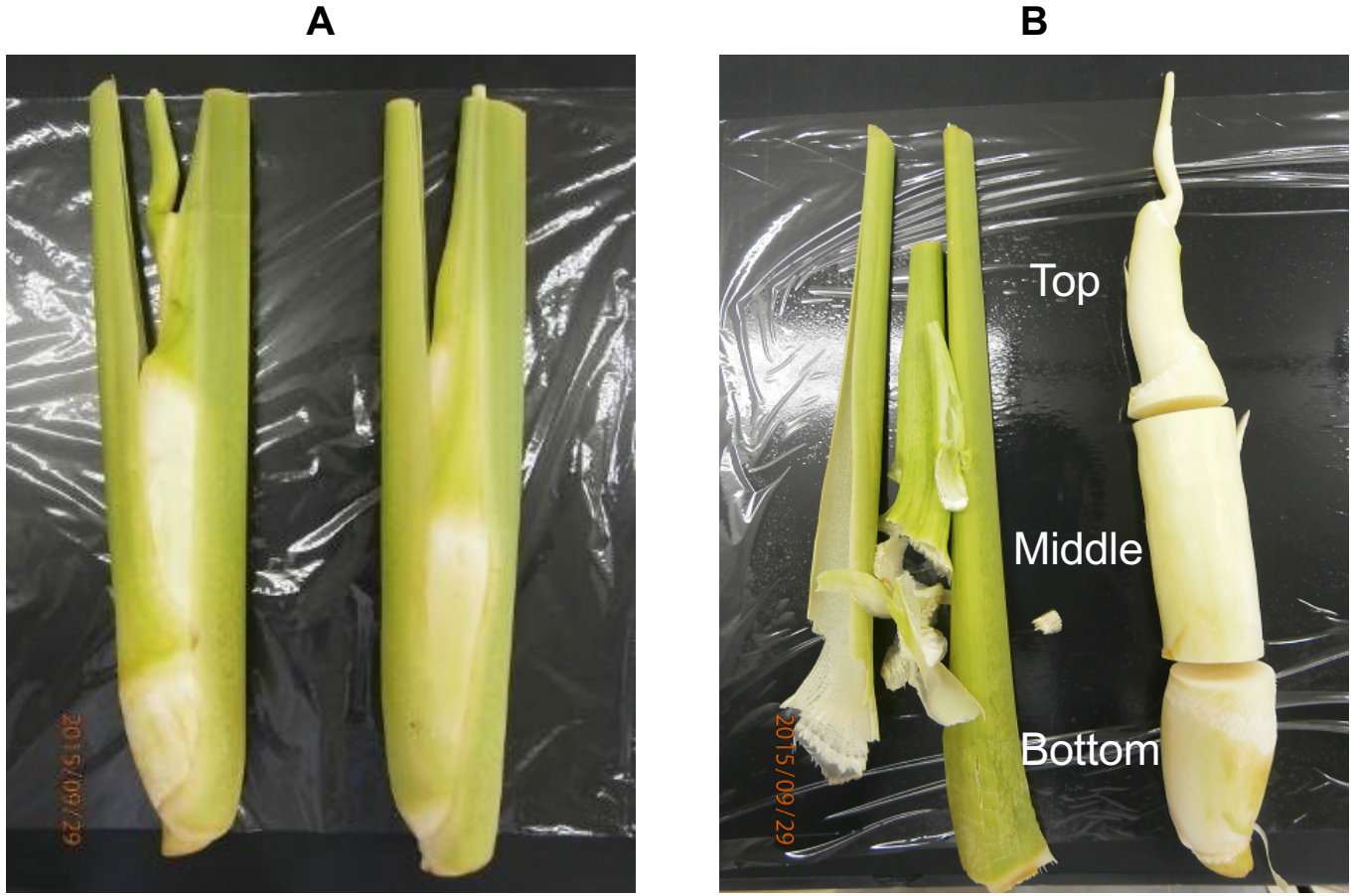
Fig. 2 Samples from different stages of swelling ranging from smaller (younger) to larger (older) and indicated by Nos. 1 to 5 (left, homogenized), in which No. 5 displays the formation of black spores (right).

Fig. 3 ARISA microbial profiles from edible galls at different stages of swelling (Nos. 1-5) with a pair of two-primer sets: Fun-1 for fungi and Bac-2 for bacteria.

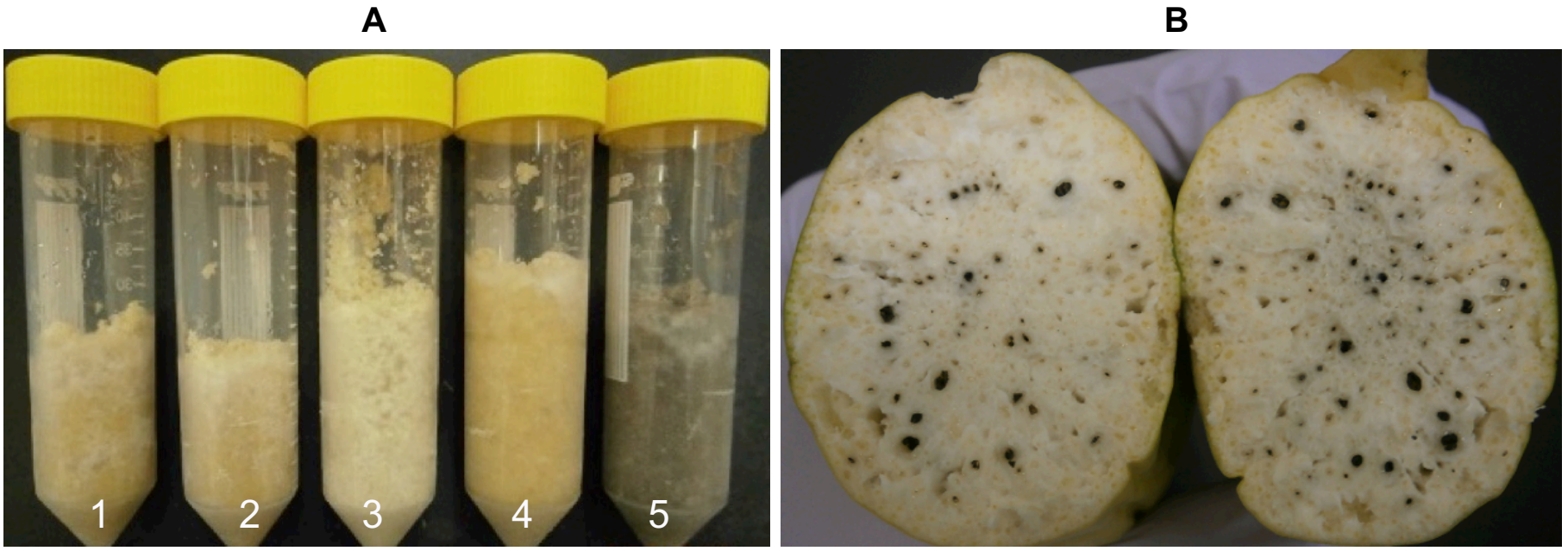
Fig. 4 ARISA microbial profiles from different parts (top, middle and bottom) of edible gall and a *U. esculenta* clone with a pair of two-primer sets: Fun-1 for fungi and Bac-2 for bacteria.

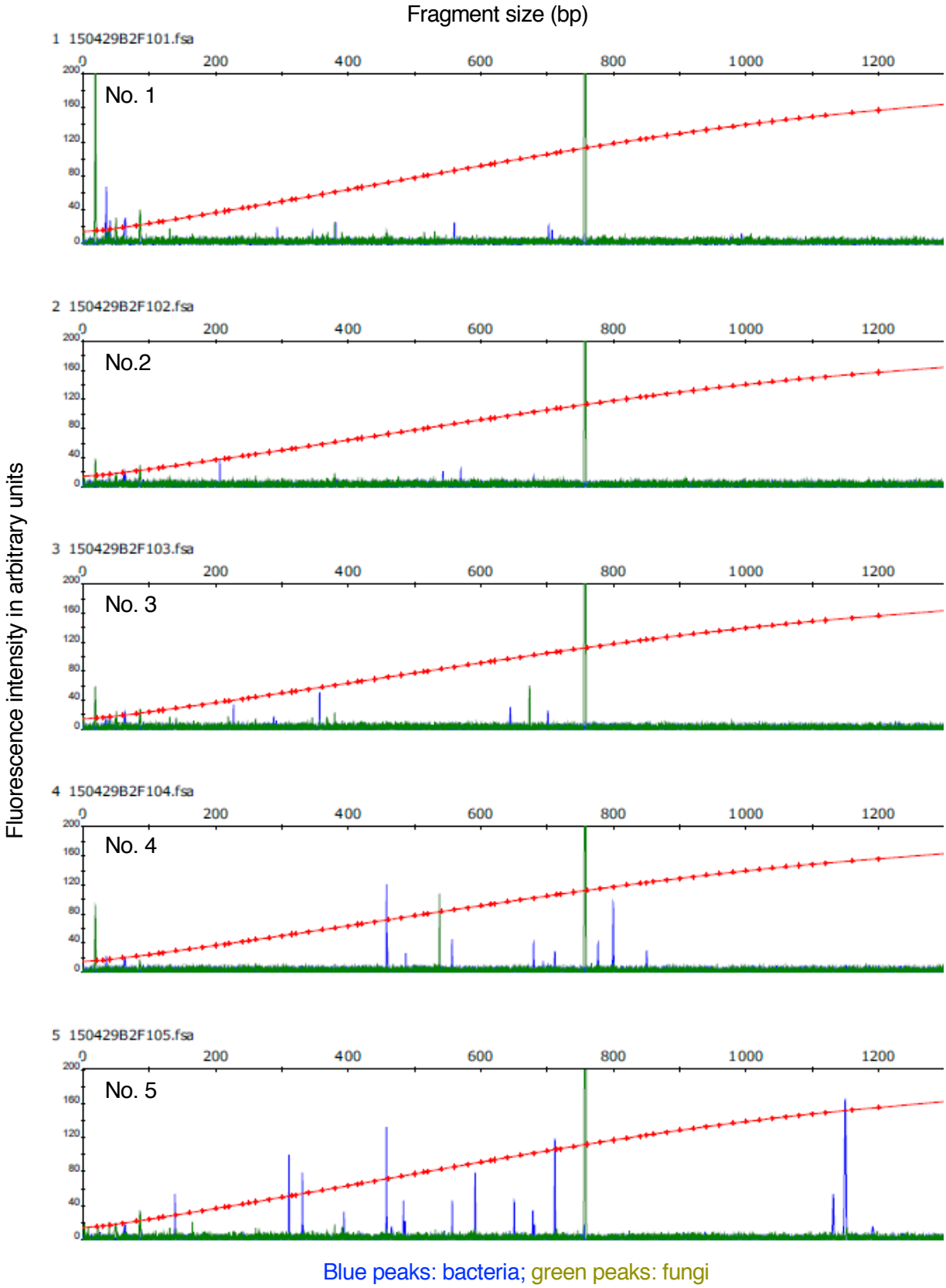
Fig. 5 Cell numbers of *U. esculenta* determined by qPCR in edible galls at different stages of swelling (left) and in edible gall parts (right).

(Fig. 1)



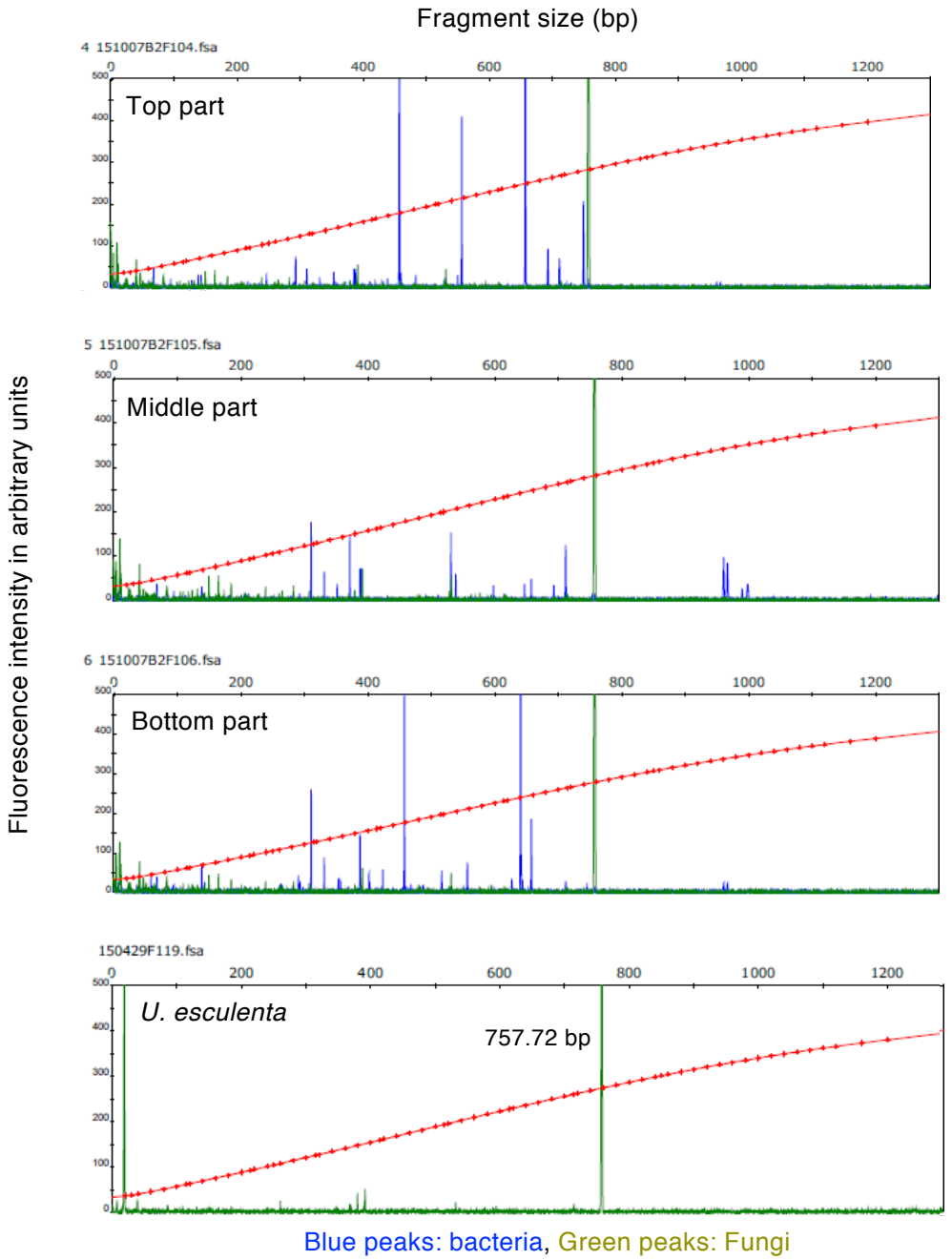
(Fig. 2)



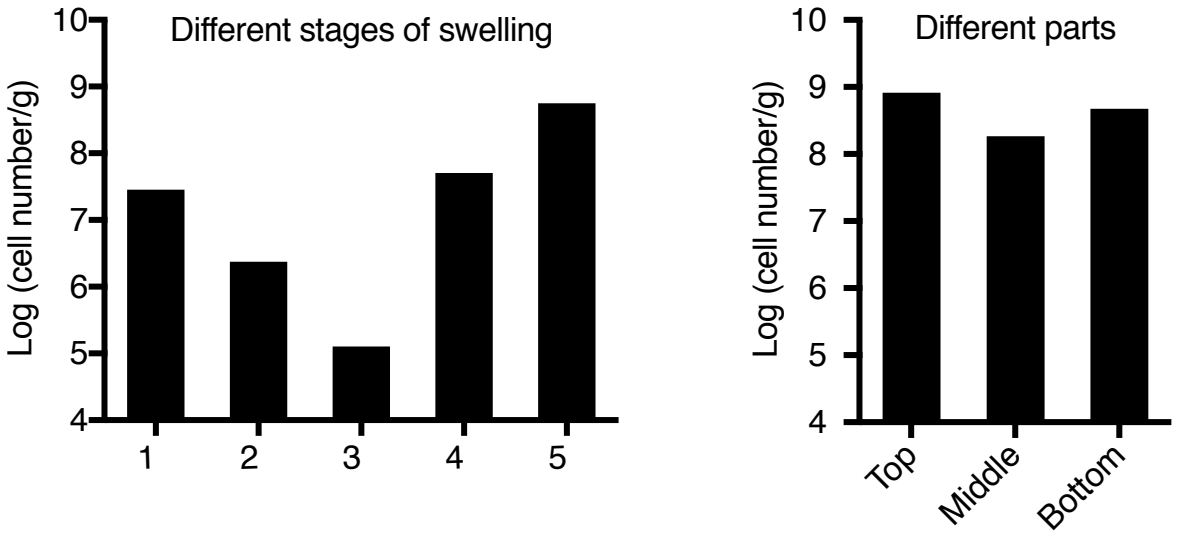

Fragment size (bp)
Fluorescence intensity in arbitrary units
1 150429B2F101.fsa
No. 1
2 150429B2F102.fsa
No.2
3 150429B2F103.fsa
No. 3
4 150429B2F104.fsa
No. 4
5 150429B2F105.fsa
No. 5
0
200
400
600
800
1000
1200
Blue peaks: bacteria; green peaks: fungi

(Fig. 3)

(Fig. 4)


Fragment size (bp)
4 151007B2F104.fsa
Top part
5 151007B2F105.fsa
Middle part
6 151007B2F106.fsa
Bottom part
150429F119.fsa
U. esculenta
757.72 bp
Fluorescence intensity in arbitrary units
Blue peaks: bacteria, Green peaks: Fungi

(Fig. 5)


Different stages of swelling
Log (cell number/g)
10
9
8
7
6
5
4
1
2
3
4
5
Different parts
Log (cell number/g)
10
9
8
7
6
5
4
Top
Middle
Bottom