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Henon bamboo flowering recorded first time in 120 years revealed how *Aciculosporium take* affects the floral organs of the host

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ABSTRACT

Flowering of Henon bamboo (*Phyllostachys nigra* var. *henonis*) was observed in Japan in 2020s. We estimated that the observation of flowering was recorded for the first time in 120 y. Additionally, stromata of *Aciculosporium take* have also been observed in the flower buds, or spikelets, of Henon bamboo. *Aciculosporium take* usually forms stromata at the vegetative shoot apex, which presumably originated from ancestral pathogens affecting floral tissues. However, given the infrequent occurrence of bamboo flowering events, it is unclear whether *A. take* still retains the ability to colonize ovaries of flowers. To ascertain the location where the fungus forms stromata, anatomical and histological analyses were performed. Because flower buds, including floral organs, are fragile, tissue sections were prepared by adhering them to cellophane tape, and subsequently examined using a triple fluorescent staining method. The findings showed that the fungus did not invade the ovaries but formed stromata from the apical rudimentary floret within the flower buds.

Keywords: *Clavicipitaceae*, endophytic fungi, mass flowering, ovary, witches' broom

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1. Introduction

Aciculosporium take (*Ascomycota*, *Hypocreales*, *Clavicipitaceae*) is the causal agent of witches' broom disease of many bamboo species; it inhabits in the intercellular spaces of meristematic tissues of the shoot apices and lateral buds (Tanaka, 2009). The shoot apex, where the fungus colonizes, continues to grow with small leaves and stops growing by forming stromata at the tip. Lateral buds start to grow due to release from apical dominance, and this process is repeated, resulting in witches' broom symptoms (Tanaka, 2009, 2010). In contrast, two species of the genus *Aciculosporium* (*A. monostipum* and *A. phalaridis*) parasitize the ovaries of their hosts and form stromata (Sullivan et al., 2001; Walker, 2004). In this respect, they exhibit similarities to the closely related *Claviceps* species; however, unlike *Claviceps* species, which colonize only floral organs, *Aciculosporium* species colonize host plant tissues systemically. These floral pathogens that invade ovaries to form stromata inhibit seed formation in host plants. On the other hand, the bamboos that are host of *A. take* are known to seldom flower, and their flowering frequency may be longer than 100 y. That is, because bamboo rarely flowers to reproduce by seed, the characteristics of stromata formation at the shoot apices appear to be an evolutionarily adaptation, probably to compensate for the low frequency of host flowering. Also, even if *A. take* forms stromata at the shoot apices, its impact on the vegetative reproduction of the host is minimal. However, *Aciculosporium oplismeni*, found in recent years, forms ascostromata at the shoot apex and prevents the host *Oplismenus undulatifolius* from flowering (Tanaka et al., 2021). *Aciculosporium siamensis*, another recently described species, is not known in detail, except that it forms stroma on the leaf sheaths of a poaceous plant (Mongkolsamrit et al., 2021). To consider the evolutionary significance of this trait, it was necessary to clarify whether *A. take* still retains the ability to colonize ovaries of its host flowers, but due to lack of flowering events, no information has been available.

In 2022, we encountered massive flowering of a bamboo species (Henon bamboo, *Phyllostachys nigra* var. *henonis*) and formation of stromata of *A. take* on the flower buds in Hyogo prefecture, Japan. Its mass flowering has been reported in various regions of Japan since around 2017 (Kobayashi et al., 2022; Maeda et al., 2023; Sakata et al., 2022; Yamada et al., 2023). Henon bamboo is estimated to flower once every 120 y in Japan, and the last observed Henon bamboo flowering in Hyogo prefecture was in 1904 and 1905 (Kawamura, 1911). In addition to documenting this rare phenomenon, this study aimed to examine the location of the fungus within the flower buds and clarify where the stromata are formed. Consequently, a method was developed to prepare sections of flower buds while preserving their fragile structure, facilitating the observation of the fungus within the flower buds.

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2. Materials and Methods

2.1. Recording and sampling

A survey of flowering Henon bamboo was conducted in Kobe, Miki, Sanda, Takarazuka, and Tanba-Sasaya cities in Hyogo prefecture, Japan from Jun 2022, and flowering Henon bamboo was observed in all communities found. Most of them were infected with *A. take*, and if stromata were formed on the flower buds, they were collected (Fig. 1). These voucher specimens were donated to the Museum of Nature and Human Activities, Hyogo (HYO), (Supplementary Table S1). Samples for sectioning were collected along the Shijimi River in Miki City on 16 Dec 2022 (HYO; C1-284336– C1-284339). The inflorescences, spikelets, and florets were disassembled and observed (Figs. 2, 3; Supplementary Figs. S1, S2). The flower buds with stromata were picked up by visual observation, and the fungus was identified by microscopic observation of the unique needle-shaped conidia with swollen ends (Supplementary Fig. S3A). To ensure identification, the fungus was isolated from the stroma and cultured to confirm the unique cultured conidia with branched appendages (Supplementary Fig. S3B) (Tsuda et al., 1997). An isolate was deposited in the NITE Biological Resource Center (NBRC 116683). Following a previous study (Tanaka et al., 2021), the DNA sequence of the internal transcribed spacer regions was confirmed to be 100% identical to the known sequence of *A. take* (LC571753).

2.2. Sectioning

Henon bamboo flower buds infected with *A. take* were immersed in FAA [4% (v/v) formaldehyde, 5% (v/v) glacial acetic acid, and 50% (v/v) ethanol], placed under low pressure to carefully remove air from the samples, and soaked for several days. The fixed samples were dehydrated in an ethanol series and placed in 99.5%

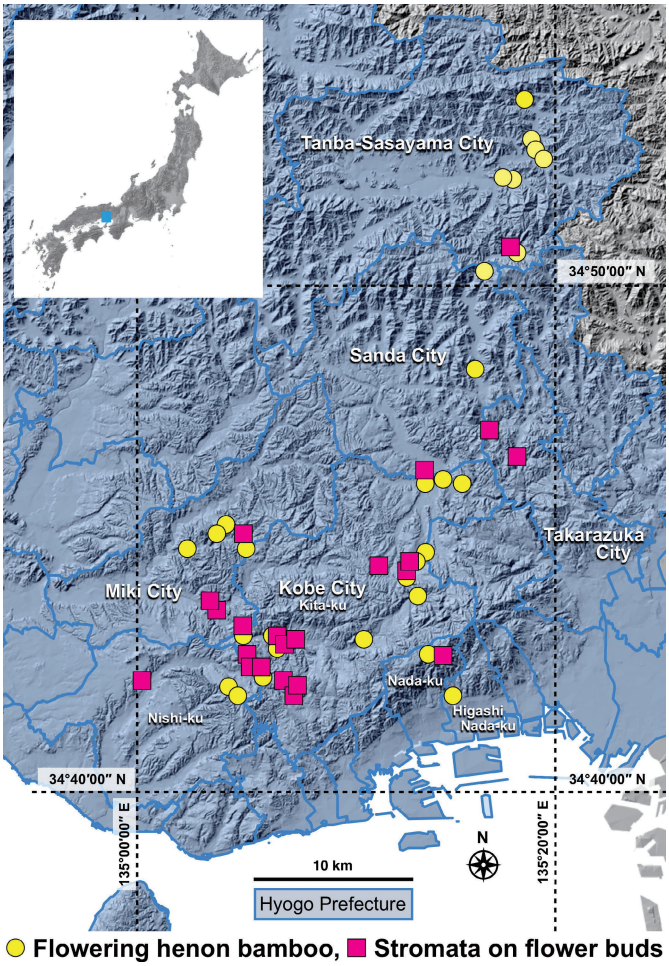

Fig. 1 –A map depicting the surveyed area (part of Hyogo prefecture, Japan). This map is based on the shaded-relief map published by Geospatial Information Authority of Japan (GSI Japan). Yellow circles indicate the locations of flowering Henon bamboo. Magenta squares denote the locations of flowering Henon bamboo with stromata of *Aciculosporium take* on the flower buds.

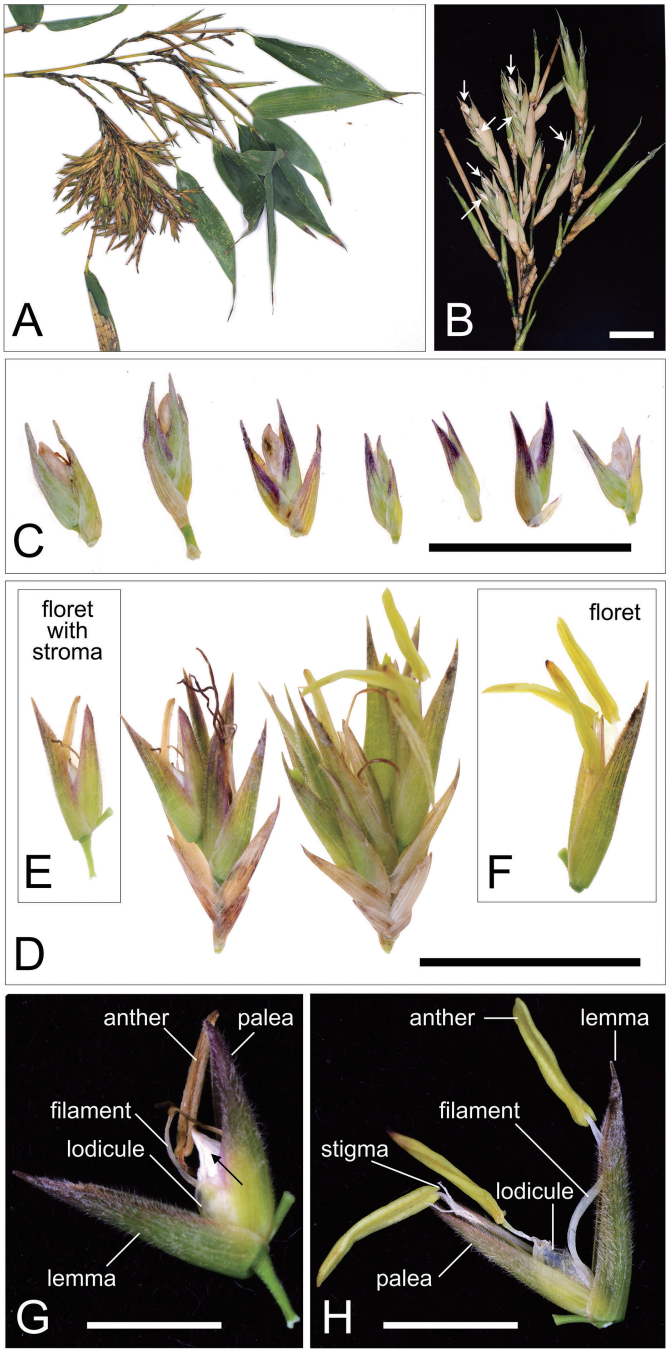

Fig. 2 – Flowering henon bamboo (*Phyllostachys nigra* var. *henonis*) infected with *Aciculosporium take*. A: Henon bamboo with infected flower buds. B: Inflorescences containing *A. take* stromata. Arrows indicate stromata. C: Stromata of *A. take*. D: Spikelets with or without stroma. E: Floret with stroma. F: Floret without stroma. G: Floret structure with stroma. H: Floret structure. *Bars*: B–F 1 cm; G, H 5 mm.

Fig. 3 – Flower buds of Henon bamboo with or without stromata of *Aciculosporium take*. A–C: A normal spikelet containing two florets and one rudimentary floret. D–G: Flower bud containing six leaf buds and one normal spikelet. G: Magnified view of the area marked in F. A spikelet containing two florets and one rudimentary floret. H–K: A spikelet containing one floret and stroma. L–O: Flower bud containing two spikelets. Each spikelet has one stroma at the tip. P–S: A shoot containing four lateral shoots with stromata at the tip of each. T–W: A shoot containing three lateral shoots with a stroma at the tip of each. Arrows indicate stromata. Abbreviations: a anther, F1 first floret, F2 second floret, lb leaf bud, ov ovary, rf rudimentary floret. *Bars*: 1cm.

ethanol until chlorophyll was removed. The ethanol in which the discolored samples were immersed was replaced with t-butyl alcohol. Paraffin (Paraplast Plus; Monoject Scientific, St. Louis, MO, USA) was added to the t-butyl alcohol at 60 °C and allowed to evaporate slowly at 60 °C. The samples were embedded in paraffin using the conventional method. For longitudinal sections of the flower buds, the paraffin blocks were mounted on a rotary microtome (RM2125RT; Leica, Wetzlar, Germany), carefully angled, and trimmed to the area of interest. A piece of cellophane tape (no bland, distributed by Daiso Industries Co. Ltd, Hiroshima, Japan) was placed on the exposed surface, and a 10 μm thick section was made. To prevent the cellophane tape from curling in later steps, the edge of the adhesive surface was attached to the edge of a glass slide. This sectioning procedure was repeated. The sections attached to the adhesive surface of the cellophane tape were deparaffinized twice for 10 min in Lemosol (Wako Pure Chemical, Osaka, Japan) and hydrated in an ethanol series.

2.3. Fluorescent staining

A triple staining method was used to differentiate the fungus from its host plant tissues (Figs. 4, 5; Supplementary Fig. S4). The sections were first treated with a few drops of 10% KOH solution for 30 s and rinsed with water. The sections were stained with trypan blue [Nacalai Tesque, Kyoto; 0.01% in 50% (v/v) glycerol (w/v)] for several minutes; this step was necessary to suppress the autofluorescence of plant tissues. After washing the stained sections with water, the sections were stained with WGA-Alexa Fluor 488 conjugate (Thermo Fisher Scientific, Waltham, MA, USA; 0.5% in distilled water) and DAPI (Nacalai tesque; 0.1% in distilled water) for several minutes. WGA-Alexa Fluor 488 was used to stain the fungal cell walls within the host plant tissues. This fluorescent dye-conjugated lectin was used to observe fungi within host plant tissues (Tanaka & Ono, 2018). DAPI was also used to stain nuclei. Some sections were stained with periodic acid-Schiff (Fig. 5C). After rins-

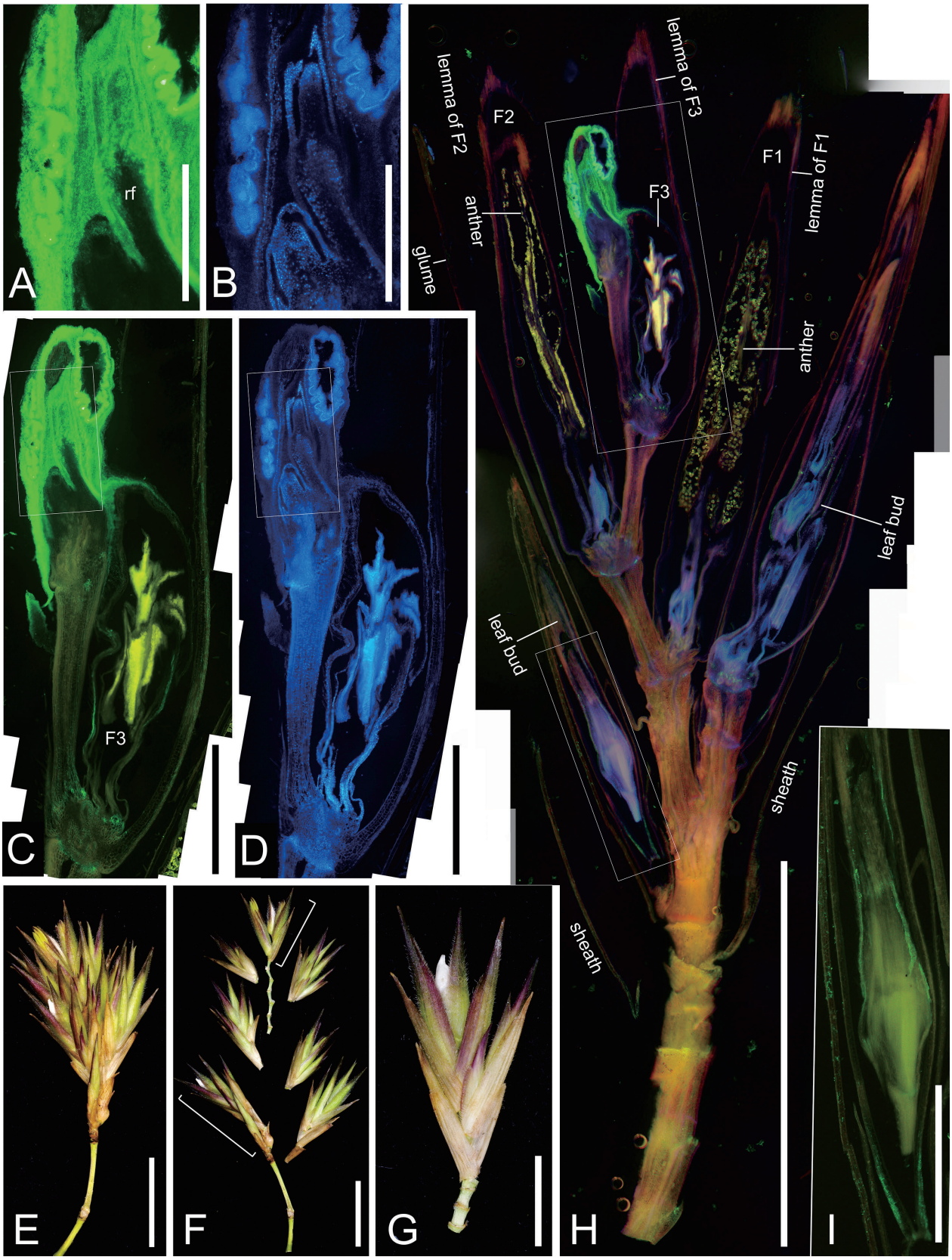

Fig. 4 – Henon bamboo flower buds with stroma of *Aciculosporium take*. A–D, H, I: Fluorescent stained sections. Green coloration indicates fungal cell wall stained by WGA Alexa fluor 488. Blue coloration indicates DNA stained by DAPI. Red coloration indicates cellulose stained by Trypan blue. Yellow coloration indicates autofluorescence of pollens. A, C, I: Blue excitation. B, D: UV excitation. A, B: Enlarged views of the areas marked in C and D, respectively. C, D: Stromata. Enlarged view of the area marked in H. E: Inflorescence. F: Disaggregated inflorescence. G: One of flower bud in F. H: Fluorescent stained section of the flower bud indicated in G. Merged view of Blue, Green and UV excitation. I: Leaf bud. Enlarged view of the area marked in H. Abbreviations: F1–F4 first to third florets, rf rudimentary floret. *Bars*: A, B 0.5 mm; C, D, I 1 mm; E–H 5 mm.

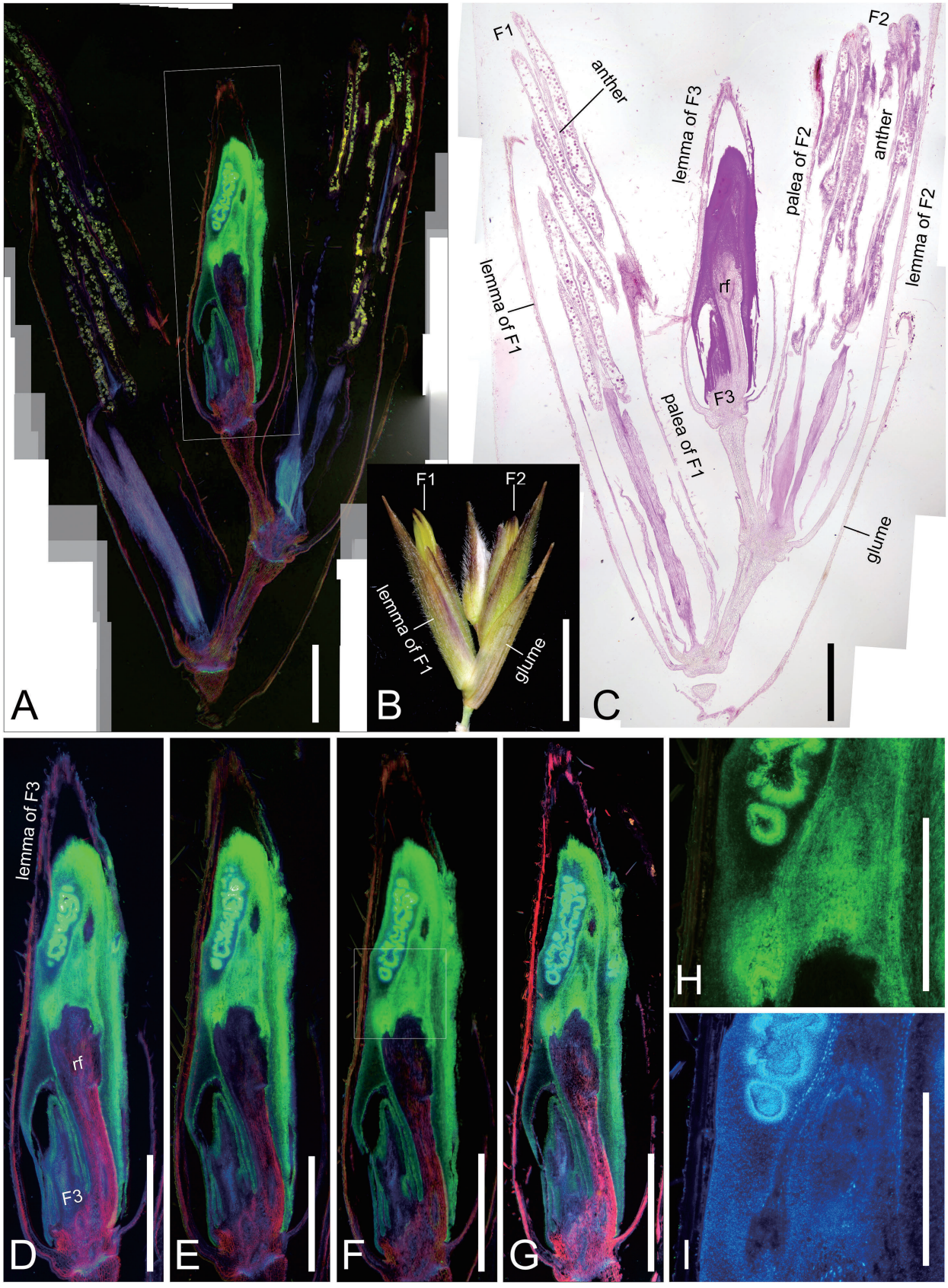

Fig. 5 – Serial sections of a Henon bamboo spikelet with stroma of *Aciculosporium take*. A, D–I: Fluorescent stained sections. Green coloration indicates fungal cell wall stained by WGA Alexa fluor 488. Blue coloration indicates nuclei stained by DAPI. Red coloration indicates cellulose stained by Trypan blue. Yellow coloration indicates autofluorescence of pollen. B: One of spikelet in Fig. 4F. C: Section stained with periodic acid–Schiff. D–G: Serial sections. F: Enlarged view of the area marked in A. H, I: Enlarged view of the area marked in F. Abbreviations: F1–F3 first to third florets, rf rudimentary floret. *Bars*: A, C–G 1 mm; B 5 mm; H, I 0.5 mm.

ing the stained tissues with water, the pieces of cellophane tape to which the sections were attached were cut off. The pieces of cellophane tape were placed on another glass slide with the section side facing up. The pieces of cellophane tape with sections were mounted with VectaMount AQ (Vector Laboratories, Burlingame, CA, USA) and covered with coverslips. Epifluorescence images were captured using a microscope (Nikon Eclipse Ni, Nikon, Tokyo) and a standard HG lamp. Alexa Fluor 488, DAPI, and trypan blue signals were obtained using B-2A, UV-1A, and G-2A filters, respectively. Image processing and overall general adjustments were performed using Photoshop Elements 2023 (Adobe Systems, San Jose, CA, USA). Multiple images were combined into one large image using the Photomerge Panorama function.

3. Results and discussion

3.1. Flowering Henon bamboo infected with A. take

Flower buds with *A. take* stromata were observed on the flowering Henon bamboo in Kobe, Miki, Sanda, Takarazuka and Tanba-Sasayama cities in Hyogo prefecture, Japan during the observation period from Nov 2022 to Mar 2024 (Figs. 1, 2; Supplementary Table S1). Within a Henon bamboo community, a small number of ramets began to flower in the winter season, and the number of flowering ramets gradually increased through the spring season, while the inflorescences turned brown and died through the summer season. Since the infected flower buds were found on the Henon bamboo, where *A. take* had already formed stromata at the vegetative shoot apices, it was considered that *A. take* had systemically parasitized and formed stromata on the flower buds. On the other hand, it is also possible that the infection was caused by raindrop-dispersed transmission of conidia from the surrounding *A. take* after the flower buds had formed. When the flower buds of infected Henon bamboo were observed, the flower buds on branches containing infected flower buds were smaller overall than normal flower buds (Fig. 2B). Some flower buds had a stroma, but not all flower buds on the branches with inflorescences had stromata.

3.2. Anatomical study of Henon bamboo flower buds infected with A. take

The stromata on each floret resembled whitish ergot sclerotia (Fig. 2C). In some cases, stamens with yellow anthers were observed emerging from the flower buds, although the stromata were visible (Fig. 2E, G). Each perfect floret of Henon bamboo was comprised of a lemma, a palea, three lodicules, three stamens with yellow anthers, and a pistil with three stigmas (Fig. 2G, H). In general, each spikelet of Henon bamboo flower buds contains 1–3 perfect florets covered by a glume and the apical rudimentary floret has only a lemma and rarely immature stamens (Fig. 3A–G; Supplementary Fig. S1). This pattern also applies to leaf development in the shoot of Henon bamboo; a shoot develops approximately three leaves, and the tip of the shoot does not develop leaves. When the infected flower buds were dissected, each spikelet had only one stroma (Fig. 3H–W; Supplementary Fig. S2). Although the stromata appeared to be formed on the lateral buds (Fig. 3P, T), they were already elongated shoots or flower buds and formed stroma at the tips (Fig. 3Q–S, U–W). However, it was impossible to determine whether the fungus had invaded the ovaries without examining the inside of the tissue because the stamens were unaffected, and, at first glance, the stroma appeared to emerge from the ovary.

3.3. Histological study of Henon bamboo flower buds infected with A. take

Sections of infected Henon bamboo flower buds were prepared for histological examination. It is difficult to prepare longitudinal sections of whole flower buds using conventional methods. This is because the flower buds are relatively large, the floral organs are fragile, and the sections may become distorted during the sectioning process or detach from the glass slide during the staining process. Therefore, the tape transfer method was employed. Methods for transferring sections to adhesive tape have been developed (Bonga, 1961; Kawamoto, 2003; Ogawa et al., 2003; Palmgren, 1954; Saho, 1973). In many cases, the sections were transferred to special tape and then to glass slides for observation. In this study, a familiar cellophane tape was used to directly observe the tissue sections adhered to the cellophane tape with fluorescent staining. Depending on the manufacturer of the cellophane tape, the adhesive may melt after deparaffinization, making observation difficult; therefore, it must be tested in advance. Furthermore, the piece of cellophane tape would curl up during the hydrolysis process, so the pieces of cellophane tape must be attached to something to prevent the sections from peeling off.

The triple fluorescent staining method distinctly differentiated the fungus from the plant tissues in the flower buds (Figs. 4, 5; Supplementary Fig. S2). Trypan blue stained the cellulose of plant cell walls and suppressed the autofluorescence of plant tissues. Although trypan blue can stain the chitin of fungal cell walls, it did not significantly interfere with the staining of chitin by WGA-Alexa Fluor 488. Observing the nucleus size by nuclear staining with DAPI helped distinguish fungi from plants. Since these fluorescent dyes have different excitation and fluorescence wavelengths, this triple staining method was sufficient to observe fungi in plant tissues. In flower buds, pollen autofluorescence was visible with blue excitation and yellow emission but was distinguishable from fungi due to different fluorescence wavelengths (Figs. 4H, 5A).

The location of colonizing *A. take* in flower buds was revealed by a histological examination (Figs. 4, 5; Supplementary Fig. S4). The stromata were formed within the meristematic tissue of apical rudimentary florets (Figs. 4A–D, 5D–I; Supplementary Fig. S4C–F), which did not develop floral organs. In other words, they followed the normal pattern of stromata formation of *A. take*, which is to form stromata at the shoot apex. Therefore, *A. take* does not invade the ovaries of the host. The stroma appeared to be formed in the ovaries of the flower buds because the mycelia forming the stroma at the tip of the flower buds spread epiphytically and enveloped the second floret from the tip (Figs. 4A, B, 5D–G; Supplementary Fig. S4E, F), and sometimes even the third floret from the tip (Fig. 4C, D). Detailed observations revealed that the fungus was observed within the meristematic tissue of the rudimentary floret at the tip, but the fungus was observed on the exterior of the floret tissue second from the tip (Figs. 4A, B, 5D–G). Since the shoots of a flower bud are very small, unlike those shoots that produce leaves, the stroma even covers several florets. In some sections, the fungus appeared inside the second floret from the tip (Fig. 5C, D), but serial sections confirmed the presence of stroma outside of the tissue (Fig. 5E–G). Furthermore, the fungus was observed in the tissues of the palea of the third floret (Fig. 4C) and in the tissues of juvenile leaves of the leaf buds (Fig. 4I).

4. Conclusions

The infrequent flowering event of Henon bamboo, which occurs once every 120 y has allowed us to elucidate the interaction of

A. take with the host's flower buds: *A. take* has evidently lost its ability to invade the ovaries of its hosts. In other words, *A. take* has acquired the ability to form stromata at the shoot apices of vegetative shoots and flower bud shoots without interfering with the seed production of its host. This change in characteristics likely occurred during the evolutionary process of the genus *Aciculosporium*. The tissue sectioning method with a triple fluorescence staining method in this study is well-suited for observing colonizing fungi in fragile plant tissues and will prove useful for future investigations of fungus–plant interactions.

Disclosure

The authors declare no conflicts of interest.

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Supplementary Materials

Supplementary online materials are available at https://doi.org/10.69199/data.mycosci.26425993, https://doi.org/10.69199/data.mycosci.26495473, https://doi.org/10.69199/data.mycosci.26425993, https://doi.org/10.69199/data.mycosci.26495491, https://doi.org/10.69199/data.mycosci.26495497.

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