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3 **Dark septate endophytic fungi associated with pioneer grass inhabiting volcanic deposits**
4 **and their functions in promoting plant growth**

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12 **Running Headline:** Endophytic fungi in volcanic deposits

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14 **Type of the Study:** Original Research Article

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23 **Dark septate endophytic fungi associated with pioneer grass inhabiting volcanic deposits**
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31 **Abstract**

32 Growth of the pioneer grass *Miscanthus condensatus,* one of the first vegetation to be
33 established on volcanic deposits, is promoted by root-associated fungi, particularly dark septate
34 endophytes (DSE). Fungal taxa within DSE colonize the root of *Miscanthus condensatus* in
35 oligotrophic *Andosol*, and their function in plant growth promotion remains largely unknown.
36 We, therefore, comprehensively assessed the composition of the DSE community associated
37 with *Miscanthus condensatus* root in volcanic ecosystems using the approaches of both
38 metabarcoding (next-generation sequencing) and isolation (culturing). Also, their promotion
39 effects of DSE on plant growth (rice as a proxy) were evaluated by inoculation of core isolates
40 to rice roots. Here, we found: i) 70% of culturable fungi that colonized *Miscanthus condensatus*
41 phylogenetically belonged to DSE, ii) 7 orders were identified by both sequencing and culturing
42 methods, and iii) inoculation of DSE isolates (*Phialocephala fortinii, P. helvetica, and*
43 *Phialocephala sp.*) validated their effects on rice growth, particularly under an extremely low
44 pH condition (compared to control without inoculation, rice biomass enhanced by 7.6 times
45 after inoculation of *P. fortinii*). This study helps improve our understanding of the community
46 of *Miscanthus condensatus*-associated DSE fungi and their functions in promoting plant growth.

Key words: *volcanic deposits*, *pioneer grass*, *Miscanthus condensatus*, *culture-non-culture approaches*, *dark septate endophytic fungi*, inoculation, plant growth promotion

Introduction

Numerous studies demonstrated that symbiotic fungi play a significant role in the establishment of pioneer vegetation in harsh environments or agricultural soils with extremely low pH. The association of these fungal micro-organisms that promote plant colonization is significant in extreme conditions. As these fungal symbionts help plant survival mainly by: improving host nutrient uptake (Usuki and Narisawa, 2007; Yadav *et al*., 2009), defending against pathogens (Busby *et al*., 2016), promoting tolerance to abiotic stress (Rodriguez *et al*., 2008; Gill *et al*., 2016), and modifying trophic interactions (Clay, 1996; Omacini *et al*., 2001; Bultman *et al*., 2003).

One of the most common groups of monocotyledonous root endophytes is dark septate endophytes (DSE), which usually colonize in tissues intracellularly and intercellularly of more than 600 living herbaceous and woody plant species (Jumpponen and Trappe, 1998). DSE, which is characterized by their morphology of melanized, septate hyphae and structure like microsclerotia, also confer the ability to improve plant performance through enhanced nutrient uptake, and increased ability to withstand adverse environmental conditions (Khastini and Jannah, 2021). Increasing evidence shows that DSE gradually become the most prevalent root colonizers under extreme environmental conditions of different ecosystem (Haruma *et al*., 2021; Yu *et al*., 2021). For example, Huusko *et al*. (2017) reported DSE-dominated colonization in *Deschampsia flexuosa* roots along a postglacial land uplift gradient. Gonzalez Mateu *et al*. (2020) reported that DSE inoculation *Phragmites australis* had higher aboveground

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80 biomass under mesohaline conditions. DSE, e.g., *Phialocephala fortinii*, promote host plant
81 growth and adaptation to the hostile environment by: i) increasing resistance to heavy metal
82 contamination and heat/drought stress via producing melanized cell walls (Li *et al*., 2018;
83 Haruma *et al*., 2021) and, ii) facilitating uptake of nutrients such as nitrogen and phosphorous
84 (Jumpponen *et al*., 1998; Surono and Narisawa, 2017).

85 Wild plant species may live in symbiosis with mycoflora that may have been lost during
86 breeding of the cultivars used in agriculture (Yuan *et al*., 2010). Whilst, some of symbiotic
87 fungi, that can assist plants to adapt to a given stress in a natural habitat, might increase
88 tolerance of crop species to that stress in an agriculture system. Thus, from an agricultural point
89 of view, the plant symbiotic fungi could be seen as an extended source for crop adaptation and
90 growth in agronomy. In attempts to domesticate "wild" symbiotic fungi (associated with
91 genetically wild type plant), some of these DSE species in natural system have been
92 successfully transferred to agricultural species from their original host, providing benefits to
93 the inoculated crops (Toju *et al*., 2018).

94 Rice (*Oryza sativa*) is the principal food grain crop (one of the four major food crops) for
95 more than 3 billion people, and its consumption exceeds 100 kg per capita annually in many
96 Asian countries (Yuan *et al*., 2010). During the last several decades, there have been major
97 climatic events, including global warming, soil acidification, etc, that influenced agricultural
98 productivity of rice around the world. Soil pH is a highly sensitive factor to determine plant
99 survival, distribution, and interactions with microorganisms, which are vital for the availability
100 of essential nutrients and plant growth (Luo *et al*., 2013). About 13% of the world's rice is
101 produced in acid soil. Compared with other crops, rice has relatively stronger Al toxic resistance
102 (Famoso *et al*., 2010), and is also the most complex cereal crop with Al resistance genes (Ma
103 *et al*., 2002). Nevertheless, as for other crops, heavy metal toxicity in acid soil limits rice growth

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- 删除了: Acidic soils occupy around 40-50% of the world's potentially arable land. Plants commonly encounter deficient and toxic levels of mineral elements (soluble ionic Al, mainly Al^{3+})when grown in acidic (pH<5) soil.
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and nutrients uptake, and subsequently reduces grain yield (Chen *et al*., 2020). The optimal pH range for rice growth is 5.0-8.5, which shows the likely reduction of yield in the soil with the extended pH range (Ma *et al*., 2002). To improve these soil acidity, liming is often used but is practically difficult and unsustainable.

Microorganisms inoculation is a sustainable approach to potentially promote plant resistance to acidic stress. For instance, plant-associated fungi, such as arbuscular mycorrhizal fungi (AM fungi), reportedly play a key role in the protection of plants in acidic soils (Toju *et al*., 2018). Yet, high concentrations of H^+ and Al^{3+} can inhibit hyphal growth and spore germination in AM fungi, thereby decreasing the possibility of colonizing plant roots (Clark, 1997; Van Aarle *et al*., 2002; Postma *et al*., 2007). Comparably, DSE show marked potential to help host plants resist acidity because of their higher H^+ tolerance than other colonizing fungi (Postma *et al*., 2007). Still, there is a lack of reports of DSE improving host crop (e.g., rice) growth under acidic conditions, especially an extremely acidic condition (pH 3.0).

Re-vegetation in volcanic soil, characterized by a dominance of biological processes, is difficult due to: i) strong acidity of volcanic deposits, ii) high concentration of toxic elements, and iii) deficiencies in essential nutrients. *Miscanthus sinensis,* an unique pioneer grass plant during recovery after volcanic eruption, is the first to be established on volcanic deposits, and frequently found as primary vegetation in lahar deposited by volcanic eruptions (Watanabe *et al*., 2006; Hirata *et al*., 2007; An *et al*., 2008; Ezaki *et al*., 2008). This is because *M. sinensis* can tolerate a wide range of environmental stresses due to the trait of C4 photosynthesis, leading to high productivity and low-nutrient requirement (Stewart *et al*., 2009). Apart from *Miscanthus* traits that adapt to the volcanic soil, the root-associated fungal communities are widely reported to benefit the growth and promote the adaptation of host plants to stress, such as aridity (Wu and Xia, 2006), salinity (Porcel *et al*., 2012), and oligotrophic conditions (Jeewani *et al*., 2021).

133 A better understanding of plant-microbe interactions, therefore, can help improve our
134 understanding of vegetation recovery and plant growth promotion including agricultural
135 application scene. The isolation and culture of fungal species, therefore, are indispensable as
136 they complement taxonomic databases and validate taxa revealed by sequencing. Bai *et al*.
137 (2015) established *Arabidopsis* root-derived bacterial culture collections representing the
138 majority of species that were reproducibly detectable by culture-independent community
139 sequencing. Laval *et al*. (2021) investigated fungal and bacterial communities in soils receiving
140 wheat and oilseed rape residues, and confirmed the feasibility of combined culture-unculture
141 approaches that revealed consistent community profiles. The role of keystone taxa revealed by
142 the sequencing data-based co-occurrence network can be further validated by culturing and
143 followed inoculation. For example, isolation was used to test whether the interaction between
144 micro-organisms predicted by metagenomic sequencing actually occurs (Laval *et al*., 2021). By
145 isolation and inoculation, (Zheng *et al*., 2021) identified the strong decomposition ability of
146 keystone taxa such as the genera *Chryseobacterium* (bacteria), *Fusarium*, *Aspergillus*, and
147 *Penicillium* (fungi), which are consistent with the keystone taxa revealed by the co-occurrence
148 network. The combination of sequencing and culturing methods, therefore, is powerful for the
149 identification of putative taxa (either individually or creation of synthetic communities). Yet,
150 studies on DSE in volcanic ecosystems by culture-unculture approaches are lacking, and
151 inoculation to validate the function in rice growth still awaits further investigation.
152 For this purpose, both culture-dependent and culture-independent approaches were
153 adopted, to comprehensively reveal the fungal communities of *Miscanthus*-associated,
154 particularly DSE, from volcanic ecosystems. Their functions in promoting plant growth (via
155 isolation-inoculation) in different pH soils were further evaluated. This study, therefore, aimed
156 to: i) reveal the fungal taxa associated with the roots of *M. condensatus* during vegetation

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216 recovery by a combination of sequencing and culturing approaches, and ii) inoculate the major
217 food crop i.e., rice with these indigenous isolates (overlapped with sequencing-revealed taxa)
218 to evaluate their effects on rice growth, in particularly under low pH condition. We
219 hypothesized that prevalent colonization by DSE fungi occurs in the pioneer grass *M.*
220 *condensatus* inhabiting volcanic deposits near the crater of Miyake-jima, due to DSE's traits of
221 preferential colonization under oligotrophic and acidic conditions.

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223 **Materials and Methods**

224 **Study site description and root sampling**

225 Miyake-jima (55.5 km^2 in area; highest point, 775 m), a basaltic volcanic island (34º05′ N,
226 139º31′E; Fig. 1), belongs to the Fuji volcanic southern zone in the East Japan volcanic belt.
227 As a model active basaltic volcanic island with an eruption in 2000, it ejected large amounts of
228 volcanic ash and gases such as sulfur dioxide and hydrogen sulfide (60% of vegetation on the
229 island was affected) (Yamanishi *et al*., 2003; Guo *et al*., 2014). As a result of SO_2 gas exposure,
230 volcanic ash deposits were acidified due to SO_4^{2-} absorption. They were characterized by strong
231 acidity, with high levels of exchangeable Ca^{2+} and Al^{3+} (Fujimura *et al*., 2016). Mount Oyama
232 is an active volcano, located in the center of the island. A large amount of volcanic SO_2 gas
233 (~54 kt d^{-1}) was ejected immediately from a newly created summit caldera after the latest
234 eruption in 2000 (Fujimura et al., 2016). The SO_2 gas exposure declined slowly after the
235 eruption, and as a result of this exposure, the volcanic ash deposits were acidified due to SO_4^{2-}
236 absorption. They were characterized by strong acidity [pH (H_2O), 3.1-4.0], with high levels of
237 exchangeable Ca^{2+} (33.5-115 cmol kg^{-1}) and Al^{3+} (0.8-10.2 cmol kg^{-1}) (Fujimura *et al*., 2016).
238 At 18 years after the eruption, the patchy vegetation of a pioneer grass, *Miscanthus condensatus*,
239 was established at site OY near the Miyake-jima summit crater (34º04′ N, 139º31′ E; 553m

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251 a.s.l; Fig. 1). The rhizosphere soils of *Miscanthus condensatus* were collected at site OY in
252 November 2017, and March and September 2018. From each period, three healthy specimens
253 of *M. condensatus* were collected, kept in sterile plastic bags, and immediately stored on ice.
254 Samples were divided into two portions, and: 1) kept at 4ºC and processed within 48 h after
255 collection for isolation, and 2) kept at -20 ºC until DNA extraction and molecular analysis.
256

257 **Root surface sterilization and culturable endophytic fungal isolation**

258 In order to remove adhering soil and free-living microbes, root surface sterilization was
259 performed by modifying the method of Sahu, *et al.* (2022). Root samples were gently rinsed
260 with tap water. Individual roots were severed aseptically in 1-cm-long sections with a sterile
261 scalpel and put into 50-mL conical centrifuge tubes. Then, they were superficially sterilized
262 with 0.005% Tween 20 and then rinsed with sterilized distilled water before the aseptic stepwise
263 sterilization process was carried out. Root sections were treated with 70% ethanol for 1 min,
264 with a further step in the above process, 1% sodium hypochlorite was added and sterilized for
265 5 min. Finally, sections were rinsed with sterilized distilled water three times (Sahu, *et al.*,
266 2022).

267 After surface sterilization, the final wash was spread plated onto 1/2 Corn Meal agar
268 medium (Cornmeal, Difco 25 g L^{-1}, Bacto agar, Difco 15 g L^{-1}) to confirm the disinfection and
269 incubated for 2 weeks at 23ºC to examine for the presence of a growth colony. Root sections
270 were dried with sterile filter paper overnight and then placed onto cornmeal agar medium
271 containing 0.1 mg kg^{-1} streptomycin and incubated at 23ºC for 2 weeks. When endophytic
272 fungal growth was observed, the mycelia were immediately transferred to a new plate. An
273 isolate was transferred only when the probability of a good pure culture was considered high.
274 Thus, when the strains originated very close to each other and in later stages, when they

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280 overgrew earlier strains, they were left untransferred and not calculated into the total number
281 of the isolates. After incubation, pure cultures were obtained by transferring single hyphae to
282 cornmeal malt yeast agar medium (CMMY; Malt extract 10 g L^{-1}, Yeast extract 2 g L^{-1},
283 Cornmeal 8.5 g L^{-1}, Bacto agar 7.5 g L^{-1}).
284
285 **Identification of fungal isolates and phylogenetic analysis**
286 Genomic DNA from each fungal isolate was extracted from mycelium using Prepman
287 Ultra Sample Preparation Reagent Protocol (Applied Biosystems, California, USA). The
288 universal primer pairs of ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') hybridize at the end of
289 18S rDNA, and the primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') that hybridizes at the
290 beginning of 28S rDNA was used to amplify fungal isolates (Mahmoud and Narisawa, 2013).
291 PCR amplification was carried out in a 50-µL reaction mixture containing 1 µL fungal genomic
292 DNA, 2.5 µL of each primer, 5 µL of 10×Ex Taq buffer, 4 µL of dNTP, 0.25 µL of Ex *Taq*
293 DNA polymerase, and 34.75 µL of sterilized MilliQ water under thermal conditions of 4 min
294 at 94ºC, 35 cycles of 94ºC for 35 s, 52ºC for 55 s, and 72ºC for 2 min, and a final extension of
295 72ºC for 10 min using a Takara PCR Thermal Cycler Dice (Takara Bio INC., model TP 600,
296 Japan). The PCR products were purified and sequenced using an Applied Biosystems 3130*xl*
297 DNA sequencer. All sequences obtained were compared with similar DNA sequences retrieved
298 from the Genbank database using the NCBI BLASTN program.
299
300 **Illumina MiSeq sequencing for culture-independent identification**
301 Roots of samples which collected in November were added to 10-mL aliquots of sterile
302 distilled water and macerated with a pestle and mortar for DNA extraction with DNeasy Plant

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304 Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA was purified
305 using Ultra Clean DNA Purification Kit (MOBIO, Carlsbad, CA, USA). Then, DNA was eluted
306 in 50 µL of Tris and EDTA buffer. A NanoDrop spectrophotometer (NanoDrop Technologies,
307 Wilmington, DE, USA) was used to quantify the DNA concentration. Finally, DNA samples
308 were stored at -80ºC before molecular analysis. The second nuclear ribosomal internal
309 transcribed spacer (ITS2) region of the rRNA operon was targeted using the fungal-specific
310 primer pairs ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-
311 TCCTCCGCTTATTGATATGC-3') (Chen et al., 2021). PCR amplification was carried out in
312 triplicate with 50-µL reactions containing 25 µL of Premix Taq (TaKaRa, Shiga, Japan), 23 µL
313 of sterilized MilliQ water, 0.5 µL of both forward and reverse primers (125 pmol), and 1 µL of
314 template DNA. The PCR program had the following thermocycling conditions: 35 cycles of
315 denaturation at 94ºC for 30 s, annealing at 54ºC for 30 s, 72ºC for 45 s, and a final extension of
316 72ºC for 10 min. PCR products were pooled and their relative quantity was estimated by
317 running 5 µL of amplicon DNA on 1.5% agarose gel, and products were purified with QIA
318 Quick PCR Purification Kit (Qiagen, Shenzhen, China). The purified mixture was diluted and
319 denatured to obtain an 8 pmol amplicon library and mixed with an equal volume of 8 pmol
320 PhiX (Illumina) following the manufacturer's recommendations in the Illumina MiSeq reagent
321 kit preparation guide (Illumina, San Diego, CA, USA). Finally, 600 µL of the amplicon
322 mixtures were loaded with read 1, read 2, and the index sequencing primers. The paired-end
323 sequencing (each 250 bp) was completed on a MiSeq platform (Illumina). The sequencing data
324 were processed using the UPARSE pipeline
325 (http://drive5.com/usearch/manual/uparse_pipeline.html). The raw sequences were subjected
326 to quality control. The singleton and chimeric sequences were removed after dereplication, and

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the remaining sequences were categorized into operational taxonomic units (OTU) with 97% similarity and then assigned taxonomy using the UNITE database (https://unite.ut.ee/) .

Inoculation

The experiment was conducted as a complete randomized factorial design with two factors. The first factor had four levels: non-inoculation control or inoculation with three dominant isolates (*Phialocephala fortinii, P. helvetica, and Phialocephala sp.*); and the second factor had three levels: pH 3, pH 4, and pH 5. Each treatment consisted of four replicates with two plants per pot, and thus totaling 48 experimental pots in the study. Fungal inoculates were prepared by aseptically growing three dominant DSE isolates on Petri dishes with oatmeal agar medium (10 g L^{-1} oatmeal and 15 g L^{-1} Bacto agar enriched with nutrients: 1 g L^{-1} $MgSO_4.7H_2O$, 1.5 g L^{-1} KH_2PO_4, and 1 g L^{-1} $NaNO_3$). Due to the host non-specific character of DSE, rice was chosen as a host plant in this study mostly for its important role in consumed cereal in the world and it is from the same family as *Miscanthus*. Rice seeds were surface-sterilized by immersion in 70% ethanol for 2 min, and a solution of 1% sodium hypochlorite for 5 min with agitation. The sterilized seeds were gently rinsed several times with sterilized distilled water, then dried overnight, and plated onto 1% water agar medium in Petri dishes for germination at 30ºC. Following pre-germination, 2-day-old seedlings (two seedlings per plate) were transplanted as growing fungal colonies on the medium at pH 3, pH 4, or pH 5. For DSE inoculation, two 5-mm plugs excised from the edge of an actively growing colony on culture medium were inoculated at a 1-cm range close to the rice seedlings. Seedlings transplanted onto non-inoculated medium were used as controls. The whole set was placed into sterile plastic culture bottles and incubated for 3 weeks at room temperature with an 18 h:6 h (L:D) regimen and

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354 intensity of 180 μmol $m^{-2}s^{-1}$. Assessed plants were harvested and oven-dried at 40ºC for 72 h.
355 The shoot and root dry weights of treated plants were measured and compared with the control.

356 **DSE root colonization observations**

357 Root colonization by DSE fungal isolates was observed to confirm whether the selected
358 DSE colonized the inner roots endophytically. Roots were harvested from plants after 3 weeks
359 of cultivation. Root systems were washed thoroughly under running tap water to remove
360 adhering agar, then rinsed with distilled water, and used for root staining. The root samples
361 were cleared with 10% (v/v) potassium hydroxide in a water bath at 80ºC for 20 min.
362 Subsequently, roots were acidified with 1% hydrochloric acid at room temperature for 5 min,
363 then stained with 50% acetic acid solution containing 0.005% cotton blue at room temperature
364 overnight. Root fragments were placed on a slide glass and covered with a cover glass. Fungal
365 colonization was observed using a light microscope equipped with an Olympus DP25 digital
366 camera.

367 **Statistical analyses**

368 All statistical analyses were performed in the R environment (version: V4.1.2).
369 Homoscedasticiy was checked using Levene's test and normality using Shapiro-Wilk's test.
370 The differences of mean dry biomass between the analyzed traits of the seedlings in different
371 treatments in this study were calculated and analyzed statistically with two-way analysis of
372 variance (ANOVA) and Tukey's honestly significant difference test at P-values<0.05.

373

374

Results and Discussions

The core fungal taxa identified by both culture-dependent and culture-independent methods

This study compared the culture-dependent isolates with the fungal taxa revealed by culture-independent methods. Based on 97% sequence similarity, all reads were clustered into 224 OTUs, and the valid sequences were classified into five phyla, including two major dominant phyla of Ascomycota (71.5%) and Basidiomycota (17.1%), followed by Mortierellomycota, Mucoromycota, and Calcarisporiellomycota, while the cultivable endophytic fungi were classified into two different phyla of Ascomycota (97.5%) and Basidiomycota (2.50%). Fifteen and four classes were detected by culture-independent and culture-dependent approaches, respectively. Specifically, classes Sordariomycetes and Leotiomycetes (both belonging to phylum Ascomycota) were the major classes in terms of the number of OTUs. These data were in agreement with a previous study showing that Leotiomycetes and Sordarimoycetes were the major classes of endophytic fungi associated with plants (regardless of plant species, associated host tissue) in acidic, oligotrophic ecosystems and nutrient-limiting boreal and arctic areas (Arnold *et al*., 2007; Yuan *et al*., 2010; Ghimire *et al*., 2011; Luo *et al*., 2014; Knapp *et al*., 2019).

While looking at the lower level, 27 orders were found by Illumina-based sequencing analysis, and 10 of them had an average abundance over 1%. Among these orders detected by sequencing, seven orders were identified via culture-dependent methods as well (Fig. 2). Significantly higher proportions of Hypocreales (35.6%), Helotiales (21.2%), and Eurotiales (13.2%) were observed by Illumina-based analysis (Fig. 2). Through culture-dependent methods, an abundance of Helotiales (70.0%) occupied the whole community, followed by Eurotiales (15.0%) and Hypocreales (8.75%). In general, the abundant orders of fungal isolates

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401 also showed abundance in the OTU table generated by high-throughput sequencing. The
402 overlapping of taxa (Hypocreales and Helotiales) identified by both approaches suggests their
403 significance and dominance in *Miscanthus condensatus*-associated fungal communities.
404 Similarly, the key fungal and bacterial community in soils amended with wheat and oilseed
405 residues were identified via culture and non-culture approaches (Laval *et al*., 2021). Several
406 other studies also confirmed the feasibility to reveal major microbial taxa and showed the
407 marked potential of adopting the combination of both culture and non-culture approaches to
408 identify putative taxa (Laval *et al*., 2021; Bai *et al*., 2015; Zheng *et al*., 2021). Undoubtedly a
409 combination of culture-dependent and culture-independent methods might provide a powerful
410 strategy to identify and obtain novel endophytes.

411 The overlapping order Helotiales identified by both culture-dependent methods was
412 abundant in the *Miscanthus condensatus*-associated fungal community (Fig. 2). The isolates
413 including *P. fortinii*, *P. helvetica*, and *Phialocephala* sp. belonged to Helotiales species, which
414 are highly conserved and found to be co-occurring species in the root symbiont communities
415 based on Sanger sequencing (Walker *et al*., 2011; Bruzone *et al*., 2015). This study also found
416 these fungi, *i.e., Phialocephala* sp., *P. helvetica*, and *P. fortinii*, in all samples irrespective of
417 the sampling period (Table 1). Previous studies isolated *P. fortinii* from the root of *Pinus*
418 *resinosa* (Wang and Wilcox, 1985), *Vaccinium vitis-idaea*, *Betula platyphylla* var. *japonica*,
419 *Luetkea pectinate* (Addy *et al*., 2000), *Piceas abies*, *Betula pendula* (Menkis *et al*., 2004),
420 *Rhododendron* sp. (Grünig *et al*., 2008), *Chamaecyparis obtusa*, and *Rubus* sp. (Surono and
421 Narisawa, 2017). Yet, the phylogeny and ecological effects of *P. fortiniii* on plant quality still
422 remain largely unknown (Tedersoo *et al*., 2009). For example, *P. fortiniii* itself is genotypically
423 diverse and composed of at least 21 morphologically indistinguishable but genetically isolated
424 cryptic species (CSP) (Grünig *et al*., 2008). Up to seven isolates belonging to *P. fortinii* have

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been formally described as CSP (Grünig *et al*., 2008). *Phialocephala helvetica* (sub-species of *P. fortiniii*) associated with the root of *Picea abies* (Stroheker *et al*., 2021) and *Pinus sylvestris* (Landolt *et al*., 2020), is regarded as one of the most common CSP. Yet, their functions in promoting plant growth remain largely unknown.

Colonization of DSE fungal isolates in plant root

Isolating and characterizing microorganisms could provide insights into their phylogenetic identification, physiological properties, and metabolic potentials, which will help understand the formation, persistence, adaptation mechanisms, and ecological functions of microbial communities (Li *et al*., 2019). Therefore, these three most promising isolates of *Phialocephala* sp., *P. fortinii*, and *P. helvetica*, as typical DSE, were further examined regarding their effects on growth-promoting activity for plants. Based on the inoculation test, all rice seedlings exhibited healthy growth throughout the experimental period by fungal isolate × agar pH interaction (Fig. 3).

After harvesting, the roots were stained with 0.05% cotton blue to determine the endophytism of DSE isolates. Microscopic observation revealed that all DSE isolates successfully colonized hair roots of rice seedlings. The hair roots were coated with loose wefts of fungal hyphae. This feature was identical to that previously described for typical DSE, *i.e.,* they are characterized by microsclerotia, thick, and darkly pigmented septate hyphae in the hair roots. Non-inoculated plants as a control showed no DSE colonization. The root colonization pattern was similar in *P. fortinii* and *P. helvetica*, but the degree of fungal colonization of *Phialocephala* sp. was the lowest compared with those two isolates. The images show the dense networks of hyphae of DSE inter- and intra-cellularly colonizing rice roots (Fig. 4). Very few

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448 studies, however, investigated the role and ecological significance of isolated DSE underlying
449 plant growth.
450

451 **The role of isolated DSE in rice growth promotion**

452 As rice is one of the four major food crops for most Asian people, to domesticate these
453 isolated "wild" DSE can benefit agriculture production. Thus we transferred these DSE isolates
454 from their original hosts of *Miscanthus condensatus* to agricultural species (rice). DSE is widely
455 reported to be characterized with non-host specific, but different host (cross family) may have
456 different responses (in terms of morphology) to inoculated isolate. For example, *P. fortinii* is
457 frequently reported in roots and formed typical ectomycorrhizae with members of the Pinaceae
458 plants (Jumpponen *et al*., 1998). In contrast, for other family plants, *P. fortinii* is often found to
459 be an endophytic fungi. In addition, *C. chaetospira* was reported able to develop and form spiral
460 structures resembling ericoid mycorrhizas within the roots of ericaceous plants (Usuki and
461 Narisawa, 2005). Whilst *C. chaetospira* colonizing in other host family are characterized by
462 formation of microsleclerotia-aggregations of irregularly lobed hyphae and dark septate hyphae
463 growing inter- and intracellulary. Considering both plants, used in this study as a host (e.g.,
464 miscanthus and rice), belong to the same family of grass with similar host responses to DSE
465 (showing non-host specific trait), we aimed to test effects of these isolated DSE in crop (rice as
466 a proxy) growth promotion. Here, we found that shoot biomass of rice inoculated with DSE
467 isolates increased up to 7.6 times, compared with non-inoculated controls (Fig. 5). The greatest
468 shoot dry weight was recorded in plants treated with *P. fortinii*, followed by *P. helvetica* and
469 *Phialocephala* sp. Similarly, *P. fortinii* isolates were used to inoculate asparagus plants and
470 promote plant growth, e.g., shoot biomass increased by up to 53.5% (Surono and Narisawa,

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2017). The beneficial effects of *P. fortinii* on enhancing plant yield have been reported (Jumpponen *et al*., 1998; Jumpponen and Trappe, 1998).

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This improvement in plant growth may be related to the ability of these isolates to use organic nitrogen sources under nitrogen-deficient conditions. Low nitrogen uptake by plants is associated with soil acidity. The presence of *P. fortinii* associated with plant tissues demonstrated its ability to produce a variety of extracellular enzymes that break down complex forms of organic matter containing nitrogen and phosphorus (Jumpponen *et al*., 1998). For example, *Cladophialophora chaetospira* activates soil nitrogen and promotes aboveground transfer in Chinese cabbage (Usuki and Narisawa, 2007). Therefore, the most abundant DSE identified by both culture and non-culture approaches, acting as an important mycorrhizal symbiont via melanized septate hyphae formation that removed resource limitation, might promote plant growth. A labeled nitrogen study is required to validate this mechanism.

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Rice growth was markedly different depending on the combination of DSE isolates and pH. Differences in dry weight of DSE inoculated rice compared with non-inoculated rice grown at pH 3.0 (as high as 7.6 fold) were significantly greater than for those DSE inoculated rice grown at pH 4.0 and 5.0 (as high as 1.6 fold and 1.2 fold, respectively). In particular, the root dry weight of *P. fortinii*-treated seedlings was the highest at pH 3.0 with respect to that of the control. Also, we observed that inoculated species of *Phialocephala* effectively promoted plant growth, particularly under acidic conditions. The enhanced shoot biomass via DSE isolate inoculation was most marked in acidic environments, *e.g.*, with 7.6, 1.6, and 1.2 times greater shoot biomass at pH 3, pH 4 and pH 5, respectively. Less promotion of plant growth by inoculation with *Phialocephala* at pH 5 compared with 4 and 3 agar indicated that these DSE isolates likely promote plant tolerance to soil acidity.

Many researchers have reported relatively narrow ranges of pH for the presence or activity of mycorrhizal fungi in soils (Clark, 1997; Postma *et al*., 2007). This is consistent with the observation that most colonized isolates associated with plants were found in acidic agar. Similarly, the colonization of investigated plants with DSE significantly decreased with increasing soil pH (Postma *et al*., 2007). The mechanisms underlying the promotion of plant growth by DSE fungal have been addressed. DSE fungal might help adaptability of crop to acid stress, *i.e.*, low soil pH, and subsequent support of plant growth. The relatively high abundance of DSE supports host survival in stress habitats mainly via high chitin contents and forming melanized septate hyphae and microsclerotia in plant roots (Likar and Regvar, 2013). Also, it might increase the concentration of Mg, known to ameliorate Al toxicity, in the roots of *M.sinensis* to decrease Al activity (Haruma *et al*., 2021).

Here, we validated the effects of these DSE isolates on rice growth, particularly under an extremely low pH condition, e.g., compared to control without inoculation, rice biomass enhanced by 7.6 times after inoculation of *P. fortinii*. DSE show great potential to help host crop resist acidity and thus enable crop cultivation, especially in acidic soil (Postma *et al*., 2007). Acidic soils occupy up to 50% of the arable worldwide, and around 13% of paddy is acid soil. While soil acidification can be a problem for crop yield, these DSE isolates might be used as a management strategy to reduce acidic harm to crops. This, yet, awaits field investigation.

Taken together, this study helps improve our understanding of the community of *Miscanthus condensatus*-associated DSE fungi and their functions. Our findings suggest that DSE have the ability to support rice growth under an extremely acidic conditions, and the formation of melanized septate hyphae and microsclerotia-associated rice tissues might promote increases in rice growth and root biomass via removing stress and resource limitations,

and thus they show marked potential in not only re-vegetation of pioneer plants in post-volcanic ecosystems but also promotion of rice growth.

Conclusion

The present study provided detailed insights into the diversity and function of the endophytic fungal community in *Miscanthus condensates*, using both culture-dependent and -independent approaches. Here, we showed that the fungal community was dominated by isolates of *Phialocephala*, which were abundant and widely distributed in the volcanic deposits. Additionally, we validated the functions of these DSE in rice growth, particularly under acidic conditions, by adopting the approach of isolation-inoculation. Considering that these fungal isolates promote plant adaptation to acidic soil, the identified DSE, e.g., *Phialocephala. fortinii, P. helvetica*, *and Phialocephala* sp., might be potential candidates as plant growth-promoting fungi for either restoring vegetation or promoting rice growth under extreme conditions.

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813 **Tables**

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815 **Table 1.** Summary of the endophytic fungal isolates among three months of sampling in *Miscanthus condensatus*

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Phylum	Class	Blast top-hit	Sequence similarity (%)	Accession number in NCBI	Total number		
					Nov	Mar	Sep
Ascomycota	*Sordariomycetes*	*Acremonium sp.*	98	KT192555.1	4	2	0
		Sarocladium sp.	99	MG649463.1	3	2	0
		Xylariaceae sp.	97	AB741591.1	1	1	0
		Arthrinium phaeospermum	99	MH857420.1	0	2	2
	Leotiomycetes	*Phialocephala fortinii*	97	KJ817297.1	24	17	16
		Phialocephala helvetica	97	MT107593.1	21	36	37
		Phialocephala sp.	99	KT323172.1	11	14	16
		Pezicula ericae	99	NR155653.1	0	5	2
	Eurotiomycetes	*Talaromyces verruculosus*	97	MG748649.1	9	2	2
		Penicillium funiculosum	97	JQ724527.1	3	0	0
	Dothideomycetes	*Pyrenochaetopsis setosissima*	97	LT623227.1	2	2	1
Basidiomycota	*Agaricomycetes*	*Tulasnella calospora*	98	JQ713577.1	1	0	0
		Hypochnicium cremicolor	97	KP814161.1	1	0	0
		Phaeophlebiopsis peniophoroides	98	KP135417.1	0	0	3
		Phlebiopsis gigantea	98	MH114867.1	0	0	3
Dikarya	*Polyporus*	*Polyporus arcularius*	99	KP283489.1	0	1	0
Mucoromycota	*Mortierellomycotina*	*Mortierellales sp.*	97	JQ272348.1	0	2	1
					80	86	83

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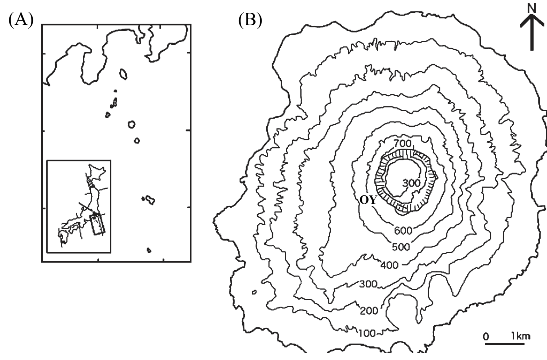

Fig. 1. (A) DEM Map showing the location of Miyake-jima in the western rim of the Pacific Ocean. (B) DEM Map showing study site OY near the summit crater in Miyake-jima

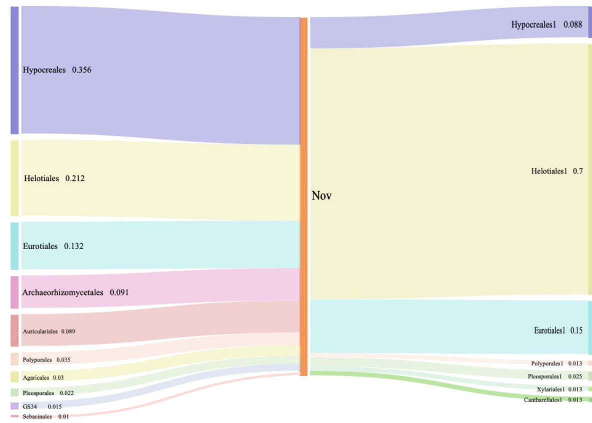

Fig. 2. Composition and relative abundance of endophytic fungi at order level by culture-independent (left) and culture-dependent methods (right)

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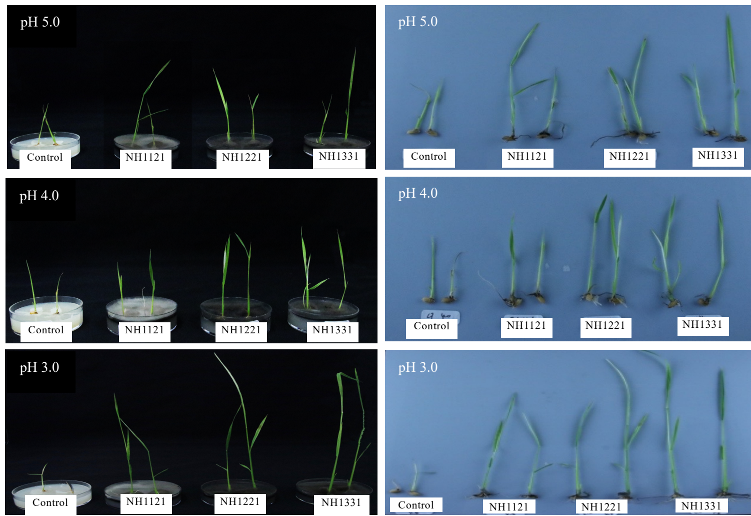

898 **Fig. 3.** Growth and development of rice plants inoculated with DSE fungal isolates under different pH conditions.

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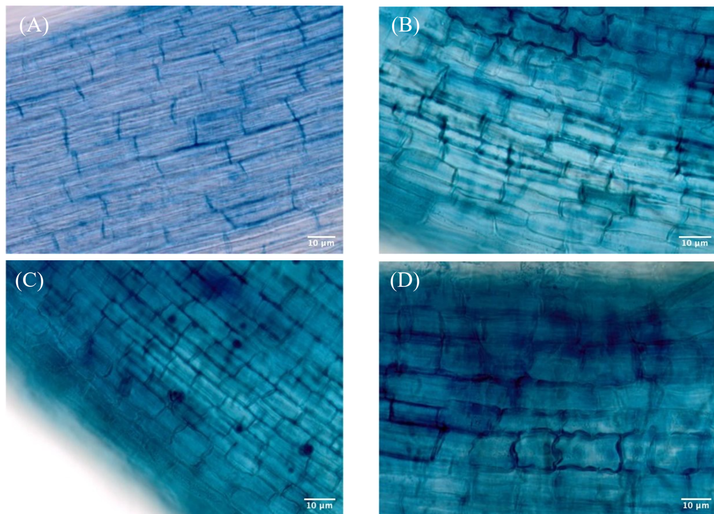

912 **Fig. 4.** (A) Non-treated DSE as control roots. (B) *Phialocephala* sp. (NH1121)-treated roots. (C) *Phialocephala helvetica*
913 (NH1221)-treated roots. (D) *Phialocephala fortinii* (NH1331)-treated roots.
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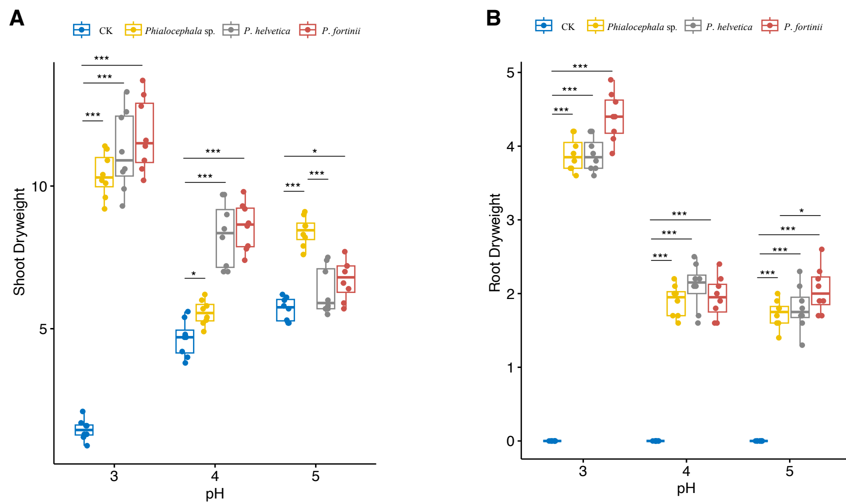
917 

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930 **Fig. 5.** Shoot and root dry weights of rice seedlings inoculated with NH1121 (*Phialocephala* sp.), NH1221 (*Phialocephala*
931 *helvetica*), and NH1331 (*Phialocephala fortinii*) after three weeks of growth on oatmeal agar either at pH 3, pH 4, or pH 5
932 (acidic conditions). There are biological replicates (n=8). Median values are lines across the box with lower and upper boxes
933 indicating the 25th to 75th percentiles, respectively. Whiskers represent the maximum and minimum values. Significance was
934 determined by ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
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