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FUSARIUM SPECIES FROM AN INDONESIAN GENOTYPE OF FOXTAIL MILLET SEEDS

Spesies Fusarium Asal Salah Satu Genotipe Benih Hotong Indonesia

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ABSTRACT

As a generalist pathogen in cereals, *Fusarium* spp. become the most threatening fungi which can conduct its saprogenesis by infecting seeds. Determination of fungal identity and the yield loss risk is needed to modify the effective controlling strategies. However, there is no report on implementing methods for controlling *Fusarium* spp. on foxtail millet (*Setaria italica* L. Beauv.). This research was undertaken from July to September 2020 and November to December 2021 under ambient laboratory conditions to identify and evaluate the pathogenicity of seed-borne *Fusarium* species in foxtail millet. One hundred colonies of seed-borne fungi were isolated from foxtail millet genotype ICERI-6 which was dominated by *Fusarium* spp. Morphological characterization by observing the structure of colonies and microscopical features indicated that the six isolates (Fu1–Fu6) were identical to *Fusarium solani, F. chlamydosporum, F. oxysporum, F. equiseti, F. proliferatum,* and *F. graminearum*, respectively. Molecular identification for the 5.8s rDNA target gene with ITS1 and ITS4 primers has confirmed that the *Fusarium* spp. were determined as mentioned species. Pathogenicity test using potato dextrose agar medium showed that the germination percentage of seed inoculated by *Fusarium* spp. was only 1.2% on average at 7 days after incubation. These species led to germination failure as the seeds were covered by fungal mycelia. Seeds that could escape from germination failure performed necrotic spots on the seedlings. These abnormalities would contribute to low productivity in the field. The study has implication in controlling seed-borne disease and that resistant variety breeding becomes important issues to be addressed for future research.

[Keywords: conidia, fusarium identification, germination failure, pathogenicity test]

ABSTRAK

Fusarium *spp adalah patogen generalis pada tanaman serealia yang menjadi ancaman utama dan mampu melakukan saprogenesis dengan menginfeksi benih. Penentuan identitas cendawan patogen serta risikonya dalam menyebabkan kehilangan hasil diperlukan dalam menyusun strategi pengendalian yang tepat. Namun, belum ada laporan mengenai metode pengendalian* Fusarium *spp. pada hotong* (Setaria italica *L. Beauv.*)*. Penelitian ini dilaksanakan pada bulan Juli hingga September 2020 dan dilanjutkan pada bulan November hingga Desember 2021 pada kondisi laboratorium, untuk mengidentifikasi dan mengevaluasi patogenisitas spesies-spesies* Fusarium *yang terbawa benih hotong. Seratus koloni cendawan yang terbawa benih hotong genotipe ICERI 6 berhasil diisolasi dan didominasi oleh berbagai spesies* Fusarium*. Karakterisasi morfologi yang dilakukan dengan mengamati struktur koloni dan ciri mikroskopik menunjukkan bahwa enam isolat (Fu1–Fu6) teridentifikasi berturut-turut sebagai* Fusarium solani, F. chlamydosporum, F. oxysporum, F. equiseti, F. proliferatum, *dan* F. graminearum*. Identifikasi molekuler pada gen target 5.8s rDNA menggunakan primer ITS1 dan ITS4 telah mengonfirmasi identitas spesies yang sama dengan hasil karakterisasi morfologi. Uji patogenisitas menggunakan media agar-agar dekstrosa kentang menunjukkan bahwa daya berkecambah benih hotong yang diinokulasi isolat* Fusarium *spp. hanya 1.2% pada 7 hari setelah inkubasi. Enam spesies ini menggagalkan perkecambahan dengan miselia menyelimuti seluruh permukaan benih. Benih yang masih dapat berkecambah menunjukkan ketidaknormalan berupa area nekrotik pada kecambah. Ketidaknormalan ini sangat berpotensi menurunkan produktivitas tanaman. Hasil penelitian ini diharapkan dapat menjadi acuan dalam pengendalian penyakit yang terbawa benih dan perakitan varietas tahan.*

[***Kata kunci:*** *identifikasi fusarium, kegagalan perkecambahan, konidia, uji patogenisitas*]

INTRODUCTION

Foxtail millet (*Setaria italica* L. Beauv.) is an economically important cereal crop grown and consumed worldwide, especially in India (Verma et al. 2015; Sharma and Niranjan 2017), China (He et al. 2015; Ni et al. 2017; Zhang et al. 2021), and other parts of Asia, the Middle East, Europe, America, and North Africa (Lata et al. 2013; Saxena et al. 2018). In Indonesia, foxtail millet is a minor cereal crop widely cultivated in the eastern part of Indonesia, such as Sulawesi, Maluku, and Nusa Tenggara. People take it as bird feed (Yulita and Ridwan

2018) and substitute corn for poultry feed (Tirajoh 2015). Foxtail millet has also been consumed and has become an alternative food because of its high fiber content. For example, at least six local foxtail millet cultivars from West Sulawesi are cultivated by local people and processed as traditional foods that are closely related to local traditions in the area (Ramlah et al. 2020). Not only for culinary purposes, but people in East Sumba also usually make porridge from foxtail millet to cure fever.

Considering its potential as alternative food and its advantages of functional compounds, special attention is needed to be performed to foxtail millet cultivation. Seed health is one-factor influencing cultivation success that is also a significant concern in crop production. According to Barret et al. (2016), seeds play a significant role in the vertical transfer of microorganisms like pathogens inter the generation of plants, hence serving as a key source of inoculum for crops. The pathogen transmission can cause various destructive effects on seed quality, including decreasing germination rate. The presence of seed-borne pathogens may cause serious problems in yield loss risk and biochemical changes in produced crops. The seed-borne pathogens could also systematically be transmitted through various mechanisms, which may increase those risks in the next planting season (Agarwal and Sinclair 1997). Therefore, evaluation of seed health status will always be preferred as the important stage to ensure a successful cultivation activity.

A genus of an ascomycete, *Fusarium* spp., threatens crop productivity and food safety regarding the production of several mycotoxins which are more considerable by its both parasitic and saprophytic abilities as seed-borne fungi. These fungi are known to be linked by various small grains that may cause several diseases such as ear blight, ear rot, head blight, stalk rot, and seed rot. Its presence and activity could be varying depending on seed variety, seed storage periods, storage practices, and biotic and abiotic favorable factors (Blanco and Aveling 2018). Fungicide seed dressing as the current controlling method has been performed on *Fusarium* spp. infection on maize but there is still a lack of reports for controlling *Fusarium* spp. on foxtail millet, both preventive and curative methods (Capo et al. 2020).

As a functional grain, foxtail millet might be highly associated with seed-borne *Fusarium* species. The identification of this pathogen is essential and related to its host specificity and cultivars differentiation. In many countries, reports have determined the species of *Fusarium* in foxtail millet. In Indonesia, *Fusarium* spp. and other fungi i.e., *Rhizoctonia, Cladosporium, Helminthosporium,* and *Curvularia* infect foxtail millet seeds with various abnormalities in the plant (Khairani and Ardie 2020). However, based on the field observations, species of seed-borne *Fusarium* from foxtail millet could be more varied with major risks and so the species identification and pathogenicity evaluation were needed regarding the potential of yield loss by *Fusarium* spp. This study aimed to explore, identify, and evaluate the pathogenicity of seed-borne *Fusarium* species in foxtail millet.

MATERIALS AND METHODS

The study was conducted in the Laboratory of Plant Mycology and Laboratory of Plant Bacteriology, Department of Plant Protection, IPB University, from July to September 2020 and November to December 2021. The foxtail millet seeds were genotype ICERI-6 harvested in July 2019 from Indonesian Cereal Research Institute, Maros, South Sulawesi, Indonesia. ICERI-6 was chosen for its desirability as an abiotic-stress tolerant genotype (Lapuimakuni et al. 2018). This genotype has water content 11%, viability 100%, vigor index of more than 33%, protein levels 12%, and carbohydrate level of 73%.

Isolation of Seed-Borne *Fusarium* spp.

One hundred seeds that showed no symptoms of abnormalities were used in this study. The seeds were surface sterilized using NaClO 1% for 10 minutes, then rinsed with sterilized water for 1 minute three times. Seeds were then dried on a sterile tissue paper for 5 minutes to remove the excess water before being placed on potato dextrose agar (PDA) medium added with chloramphenicol and incubated at room temperature (IRRI 1994). After three days, all fungal colonies were recultured on a new PDA medium and incubated for five days for initial identification. The number of each type of fungus that emerged from infected seeds was counted. *Fusarium* colonies in this finding were further investigated. Other fungal colonies were grouped and classified according to their genera followed the book of Watanabe (2010).

Morphological Identification of *Fusarium* species

The colonies were classified into genus *Fusarium* based on microscopy conidial characters and then recultured on a PDA medium to be identified morphologically. The characteristic of colonies including colony color (obverse and reverse) and colony density (cottony or velvety) were observed for differentiation on PDA medium added by lactic acid 0.1% as additional anti-bacteria (Okukawa et al. 2021). Subsequently, each

different *Fusarium* colony was identified mainly based on its macroconidia and microconidia using corn meal agar (CMA) as a semi-selective medium for spores induction (Teixeira et al. 2017). The observation was conducted using a light microscope Olympus CH3 at 400 and 1.000 optical zooms and referred to the manual for *Fusarium* spp. identification (Leslie and Summerell 2006).

Molecular Identification of *Fusarium* species

The result of morphological characterization was confirmed by the molecular approach using polymerase chain reaction (PCR) for the 5.8S rDNA target region. The primers used were a universal primer for fungi i.e. forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') which set 420-825 bp in electrophoresis (Manter and Vivanco 2007). The extraction stages were undertaken referred to methods covering *Fusarium* species (Abd-Elsalam et al. 2003). The PCR reaction was performed using Dream TaqTM Green PCR Mastex Mix (Thermo Scientific, US). Amplification after initial denaturation (94 °C 2 min) was followed by 35 cycles of denaturation (94 °C 50 sec), annealing (55 °C 1 min), extension (72 °C 1 min); and the final extension was at 72 °C (5 min) (Kaewchai et al. 2010). The PCR product was resolved by electrophoresis in 2% agarose gels (1st BASE, SG) using a loading buffer Gel-RedTM Nucleic Acid Gel Stain (Biotium, US) and a kilobase size marker (Geneaid Biotech, TW). The results were then sequenced by 1st BASE Laboratory, Apical Scientific Sdn. Bhd., Malaysia, then analyzed using Bioedit and MEGA X to be aligned to the database in GenBank.

Pathogenicity Test

The pathogenicity test was conducted to confirm the ecological status of *Fusarium* species in plant-pathogen interaction. As many as 30 ml PDA medium were placed into an autoclaved glass bottle. The 7 days-old *Fusarium* spp. colonies (1 cm) were inoculated on top of the PDA medium and incubated for 5 days at room temperature. The foxtail millet seeds were treated with pre-soaking at 37 °C for 30 minutes and continued with hot water treatment of 52 °C for 20 minutes as an early controlling method and suppressing the infection by the pathogen (Khairani and Ardie 2020). After 5 days, surface-sterilized foxtail millet seeds (in a 1% NaClO solution) were placed on the grown fungal colonies (6 seeds for each bottle, and 4 replications) (Fernandez and Chen 2005). Incubation was taken for 7 days before the evaluation was undertaken. As a control, the PDA medium was placed into autoclaved glass bottle without fungal inoculation. Germination failure, necrotic spots, seedling rots, and other abnormalities were recorded to determine that a species performed as plant pathogenic fungi. Normal growth of foxtail millet seedling indicated the performance of non-pathogenic fungi.

RESULTS AND DISCUSSION

Isolation of Seed-Borne *Fusarium* spp.

Fungal colonies from foxtail millet seeds started to grow on 2 days after isolation (DAI) and a few colonies appeared on 5 DAI. Based on the observation, all the 100 seeds were infected means that no one of the seeds was free from fungal infestation. There was no more than one colony appeared from one seed. Because some of the fungal genera have similar colonies characters in the initial growth, the differentiation of colonies was started at 4-5 DAI. Since this was a rapid detection test, this method was not able to detect fungi that may grow over the longer observation period. To detect slow-growing fungi, modification of media composition could be undertaken using additional inhibitors for fast-growing fungi.

The identified 6 fungal genera corresponded and were dominated by *Fusarium* spp. (34%) followed by *Curvularia, Penicillium, Aspergillus, Cladosporium*,

Table 1. Fungi composition in the isolated seed-borne fungi colonies on foxtail millet seeds.

Number of seeds tested	Number of seeds contaminated with fungi	Genera of fungi infecting the seeds	Percentage (%) of infected seeds
100	100	*Aspergillus*	13
		Cladosporium	9
		Curvularia	21
		Fusarium	34
		Penicillium	16
		Rhizoctonia	7

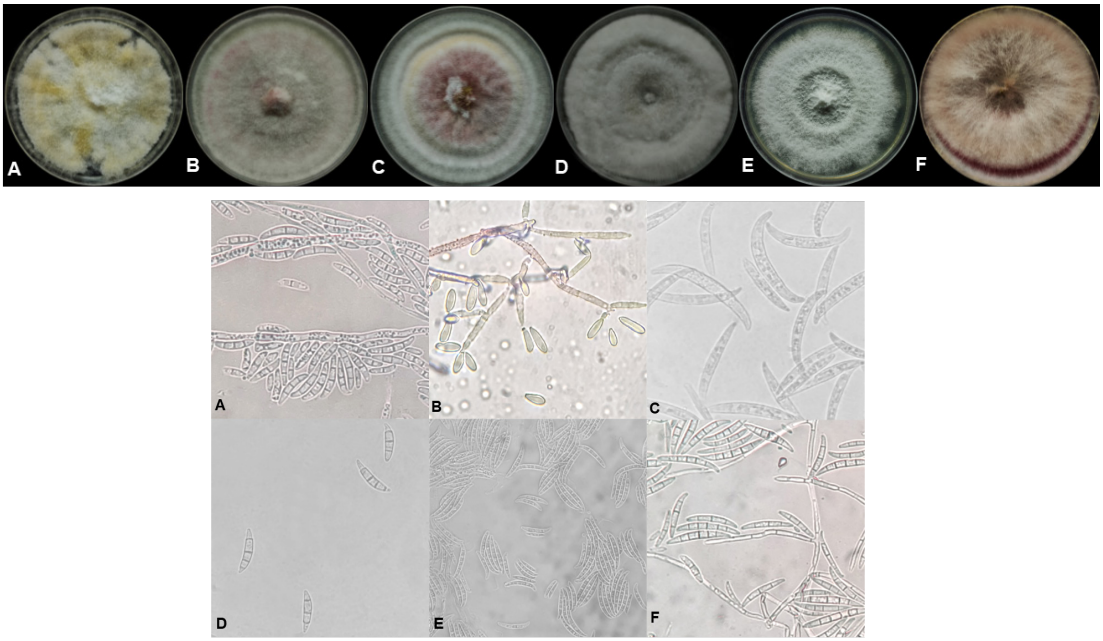

Figure 1. Morphological characters of *Fusarium* species isolated from foxtail millet (A) *F. solani,* (B) *F. chlamydosporum,* (C) *F. oxysporum,* (D) *F. equiseti,* (E) *F. proliferatum, and* (F) *F. graminearum*. Above: *Fusarium* colonies on potato dextrose agar medium; Below: microscopical observation of macroconidia (A, C-F) and microconidia (B) on cornmeal agar medium.

and *Rhizoctonia*, respectively, (Table 1). The majority of fungi found were common fungi isolated from millet seeds as reported in previous studies (Abdel-Hafez et al. 2017; Yago et al. 2018) . Odeph et al. (2021) also reported that the fungal community may appear and be associated with an infection complex.

Based on Table 1, *Fusarium* spp. was dominant. Several reports have revealed the pathological role of *Fusarium* spp. in foxtail millet. *Fusarium* species have been detected and caused severe disease of important tissues or organs of foxtail millet in some cultivated areas. According to Li et al. (2015) *Fusarium graminearum* caused ear rot in foxtail millet in China. Zheng et al. (2019) reported that *F. equiseti* was identified as the pathogen causing ear rot in northwest regions of China. This *F. equiseti* was isolated from the ears of foxtail millet with severe symptoms (ears with pink or salmon-colored mold at the ear tip of plants). At the same time, Xu et al. (2019) reported that *F. equiseti* infected the panicle of foxtail millet and caused panicle wilt and rot with an incidence of about 85% to 100% in Liaoning Province and Chifeng City, Inner Mongolia, China. Furthermore, Kong et al. (2022) had informed that another species of *Fusarium*, i.e. *F. asiaticum*, was responsible for panicle rot disease that destroyed more than 85% of panicles in the commercial field.

Although there was much research about *Fusarium* in foxtail millet, there was a lack of reports from Indonesia about the diversity of *Fusarium* species and the risk to plant productivity. Furthermore, its position as a seed-borne pathogen also has not been widely explored. Since *Fusarium* spp. plays the role as a major group of fungi to which need high concern, a more detailed determination of its identity and the ecological role was conducted, especially for pathogenic species.

Morphological Identification of *Fusarium* species

The 34 isolates of *Fusarium* species grew rapidly in a PDA medium at 26–28 °C temperature. The isolates were classified on their morphological appearance especially colony color and colony density. Multiple stages of confirmation were needed to make sure that these isolates were free from contamination. There were some changes in *Fusarium* pigmentation compared to its first occurrence from inside the seed tissue, because of reculturing stages in synthetic medium. The classification was resulting six different colonies namely Fu1, Fu2, Fu3, Fu4, Fu5, and Fu6.

The colony color varied from white cream to pink-violet. Fu2 and Fu6 were velvety while the remained were cottony. Macroconidia were found in all colonies except for Fu2 and microconidia were absent in Fu4 and Fu6 (Table 2 and Figure 2). Further identification of the macroscopy and microscopy morphological characters showed that Fu1–Fu6 were identified as *Fusarium solani, F. chlamydosporum, F. oxysporum, F. equiseti, F. proliferatum,* and *F. graminearum*, respectively (Table

Table 2. Characteristics of the *Fusarium* species isolated from millet seed genotype ICERI 6 cultured on potato dextrose agar.

Species	Colony Appearances			Microscopical characteristics	
	Obverse	Reverse	Density	Macroconidia	Microconidia
F. solani	Cream	White	Cottony	Straight, wide, 3–4 septae, blunt end	Ellipsoid, long phialide, 1 septae
F. chlamydosporum	Pink	Pink	Velvety	Absent	Clustered, tree-like, no septae
F. oxysporum	Violet	Violet	Cottony	Slightly curved, 3 septae, foot-shaped	Ovoid, short phialide, no septae
F. equiseti	White	Yellow	Cottony	Whip-like, 5–6 septae, foot-shaped	Absent
F. proliferatum	Beige	Orange	Cottony	Slender, 3–5 septae, slightly curved	Oval, 0-1 septate
F. graminearum	Pink	Red	Velvety	Moderately curved, thick-walled, 5–6 septae	Absent

Figure 2. Visualization of the pathogenicity test results of *Fusarium* spp.on foxtail millet; (A) healthy seedlings with no symptoms, (B) necrotic in seedlings arrow, (C) mycelia covered the seeds with the symptoms of germination failure.

2) corresponding to 35.3%, 11.8%, 8.8%, 14.7%, 20.6%, and 8.8%, respectively. The compositions of mentioned species were 35.3%, 11.8%, 8.8%, 14.7%, 20.6%, and 8.8%, respectively.

Molecular Identification of *Fusarium* species

Based on DNA amplification using primer ITS1 and ITS 4, these six isolates were detected at the size of 500–600 bp. These were similar to the report by El-Rabbat et al. (2018) which investigated 18 *Fusarium* species from grains in Egypt. DNA sequencing confirmed that our isolates were *F. solani*, *F. chlamydosporum*, *F. oxysporum*, *F. equiseti*, *F. proliferatum*, and *F. graminearum* with >99% homology (Table 3). Fard et al. (2014) reported more varying species i.e., *F. solani*, *F. fujikuroi*, *F. diversisporum*, *F. verticilloides*, *F. semitectum*, *F. equiseti*, *F. crookwellense*, and *F. acuminatum* as important fungal pathogens to foxtail millet production in Iran.

Pathogenicity Test

The performance of foxtail millet seeds on PDA medium in a glass bottle showed that the seeds in uninoculated media started to germinate after 3 days and grew normally to emerge a leaf in 8 days. However, seeds inoculated with *F. solani, F. equiseti, F. proliferatum*, and *F. graminearum* could not be germinated at all. Confirmation was conducted by observing the ungerminated seeds and found that the *Fusarium* colonies covered not only the upper surface but also the lower surface of seeds which may inhibit the seeds from surrounding growing resources.

Only a few seeds inoculated with *F. chlamydosporum* and *F. oxysporum* at the percentage of 3.3% and 4.2%, respectively, were germinated but have a necrotic area in

Table 3. Nucleotide homology of 5.8S rDNA gene of *Fusarium* species compared to other fungi in Genbank NCBI.

Fusarium species	Accession code	Homology (%)	Query cover (%)
F. solani	MT448894.1	99.61	99
F. chlamydosporum	MN882831.1	99.05	99
F. oxysporum	MK841429.1	99.06	98
F. equiseti	MT937067.1	99.22	99
F. incarnatum	MK748309.1	99.42	100
F. graminearum	MN017275.1	99.27	99

Table 4. *In vitro* pathogenicity test of *Fusarium* spp. to seeds of foxtail millet.

Species	Germination (%)	Seedling abnormality description
F. solani	0	Germination failure, mycelia covered the seeds
F. chlamydosporum	3.3	Germination failure, few have a necrotic area
F. oxysporum	4.2	Germination failure, few have a necrotic area
F. equiseti	0	Germination failure, mycelia covered the seeds
F. proliferatum	0	Germination failure, mycelia covered the seeds
F. graminearum	0	Germination failure, mycelia covered the seeds

the tip of seedlings (Table 4 and Figure 2). On average, we only have 1.2% of seeds that could germinate. This is a very low value of germination percentage that which still did not show any increase until 14 days so, we conclude that abortion has occurred on foxtail millet seeds triggered by *Fusarium* spp. This germination failure is needed to be furtherly investigated because this becomes a serious concern in plant productivity and seed production.

This observation result indicated that our six *Fusarium* species were pathogenic. Pathogenic *Fusarium* species were known to produce several substances that caused germination failure and seed abortion. For example, deoxynivalenol toxins were reported to be highly correlated with reduced germination capacity (Tekle et al. 2013). Amza (2018) summarized that in the pre-emerging stage, seed abortion is one of the negative impacts of seed-borne fungi besides seed rot, reduced seed size, and many physiological changes, especially for *F. graminearum*. Even if the infected seeds have successfully escaped the germination failure in the pre-emerging stage, *F. solani, F. oxysporum*, and *F. equiseti* lead seed to be susceptible to damping-off (Berg et al. 2017). Separated observation showed that *Fusarium* species caused rotten seeds which may lead to lower productivity and may also increase the number of infested produced seeds. Further confirmation is needed to investigate the importance of seed treatment to prevent production losses. This research revealed the varying *Fusarium* species from foxtail millet seeds in Indonesia with its initial evaluation of their pathogenicity which might be considered on yield loss measurement.

Besides the agronomic yield loss, the presence of this *Fusarium* species leads us to evaluate the grain safety. *Fusarium* species not only could reduce the proteins and other beneficial substances in foxtail millet but also mycotoxigenic. For example, Choi et al. (2021) revealed that *F. graminearum*, *F. incarnatum*, and *F. equiseti* from proso millet produced deoxynivalenol, nivalenol, zearalenone, and T-2/HT-2 mycotoxin. Other fusariotoxins besides mentioned ones, i.e., trichothecenes and fumonisins have been being attracted to unavoidable concerns as an emerging issue on the safety of agricultural commodities (Ekwomadu et al. 2021).

CONCLUSION

From 100 foxtail millet seeds, the ICERI-6 genotype was identified consisting of *Fusarium* spp. colonies dominated the seeds infection (34%), which were classified into 6 different species. The species were identified as *Fusarium solani, F. chlamydosporum, F. oxysporum, F. equiseti, F. proliferatum,* and *F. graminearum* confirmed by morphological and molecular characterization. The fungi *F. solani* (35%) and *F. proliferatum* (21%) were dominant followed by other species. All the six species were pathogenic as indicated by germination failure and seedlings with necrotic spots. Since the very low germination percentage and abnormalities occurred owing to the infection, *Fusarium* species became a serious threat to foxtail millet productivity.

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