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Short Notes

Fusarium annulatum causes Fusarium rot of cantaloupe melons in Spain

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Summary. During the summer of 2018, there was high incidence of fruit rots of cantaloupe melons (*Cucumis melo* var. *cantalupensis*) in Murcia province, south-eastern Spain. The fruits showed development of whitish mycelium and pulp softening. Morphological and molecular analysis of the internal transcribed spacer (ITS), translation elongation factor 1-α (*TEF1-α*) and the second largest subunit of RNA polymerase (*RPB2*) genes confirmed *Fusarium annulatum* as the causal agent of the disease. A phylogenetic study indicated that *F. annulatum* is in the *Fusarium fujikuroi* species complex (FFSC). Pathogenicity of the isolate was determined on healthy fruit verifying Koch's postulates. The first symptoms of fruit rot were observed 3 d after inoculations at 28°C. Fruit infections only occurred in artificially wounded melons, and *F. annulatum* was re-isolated from the wounds. This disease appeared after fruit harvesting, and could generate substantial economic losses mainly in fruit destined for foreign markets due to long transportation times. This is the first report of melon fruit rot caused by *F. annulatum* in Spain.

Keywords. *Cucumis melo*, fungal pathogen, pathogenicity test, first report, postharvest disease.

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INTRODUCTION

Melon (*Cucumis melo* L.) is widely cultivated in south-eastern Spain. Total melon production for Spain during 2018 was 664,353 t, the Region of Murcia being the largest producer, with 220,768 t obtained from the 5,576 ha dedicated to melon cultivation (MAPA, 2018).

Fusarium species are important cucurbit pathogens, causing diseases in host fruits, stems and roots (Chehri *et al*., 2011), and several *Fusarium* species have been reported as causal agents of fruit rot diseases. These include; *F. equiseti* (Corda) Sacc., *F. pallidoroseum* syn. *F. semisectum* (Cooke) Sacc., *F. solani* (Mart.) Sacc. (Snowdon, 1991); *F. graminum* Ces., *F. acuminatum* Ellis & Everh, *F. culmorum* (W.G. Smith) Sacc., *F. moniliforme* J. Sheld, *F. pallido-*

roseum syn. *F. incarnatum* (Roberge) Saccardo, *F. scirpi* syn. *F. longipes* Wollenweber & Reinking, *F. oxysporum* f. sp. *melonis* Snyder & Hansen (Zitter *et al*., 1998), *F. graminearum* Schwabe, *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg, and *F. roseum* syn. *F. sambucinum* Fuckel (Kim and Kim, 2004).

Regarding the *Fusarium* species affecting cucurbits in Spain, the following species have become particularly important: *F. oxysporum* (Martínez *et al*., 2003), and *F. solani* f. sp. *cucurbitae* Snyder & Hansen on watermelon grafted to pumpkin (Armengol *et al*., 2000), zucchini (Gómez *et al*., 2008) and melon (Gómez *et al*., 2014). In many studies only Fusarium rots caused by *Fusarium* sp. are mentioned, without specifying the pathogenic specifies, so that diagnostic information is lost. Thus, increasing knowledge of the diversity of species that cause melon rots through accurate diagnoses of the causal pathogens is relevant, because this would allow improvement of disease management practices.

Previous studies have not been carried out in Spain on the postharvest fruit rots of melons, caused by *Fusarium* sp., which can be favoured by the climatic conditions of the Mediterranean Basin. These diseases affect melon plantations and the subsequent marketing of melon fruits.

The present study aimed to identify the *Fusarium* species causing fruit rots in cantaloupe melons (*C. melo* var. *cantalupensis*) fruits. For this purpose, the organism associated with the symptomatology was isolated, its morphological characteristics were described, and the species was identified by molecular methods. Koch's postulates were applied by means of pathogenicity tests of the percentage of infection in inoculated healthy fruit, as well as the severity of the resulting disease.

MATERIALS AND METHODS

Sampling and fungal isolation

Melon producers in Campo de Cartagena, Murcia province, Spain (37°36'18.4"N, 0°59'10.4"W), supplied cantaloupe fruits showing rot symptoms to the Phytopathology Laboratory of the Crop Protection Group of the Polytechnic University of Cartagena (UPCT) (Grupo de Protección de Cultivos de la Universidad Politécnica de Cartagena - UPCT). The fruits were harvested during July 2018, from different producing areas of Murcia province.

Fungus isolations were carried out using two different methods. Firstly, superficial mycelium developing on the skins of the fruits was transferred to Petri dishes containing Potato Dextrose Agar (PDA) (Scharlab), amended with 0.1 g L^{-1} of streptomycin sulphate. In addition, diseased fruit tissues were disinfected with 1% NaOCl for 5 min, then sprayed with 96% ethanol, and subsequently rinsed with sterilized distilled water. Fragments of approx. 0.5 × 0.5 cm of the infected tissues were aseptically taken and placed onto PDA. The culture plates were incubated in darkness at constant 26°C for 7 d. After this period, tips of hyphae from emerging colonies were transferred to new PDA plates to obtain pure cultures.

Morphology and molecular characterization of isolated fungi

The morphology of all isolates was similar, and the isolate MLFR-09 was used as a representative strain in for further study. Mycelium fragments (5 × 5 mm) were transferred to the centres of 90 mm diam. PDA plates (four replicates), which were incubated in darkness at 26°C to assess colony development. To allow the development of microscopic characteristics, the colonies were also grown on Spezieller Nährstoffarmer Agar (SNA) (Leslie and Summerell, 2006), incubated with a 12 h light/12 h darkness regime. Mycelium growth data, based on the perpendicular and horizontal diameters of colonies, were measured with a calliper. The average of both measurements was recorded after 4 d of incubation on PDA or 6 d on SNA. The colour of the colonies was evaluated after 14 d of growth. After 10 days of incubation on both media, samples were prepared for microscope observation. The criteria of Bugnicourt (1952), Nelson *et al*. (1983), and Yilmaz *et al*. (2021), taking into consideration presence of conidiophores and conidia on aerial mycelium, conidiogenous cells, and production of sporodochia and chlamydospores. The average, maximum and minimum measurements were recorded for each identified structure (n = 30). Morphological features were observed using an Olympus BX50F optical microscope, and photomicrographs were taken of each observed fungal structure using an Olympus SC20 camera.

Molecular identification and phylogenetic analysis

To confirm the identity of isolate MLFR-09, partial gene fragments of the ITS region, translation elongation factor 1-α (*TEF1-α*), and the second largest subunit of RNA polymerase (*RPB2*) were analysed. Total genomic DNA was extracted from fresh mycelium using the Animal and Fungi DNA Preparation Kit (Jena Bioscience).

The ITS region was amplified with the primer pair ITS1 and ITS5 (White *et al*., 1990), the translation elon-

gation factor 1-α (*TEF1-α*) with ef1 (O´Donnell *et al*., 2010) and ef2 (O´Donnell *et al*., 1998), and the RNA polymerase II second largest subunit (*RPB2*) with the primer pair RPB2-5F2 (Sung *et al*., 2007) and RPB2-7cR (Liu *et al*., 1999).

DNA templates were PCR-amplified on a Veriti 96-Well Thermal Cycler (Applied Biosystems). The PCR amplifications were each carried out in a total volume of 50 µL, containing 1 µL (50 pmol μL^{-1}) of each of the universal primers, 0.25 µL (5U μL^{-1}) of Taq DNA polymerase (Takata, Clontech Laboratories, Inc.), 5 µL of 10× reaction buffer, 2.5 µL of DMSO, 4 µL of dNTP mixture (10 mM), 31.25 µL of sterile filtered water, and 5 µL of DNA sample (150-200 ng genomic DNA).

PCR conditions for ITS and *RPB2* regions were as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The PCR programme for *TEF1-α* amplification consisted of one cycle of 5 min at 94°C, 10 cycles of 45 sec at 94°C, 45 sec at 55°C, and 1.5 min at 72°C, 30 cycles of 45 sec at 94°C, 45 sec at 52°C, and 1.5 min at 72°C, and a post elongation step of 6 min at 72°C.

DNA fragments were purified using an Ultra-Clean PCR Clean-Up Kit (MoBio Laboratories Inc.). Sequencing was carried out using ABI PRISM Big-Dye Terminator Cycle Sequencing Ready reactants and an ABI3730XL sequencer (Applied Biosystems) at the Central Service of Support to Experimental Research (SCSIE) of Valencia University. Sequencing primers were the same as those used in the amplification reaction. The sequences of three repetitions of the isolate MLFR-09 were analysed.

The BLAST program was used to search homologous MLFR-09 sequences in the NCBI databases (http://www.ncbi.nlm.nih.gov/).

Pathogenicity test and evaluation of disease severity

For the pathogenicity test, healthy cantaloupe fruits at physiological maturity were used at 10 d after harvesting (≥ 10° Brix), and with diameters between 16 and 20 cm and approx. weight of 1–1.5 kg. The MLFR-09 isolate was first grown on PDA at 26°C to allow sporulation. The fruits were surface sterilized following the procedure described above. One group of fruits was wounded with a sterile needle and another group was left uninjured. A suspension of conidia (1×10^6 conidia mL^{-1}) was prepared in sterile distilled water, and 50 µL were pipetted onto the wound/surface of each healthy melon, while the experimental controls were similarly treated with sterile distilled water. Ten fruits were used per inoculation treatment, and ten were used in the control treatment. The fruits were kept at 28 ± 2°C for 7 d. Relative humidity was maintained constant by covering the fruits with plastic bags, and the test was carried out twice. Development of any symptoms was checked and recorded daily from inoculation onwards. The percentages of melons that developed disease were determined. To assess the severity of any rot in each melon, a disease severity index (DSI; Promwee *et al*., 2017) was applied, and following the scale proposed by Wonglom and Sunpapao (2020):

$$DSI\ (\%) = \frac{\Sigma\ (Score\ Amount\ of\ fruits\ )}{Maximum\ score\ \times\ Total\ number\ of\ fruits} \times 100$$

The pathogen was re-isolated and re-identified with molecular methods to verify Koch's postulates.

RESULTS

Disease description

The symptoms the disease comprised brownish areas (diam. up to 6 cm) of the melon skins, which were covered with white mycelium, on different areas of the exocarps, especially in the areas close to the stalks (Figure 1, a and b). These mycelium masses were surrounded by darkened and softened exocarps. The pathogen also colonised the inner tissues of the fruits, and internal lesions of the mesocarps reached a depth of approx. 4 cm, but which could also reach the fruit placental tissues, where sparse mycelium developed (Figure 1, c). The fruit pulp lost consistency, becoming watery as a result of cell degradation, even leading to internal browning in the areas near the skins.

Molecular and phylogenetic study of the isolated fungus

PCR amplification of ITS, *TEF1-α* and *RPB2* genes provided fragments, respectively, of approx. lengths 582, 709 and 644 bases. The PCR amplifications of the three repetitions of isolate MLFR-09 were identical, so the sequences of this isolate were deposited in GenBank under the accession number MZ355136 for ITS, OL960473 for *TEF1*-α, and OL944300 for *RPB2*. A BLAST search in GenBank (NCBI) revealed the sequence of the ITS gene region to be 100% identical to *F. annulatum* (Accession No. MH862668, reference strain CBS 738.97). Translation elongation factor 1-*α* (*TEF1-α*) showed 99.30% sequence identity with sequence

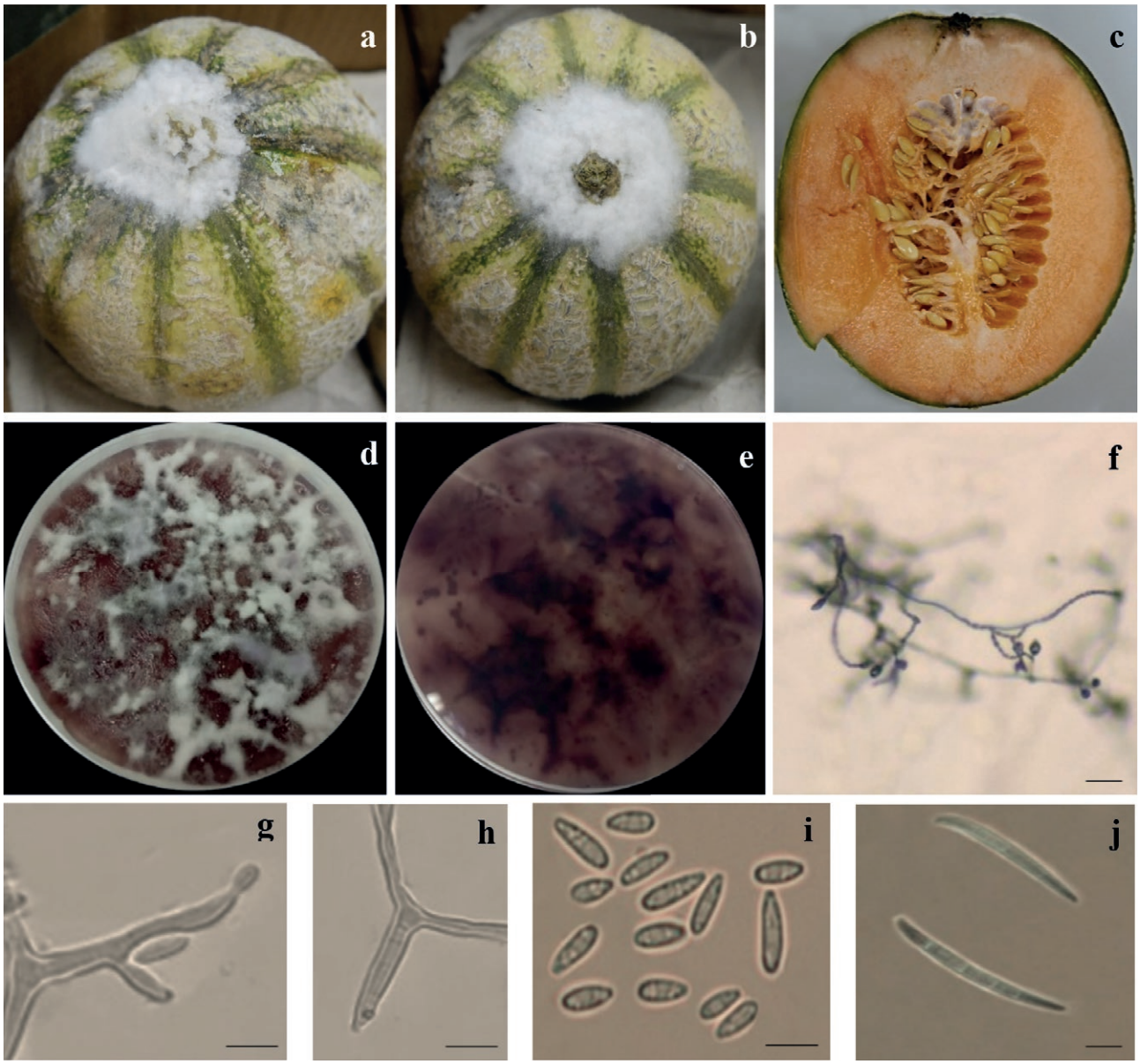

Figure 1. External disease symptoms caused by *Fusarium annulatum* in cantaloupes after natural infections (a, b), and rot of the internal tissues (c). Colony of *F. annulatum* isolate MLFR-09 on potato dextrose agar after 14 d at 26ºC, top view (d) and reverse view (e). Microconidial chains (f), polyphialide (g), monophialide (h), microconidia (i) and macroconidia (j). Scale bars: f = 20 µm, g, h, i and j = 10 µm.

MT010994, and the second largest subunit of RNA polymerase (*RPB2*) showed 99.69% sequence identity with sequence MT010983, both sequences corresponding to the CBS 258.54 type strain of *F. annulatum* (Table 1).

A BLAST search of three sequences in the *Fusarium*-ID database (Geiser *et al.*, 2004) showed that the MLFR-09 isolate belonged to the *Fusarium fujikuroi* species complex (FFSC).

Phylogenetic analysis supported the results obtained from the molecular analysis, confirming that isolate MLFR-09 was *F. annulatum*. The isolate was deposited in the culture collection (Microorganismos de la Agricultura, Poscosecha y Sostenibilidad (MAPYS), Escuela Técnica Superior de Ingeniería Agronómica (ETSIA), Universidad Politécnica de Cartagena (UPCT) (Microorganisms of Agriculture, Postharvest, and Sustainability, Higher Technical School of Agronomic Engineering, Polytechnic University of Cartagena (UPCT), Murcia, Spain).

Table 1. BLAST search for gene sequences of the *Fusarium annulatum* isolate MLFR-09, compared with the reference sequences obtained from type culture material.

GenBank accession No. (MLFR-09)	DNA target	BLAST match sequence			
		Reference accession No.	Type material	Coverage (%)	Identity (%)
MZ355136	ITS, rRNA[a]	*Fusarium annulatum* MH862668	CBS 738.97	98	100
		Fusarium concentricum MH862659	CBS 450.97	96	99.82
		Fusarium fujikuroi NR_111889	CBS 221.76	95	99.82
OL960473	*TEF1*-α[b]	*Fusarium annulatum* MT010994	CBS 258.54	100	99.30
		Fusarium globosum MT010993	CBS 428.97	100	98.31
		Fusarium fujikuroi AB725605	CBS 221.76	93	96.38
OL944300	*RPB2*[c]	*Fusarium annulatum* MT010983	CBS 258.54	100	99.69
		Fusarium concentricum MT010981	CBS 450.97	100	97.52
		Fusarium proliferatum MN534272	CBS 480.96	92	97.32

[a]ITS, internal transcribed spacer; rRNA, ribosomal gene.
[b]*TEF*-α, translation elongation factor 1-α gene.
[c]*RPB2*, second largest subunit of RNA polymerase gene.

Fungus morphology

The mean diameter of the colonies of *F. annulatum* MLFR-09 grown on PDA was 54 ± 5 mm after 4 d of incubation in darkness. Development of the isolate was also assessed on SNA after 6 d incubation, where the average diameter of the colonies was 84 ± 2 mm. The aerial mycelium developed in SNA was sparse and whitish, extending over the entire medium surface in each culture plate.

Fusarium annulatum MLFR-09 culture had characteristic violet pigmentation on PDA (Figure 1, d). On the sides, the colour was deep purple (Figure 1, e). In general, the aerial mycelium had a cottony appearance, was initially white but gradually turned violet as it aged, and becoming grey in some areas. Deep purple sporodochia were occasionally observed. The scant macroconidia had straight, thin, partitioned arrangements (3–4 septa). Macroconidium sizes ranged from 20 to 55 µm length and from 3.7 to 5 µm width; the macroconidium apical cells were blunt, and the basal cells were foot-shaped (Figure 1, j). The abundant microconidia were observed in long chains (Figure 1, f) supported by monophialides (Figure 1, h) and polyphialides (Figure 1, g). No septa were detected in the microconidia, their shapes varied from obovoid or nearly oval with a truncate bases, to fusiform, while they ranged from 5.2 to 13.5 × 2.5 to 3.2 µm (Figure 1, i). No chlamydospores were observed.

The MLFR-09 isolate was morphologically compared with the first published description of *F. annulatum* in Bugnicourt (1952), and also referenced in Nelson *et al*., (1983) and Yilmaz *et al*. (2021) (Table 2).

Pathogenicity

One week after inoculation, white mycelium covered the wounds of the fruits inoculated with *F. annulatum* MLFR-09 (Figure 2, b). No disease was detected in the uninjured melons or in the control treatment (Figure 2, a and c). Symptoms caused by *F. annulatum* MLFR-09 began to appear 3 d after inoculation. On the fruit surfaces, brown necrotic haloes initially appeared around the emerging mycelium, accompanied by the loss of structural rigidity and depression of the fruit exocarps in the same areas. The advance of the pathogen was also evident in the mesocarp of fruits, where there was a change in the colour of the pulp, which became soft with a woolly texture, reaching a depth of 3 cm after 7 d (Figure 2, d). Cross-sectioning of the fruits allowed the tissues between the mesocarp and exocarp to be analysed; the structure turned brown and oily as a result of the necrotic dehydration that had occurred.

Pathogenicity of *F. annulatum* MLFR-09 to cantaloupe fruit was confirmed, as shown in Table 3. The infection rate was 100% when the fruits were artificially wounded and 0% in the non-wounded and inoculated fruit. The mean IDS was 89.75% for wounded and 0% for non-wounded and inoculated fruit. Symptoms of fruits in which the disease developed was compared with the control treatment in which no disease appeared (Figure 2). The severity of the damage was similar to that caused to the fruits by natural infections at the time of isolation of the pathogen (Figure 1).

Microscopic analysis of the pathogen present in the wounds showed that *F. annulatum* that had been inocu-

Table 2. Morphological characteristics of *Fusarium annulatum* isolate MLFR-09, causing fruit rot in cantaloupes, compared with previous descriptions of *F. annulatum* by Bugnicourt (1952), Nelson *et al.* (1983), and Yilmaz *et al.* (2021).

Morphological characteristics	*Fusarium annulatum* grown on Corn Meal Agar (CMA)	Isolate MLFR-09 grown on Potato Dextrose Agar (PDA)
Colony top view	Aerial mycelium absent or late-developed, very light, powdery, slightly dispersed, white in colour. Blackish-purple pigments are normally formed in synthetic cultures	Aerial mycelium with cottony appearance, initially white, but gradually turning violet with age. It even had a grey colouration in some areas
Colony reverse view	*ND*	Accentuated purple colouration
Macroconidia		
Shape	Thin-walled, strongly curved and sickle-shaped, with the basal cell clearly foot-shaped	Straight and slender; the apical cell was blunt and the basal cell foot-shaped
Length (μm)	13-58	20-55
Wide (μm)	1.9-3.3	3.7-5
Number of septa	3-6	3-4
Microconidia		
Shape	Cylindrical or claviform with a truncate tip	Ovoid or nearly ovoid with a truncate tip and rarely fusiform
Length (μm)	4.7-14.4	5.2-13.5
Wide (μm)	1.7-2.3	2.5-3.2
Number of septa	0-1	0
Chlamydospores	Absent	Absent

ND = not described.

Table 3. Pathogenicity test and disease severity index (DSI) of caused by *Fusarium annulatum* isolate MLFR-09 on *Cucumis melo* var. *cantalupensis* fruits.

Inoculum	Percentage infection		Average DSI (%)	
	Wounded	Not wounded	Wounded	Not wounded
Spore suspension (MLFR-09)	100	0	89.75 ± 11	0
Sterile distilled water	0	0	0	0

lated in the pathogenicity tests was also recovered from the fruits with symptoms (data not presented). The morphological and molecular characteristics of the inoculated isolate were maintained, verifying Koch's postulates.

DISCUSSION

This research completed a morphological, molecular, and pathogenicity characterization of *F. annulatum* as a fruit rot pathogen of cantaloupe melons collected from different producing areas of Murcia province of Spain.

Fusarium annulatum is a pathogen found in tropical and temperate climates, affecting a variety of crops (Yilmaz *et al.*, 2021). According to Nelson *et al.* (1992), this fungus is considered an uncommon species, partially because *F. annulatum* has often been misidentified as *F. proliferatum* (Yilmaz *et al.*, 2021). In Spain, *F. proliferatum* has been reported as a frequent pathogen in the production of garlic, causing rots during storage (Gálvez *et al.*, 2017), while it has also been detected in strawberry crops, where it causes vascular wilt (Borrero *et al.*, 2019). In Mexico, Rivas-García *et al.* (2018) found *F. annulatum* causing rot in muskmelon, where affected fruits showed tissue darkening and thickening, with dehydrated and pinkish white exocarps. In the present case, the pink-violet colouration was not detected in the mycelium that developed in the fruit, but was detected during subsequent isolation of the pathogen on synthetic culture medium.

Fusarium annulatum was described by Bugnicourt (1952) as producing ring-shaped macroconidia. Based on this original description, Nelson *et al.* (1983) indicated that *F. annulatum* was basically a *F. proliferatum* with strongly curved macroconidia. However, Yilmaz *et al.* (2021) noted that this feature is atypical of the species because most isolates of *F. annulatum* tend to produce straight macroconidia (Figure 1, g).

Fusarium annulatum belongs to the *Fusarium fujikuroi* species complex (FFSC) (Yilmaz *et al.*, 2021), formerly designated as the *Gibberella fujikuroi* (GFC) species complex (Wigmann *et al.*, 2019). Three clades form

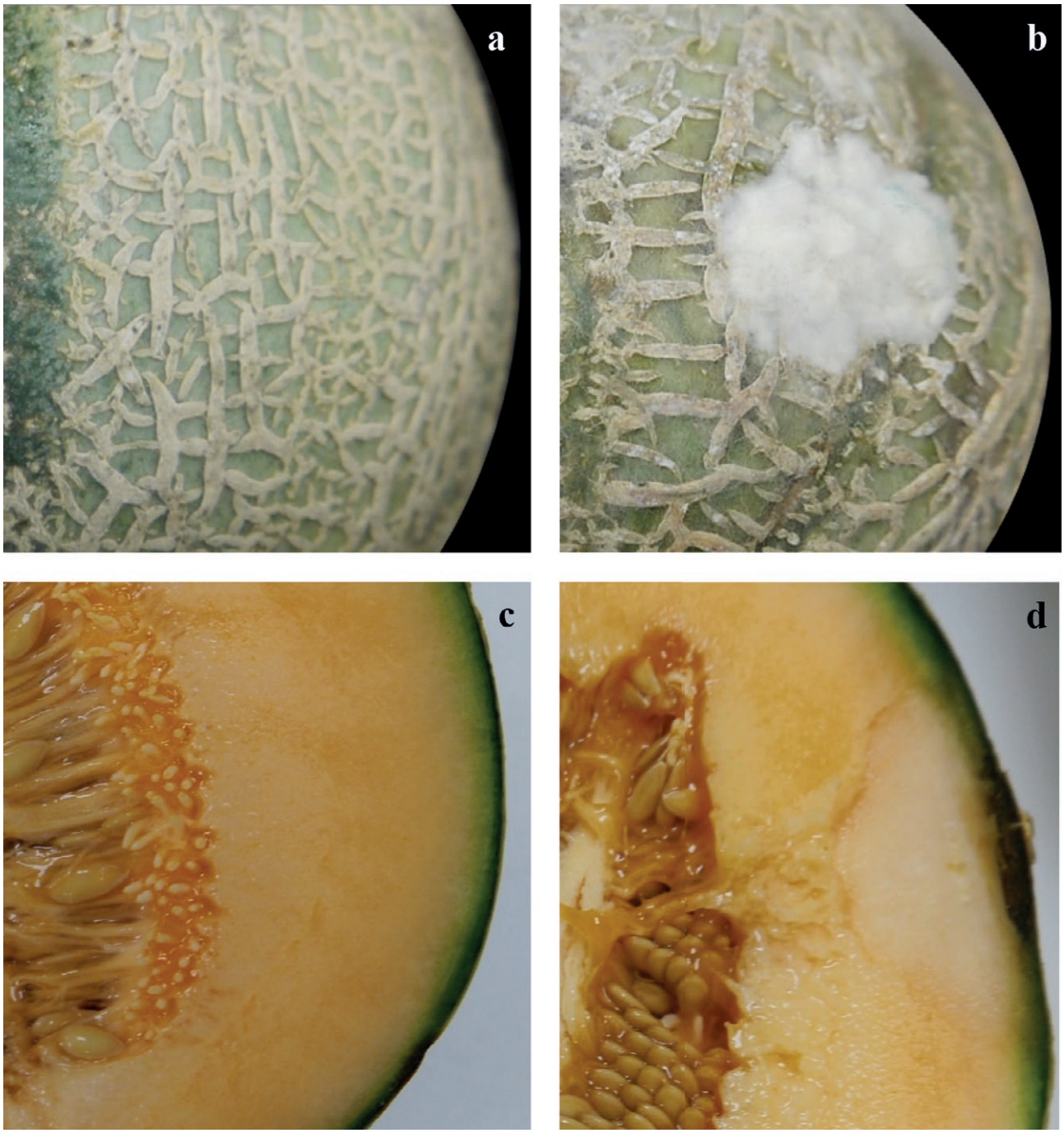

Figure 2. Morphological changes in cantaloupe (*Cucumis melo* var. *cantalupensis*) fruits, in pathogenicity tests 7 days after inoculation with *Fusarium annulatum*. a and c, Control fruit inoculated with sterile distilled water. b and d, Fruit inoculated with *F. annulatum* isolate MLFR-09.

the GFC complex, with *F. annulatum* belonging to the Asian clade (O´Donnell *et al*., 1998).

The pathogenicity test in the present study confirmed that melon fruits must be naturally wounded (mechanical injury) for the pathogen inoculations to be effective (Figure 2), as was also noted by Wonglom and Sunpapao (2020). Nuangmek *et al*. (2019) suggested that cutting the fruit peduncles during harvesting

could be the source of infections in the field. Another determining factor could be the texture of the surface tissues of some commercial melon varieties, with natural cracking facilitating pathogen infections. Fruit cracking disorders and open netting areas due to a defective synthesis of lignin and suberin are considered natural infection pathways for pathogens (Martínez *et al*., 2009). However, Champaco *et al*. (1993) commented on the possible association between the root rot caused by *Fusarium* species and fruit rot, since melons grow on the soil surface which would act as an inoculum reservoir. If pathogen penetration occurs prior to, or during, harvesting procedures, *Fusarium* survival in the soil and in the plant debris from previous crops is likely to be important.

This is the first report of *F. annulatum* causing rot in cantaloupe fruits in Spain. The results presented here provide the basis for new disease management strategies, based on the precise identification of the causal agent of this disease.

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