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Neocosmospora keratoplastica, a relevant human fusarial pathogen is found to be associated with wilt and root rot of Muskmelon and Watermelon crops in Spain: epidemiological and molecular evidences

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Abstract Some taxa of the *Fusarium solani* species complex (FSSC) have been associated with clinical infections in humans and plant diseases. Among the several fusaria that cause relevant mycoses in cucurbits in Spain, *Neocosmospora keratoplastica* is described for the first time as responsible for wilt and root rot in both watermelon and melon crops in producing areas of Valencia and Alicante provinces. Due to the ecological and systematic complexity of the group, with described clinical forms and plant pathogens practically indistinguishable from each other, both pathological evidences (including artificial inoculation bioassays) and molecular methods (multilocus phylogeny based on ITS, TEF-1α, and RPB2 regions) are provided to confirm this finding, since the presence of this soil-borne pathogen could have been probably underestimated in cucurbits-producing areas of Spain.

Keywords Cucurbits · Epidemiology · *Fusarium solani* species complex · Pathogenicity · Molecular phylogeny · ITS · TEF-1α · RPB2

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The *Fusarium solani* species complex (FSSC) is a heterogeneous assemblage of closely related species, pathogenic races or mating groups all of them included within genus *Neocosmospora*, barely distinguishable from each other morphologically and with highly variable host preferences, ranging from plant species to large vertebrates including humans (Zhang et al. 2006; Coleman 2016). In this sense, it has been shown that some *Neocosmospora* taxa usually reported as opportunistic animal pathogens are also found to be associated agricultural crop diseases and even pathogenicity on plant hosts has been demonstrated for human isolates of some of these taxa (Mehl and Epstein 2007). As pointed by some authors (Chitrampalan and Nelson 2015; Zhang et al. 2006), delimitation and recognition of taxa within the FSSC based in sexual compatibility, morphology or pathogenicity does not seem to be as accurate as that obtained by molecular methods. Concerning cucurbits, there are some *Fusarium* species

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associated with wilt and root rot of many plant species of the family, and the effects and symptoms caused by species and pathogenic variants of this important and ubiquitous soil-borne genus such as *Fusarium oxysporum* f. sp. *niveum* and *F. oxysporum* f. sp. *melonis* have been well known for a long time, both being considered as among the most important threats to these crops worldwide (Martyn 1996, 2014). On the other hand, one species of *Neocosmospora* recently delimited (Sandoval-Denis et al. 2019), *N. cucurbitae,* has also been associated with cucurbits. Two pathogenic races were described (1 and 2) for the ancient epithet *Fusarium solani* f. sp. *cucurbitae* that actually constitute separate species. Thus, race 1 (*Neocosmospora cucurbitae*) is associated with fruit rot in cucurbits, whilst isolates of race 2, currently named *Neocosmospora petroliphila*, are reported to be important human pathogens, but also associated with several diseases in plants including cucurbits (Short et al. 2013).

The present work describes for the first time the presence and precise characterization of *Neocosmospora keratoplastica*, a member of the FSSC usually associated with clinical events, causing also wilt and root rot symptoms in watermelon (*Citrullus lanatus*) and melon (*Cucumis melo*), two of the main cucurbit species cultivated in Spain. Our results show that *N. keratoplastica* and the closely related species *N. falciforme* are much more common in agronomic environments than previously thought. In this sense, *Neocosmospora keratoplastica* is commonly considered as an ubiquitous, cosmopolitan species mostly reported from infected animals (including humans) and as a component of microbial biofilms on plumbing systems, although it has been recently cited associated with several plants or inhabiting soils suggesting a wider ecological range for this species (Short et al. 2013).

Many FSSC taxa have morphological diagnostic characters that overlap each other, making discrimination between species very difficult without the help of molecular techniques (mainly through the comparison of sequences from several genomic regions) (Sandoval-Denis et al. 2019). This difficulty is also present with the taxonomic determination of non-clinical isolates coming from environmental samples (plant, soil, etc.) (Zhang et al. 2006). In recent years, efforts have been made to clarify the systematics of the group, based mainly on the recognition of lineages and phylospecies from phylogenetic reconstructions based on different genomic regions (Sandoval-Denis and Crous 2018). This type of work has allowed establishing more accurately the evolutionary relationships between taxa of the complex, the recognition and redefinition of genera, or the link between isolates of clinical origin and those coming from environmental samples.

In the growing season 2018, during a survey of fungal pathogens associated with melon and watermelon crops in experimental and commercial fields as well as research greenhouses of Valencia and Alicante provinces (Spain), occurrence of vine wilt and root rot in melon and watermelon plants was observed in several sampling areas, all of them including both non grafted and plants grafted onto *Cucurbita* rootstock. Diseased plants exhibited variable symptoms including yellowing and wilting of leaves, necrotic lesions or rotting in the base of the stems and upper part of the taproot, and collapse of the entire plant (Fig. 1).

Among these, three diseased plants were processed, coming from different sampling sites, culture types and representing different rootstock / variety combinations (Table 1). Isolations were made from both severely decayed and died plants. Small pieces (0.5–1 cm) from the cortical lesions of both lower stems and upper roots were surface disinfected for 1 min in 1.5% NaOCl, washed four times with sterilized bi-distilled water and plated onto potato dextrose agar (PDA) amended with streptomycin sulphate (0.5 g L^{-1}) to avoid bacterial contamination. Plates were incubated at 25 °C in the dark for 3–5 days.

In the three samples above mentioned, all emerging colonies from plant fragments resembled those of typical *Fusarium* colonies and were isolated and characterized by morphological and molecular methods. Subcultured pure colonies growing in PDA were firstly identified as belonging to the *Fusarium solani* species complex (FSSC) on the basis of their macroscopic features. On PDA, colonies were white-greyish to pale peach or pale yellow, reverse with pale yellow to pale salmon-yellow tones after 4 days, sometimes growing in concentric rings with mostly adpressed mycelia with scarce aerial tufts. Cream to pale yellow sporodochia were usually (not in all isolates) formed after 6–8 days of culture. Sporodochial macroconidia were sometimes abundant, narrowly cylindrical to falcate with acute, curved apexes, usually with a wide basal cell, hyaline, 3–5 septate of 40.2 (27.2–56.5) × 5.2 (3.5–7.8) μm in mean; aerial microconidia were abundant, borne on short, undifferentiated monophialides, oval, pyriform to cylindrical, straight, 0–2 septate of 12.5 (3.5–

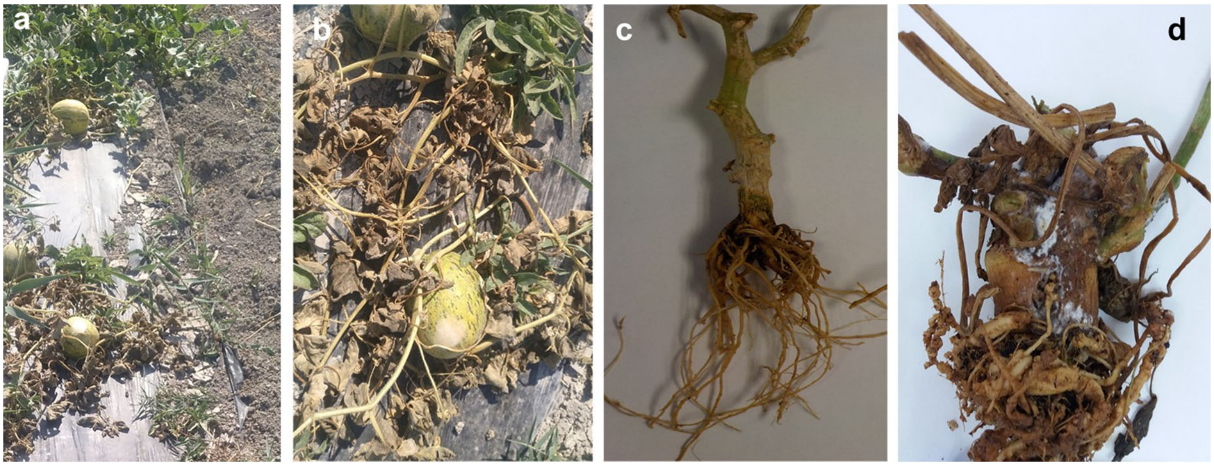

Fig. 1 Symptoms observed in melon and watermelon plants from which *N. keratoplastica* isolates were obtained. **a** and **b** wilted non grafted melon plants traditional variety "Hilo Carrete"; **c** root rot from wilted melon plant traditional variety grafted onto a snake melon variety; **d** root rot of watermelon plant grafted onto *Cucurbita* rootstock

28.5) × 3.8 (2.2–6) μm in mean. Chlamydospores were usually present, rounded to globose, either single or in pairs, mostly intercalary, thin to thick-walled, sometimes warted (Fig. 2).

Molecular characterization of the mentioned FSSC isolates was also performed. Thus, after DNA extraction, sequencing of the internal transcribed spacer (ITS) region was performed for the three isolates using ITS1/ITS4 (White et al. 1990) primer pair. In addition, fragments of the translation *elongation factor-1α (TEF-1α)* and RNA polymerase II (RPB2) gene using EF1 / EF2 (O'Donnell et al. 1998) and fRPB2-7cF / fRPB2-11aR (Reeb et al. 2004) primers respectively, were also sequenced for isolate MYC-1250. After their comparison in BLASTn and Fusarium ID Database (http://www.westerdijkinstitute.nl/fusarium/), they were identified as *Neocosmospora keratoplastica* (Geiser et al.) Sand.-Den. & Crous. Thus, ITS, T*EF-1α* and RPB2 sequences of isolate MYC-1250 showed a 99–100% homology with the mentioned taxon: e.g. MF411133 (ITS), DQ790473 (*EF-1α*) and JN235886 (RPB2), and were deposited in GenBank with accession numbers MN535800 (ITS), MN629918 (T*EF-1α*), and MN648896 (RPB2). In addition, a phylogenetic reconstruction was carried out employing ITS, EF-1α and RPB2 sequences in a combined dataset that included sequence fragments of isolate MYC-1250 and a selection of combined reference sequences of the FSSC obtained from Sandoval-Denis and Crous (2018). A multi-locus alignment with Clustal W was subjected to a Maximum Likelihood (ML) analysis in MEGA6 interface (Tamura et al. 2013), assessing branch confidence with 1000 non-parametric bootstraps. The phylogram obtained (Fig. 3) supported the previous taxonomic assignation of our isolate to *N. keratoplastica*, placing the combined ITS/TEF-1α/RPB2 sequence of isolate MYC-1250 in a monophyletic clade with the rest of *N. keratoplastica* sequences included in the analysis (supported with a 100% of bootstrap value).

For pathogenicity tests, the same isolate MYC-1250 was grown in 250-mL flasks containing potato sucrose medium for 3 days at 25 °C in the dark with constant agitation. Ten 15-day-old 'Piel de Sapo' melon seedlings were removed from the trays where they were grown with sterilized substrate (Projar Professional, Projar, Spain), and then, dipped into a suspension of 10^6 conidia/ml for 2 min, and transferred to plastic pots (Teku-tainer, Pöppelmann) with sterilized substrate. Three non-inoculated plants submerged in sterile water were used as controls. Plants were incubated in a growth chamber (25 °C; 16/8 h photoperiod). Scarce development, wilting and yellowing accompanied by dry necrosis of the central veins of some leaves as well as necrosis and thinning of the roots, followed by plant death were observed 15–20 days post-inoculation. Non-inoculated controls remained asymptomatic. The fungus was re-isolated and identified using ITS, TEF-1α and RPB2 sequences from all the inoculated plants fulfilling Koch's postulates.

Table 1 Variety/rootstock combinations, geographical origin, symptomatology, and crop type of the three plants from which *N. keratoplastica* isolates were obtained

Isolate	Variety	Rootstock	Geographical origin City (Province)	Phytosanitary status	Crop type
MYC-1168	Watermelon (local variety)	*Cucurbita*	Museros (Valencia)	Dead	Commercial field
MYC-1450	Snake Melon (local variety)	Snake Melon	Valencia (Valencia)	Severely Wilted	Research Greenhouse
MYC-1250	Melon (traditional variety Hilo Carrete)	Non grafted	Carrizales (Alicante)	Wilted	Experimental field

Neocosmospora keratoplastica belongs to the FSSC group, whose taxa are actually included under the concept of genus *Neocosmospora* (Sandoval-Denis et al. 2019). Members of the genus are ubiquitous soil-borne fungi frequently isolated from plant debris, soil, water, living plants or air and constitute one large and important group of plant pathogens. In addition, some taxa of the FSSC have been associated with human and animal mycoses (Zhang et al. 2006; Sarmiento-Ramírez et al. 2014; O'Donnell et al. 2016) through the production of a huge range of mycotoxicoses, and an increase in this type of clinical problems is currently being experienced (Sutton and Brandt 2011). Although most of these plant diseases or clinical infections were classically reported to be associated with *F. solani* s. *lato*, recent molecular systematic studies (Sandoval-Denis and Crous 2018) have demonstrated that *Neocosmospora* taxa including *N. falciforme* (O'Donnell et al. 2008), *N. petroliphila* (O'Donnell 2000), *N. keratoplastica* (Short et al. 2013) and other phylospecies recently delimited (*N. metavorans*, *N. gamsii*, *N. suttoniana*, *N. tonkinensis*, etc.) (Sandoval-Denis and Crous 2018), are commonly associated with infections in human and other animals. Interestingly, those prevalent human fusarial pathogens of *Neocosmospora* are also known to be the causal agents of plant diseases. In this sense, *N. petroliphila*, a pathogen responsible for human infections, was largely known as *F. solani* f. sp. *cucurbitae* race 1, and is also usually associated with root, stem and fruits of cucurbits, having been recently reported from *Cucurbita* in Spain (González et al. 2018). In the case of *N. falciformis* there have been some reports of the presence of decay caused by this fungus on several plant species including chickpea (Cabral et al. 2016), lima bean (Sousa et al. 2017), pistachio (Crespo et al. 2019), onion (Tirado-Ramirez et al. 2018) or even watermelon (Rentería-Martínez et al. 2018). *Neocosmospora keratoplastica* is associated mostly with infected animals or human-related environments such as plumbing systems, and has also occasionally been isolated from soil (Chehri et al. 2015) or plant seeds (Shaffer et al. 2017). Our study describes for the first time the presence in Europe of *N. keratoplastica* causing decay and root rot in cucurbits (melon, watermelon, and squash), associating this important human pathogen with plant mycoses previously assigned to *F. solani* s. lato. As some studies have pointed out (Sandoval-Denis and Crous 2018; Sandoval-Denis et al. 2019), there is a need for the precise identification of FSSC taxa based on the integration of molecular data in phylogenetic analyses coming from combined, multi-*locus* datasets, since we now have more evidence about the broad spectrum of habitats and hosts for the most clinically important species, some of which are associated with plant diseases. Moreover, the lack of precise identification of taxa, as well as the confusion existing among both clinical mycologists and plant pathologists, regarding the stability of the nomenclature of the group, has led, in the case of plant diseases, to a deficit in the knowledge of the incidence, extent and etiology of fusarioses caused by members of the FSSC. This is especially true in the case of cucurbit species, where whilst soil diseases caused by members of other common species complexes of *Fusarium* like *F. oxysporum* are well-known, little attention has been paid to FSSC-related diseases, with the exception of *N. cucurbitae* (formerly *F. solani* f. sp. *cucurbitae* race 1). The control

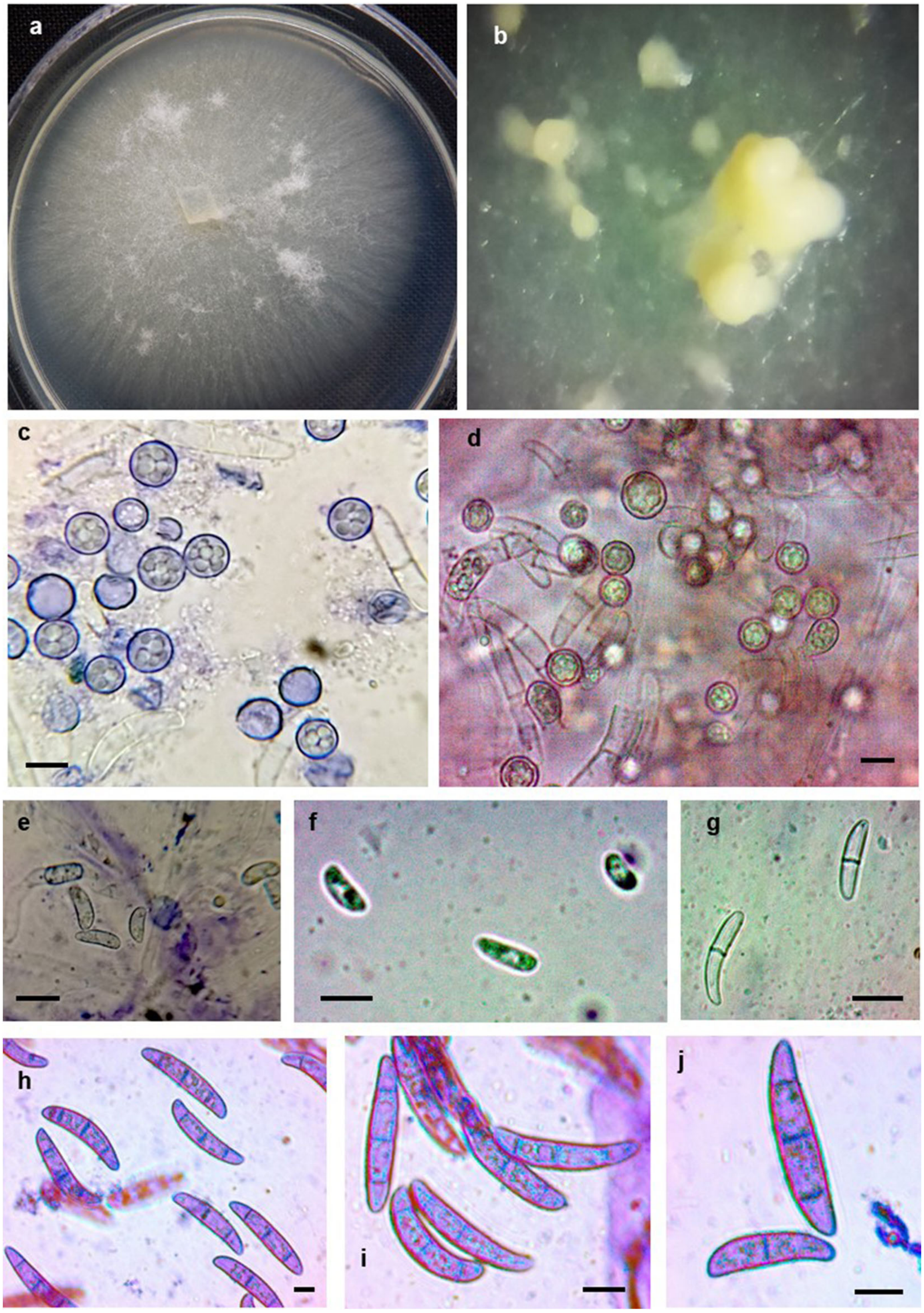

Fig. 2 *Neocosmospora keratoplastica* isolate MYC-1250. **a** colony in PDA; **b** sporodochia formed in PDA; **c** and **d** chlamydospores; **e–g** aerial microconidia; **h–j** sporodochial macroconidia. Bars = 10 μm

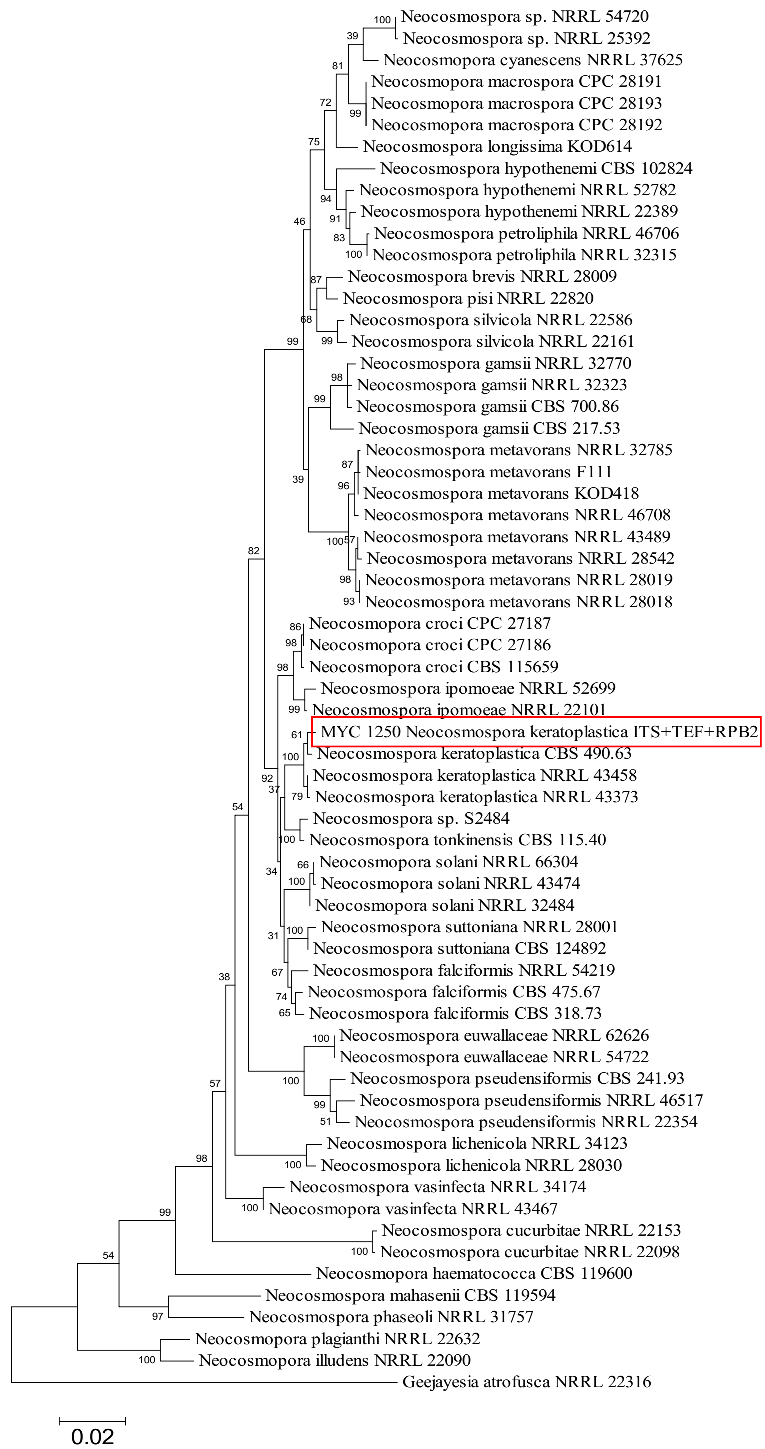

Fig. 3 Maximum Likelihood (ML) phylogram of FSSC taxa inferred from a combined ITS/TEF1α/RPB2 sequence dataset, including isolate MYC 1250. Numbers above and below braches represent bootstrap values

of Fusarium wilt in cucurbits is mainly based on the use of *Cucurbita* rootstocks known to be resistant to *Fusarium oxysporum* f. sp. *melonis* and f. sp. *niveum*, the two main pathogens associated with Fusarium wilt of melon and watermelon. The fact that members of the FSSC, such as *N. keratoplastica,* have been found causing wilting in cucurbit plants grafted onto *Cucurbita* rootstocks, suggest that there is a need to check the resistance of current rootstocks and to develop new ones with resistance to pathogens from the FSSC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This chapter does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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