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SHORT COMMUNICATION

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 either associated with clinical infections in humans and certain 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 certain producing areas of Valencia and Alicante provinces (E Spain). 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*

The so-called *Fusarium solani* species complex (FSSC) is actually considered as a heterogeneous assemblage of closely related species, pathogenic races or mating groups, 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 demonstrated that certain FSSC taxa usually reported as opportunistic animal pathogens are practically indistinguishable at the morphological and molecular level from some soil-borne isolates of the group causing certain pathologies in agricultural crops. Concerning *Fusarium* species associated with wilt and root rot in cucurbits, 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* are well known, being both considered as some of the most important threads to these crop worldwide (Martyn 1996 and 2014). On the other hand, one species of the above mentioned FSSC group, *Fusarium solani* f. sp. *cucurbitae* (*Neocosmospora cucurbitae*) has also been associated with cucurbits. There are described two pathogenic races (1 and 2) for this taxon, being race 1 (also known as *Nectria haematococca* MPI) associated with fruit rot in cucurbits, whilst isolates of race 2 (largely named as *N. haematococca* MPV) are actually included under the epithet of *Neocosmospora petroliphila* and considered as an important human pathogen, 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*, one of these FSSC taxa usually associated with clinical events, causing also wilt and root rot symptoms in watermelon and melon, two of the main cucurbit species cultivated in Spain. For this and other taxon from the FSSC group, *Neocosmospora falciforme*, can be hypothesized that their isolates could be

much more common in agronomic environments than previously thought. In this sense, *Neocosmospora keratoplastica* is commonly considered as an ubiquitous, cosmopolite 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.

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 certain genomic regions). This difficulty is also present with the taxonomic determination of non-clinical isolates coming from environmental samples (plant, soil, etc.). 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. This type of work has allowed establishing more accurately the evolutive relationships between taxa of the complex, the recognition and redefinition of certain 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 (E 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).

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.

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.

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

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

Then, some mycelia resembling those of typical *Fusarium* colonies 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 macroscopical 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 appressed??? mycelia with scarce aerial tufts. Irregular, rounded sporodochia of cream to pale yellow tones 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 wider basal cell, hyaline, 3-5 septate of 40.2 (27.2-56.5) x 5.2 (3.5-7.8) µm; aerial microconidia were abundant, borne on short, sometimes branched, undifferentiated monophialides, oval to cylindrical, pyriform, straight, 0-2 septate of 12.5 (3.5-28.5) x 3.8 (2.2-6) µm. Chlamydospores were usually present, rounded to globose, either single or in pairs, mostly intercalary, thin to thick-walled, sometimes warted (Fig. 2).

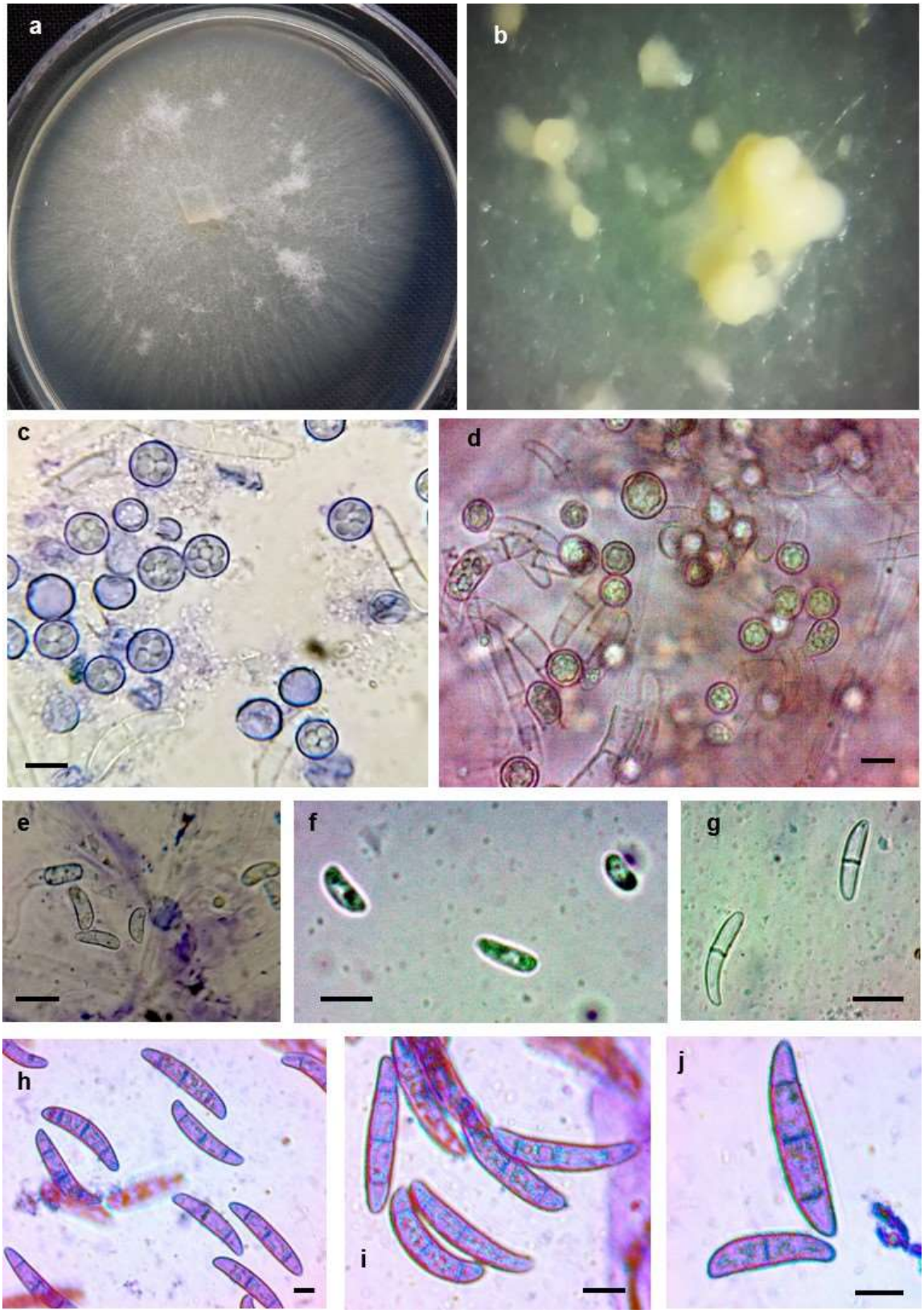
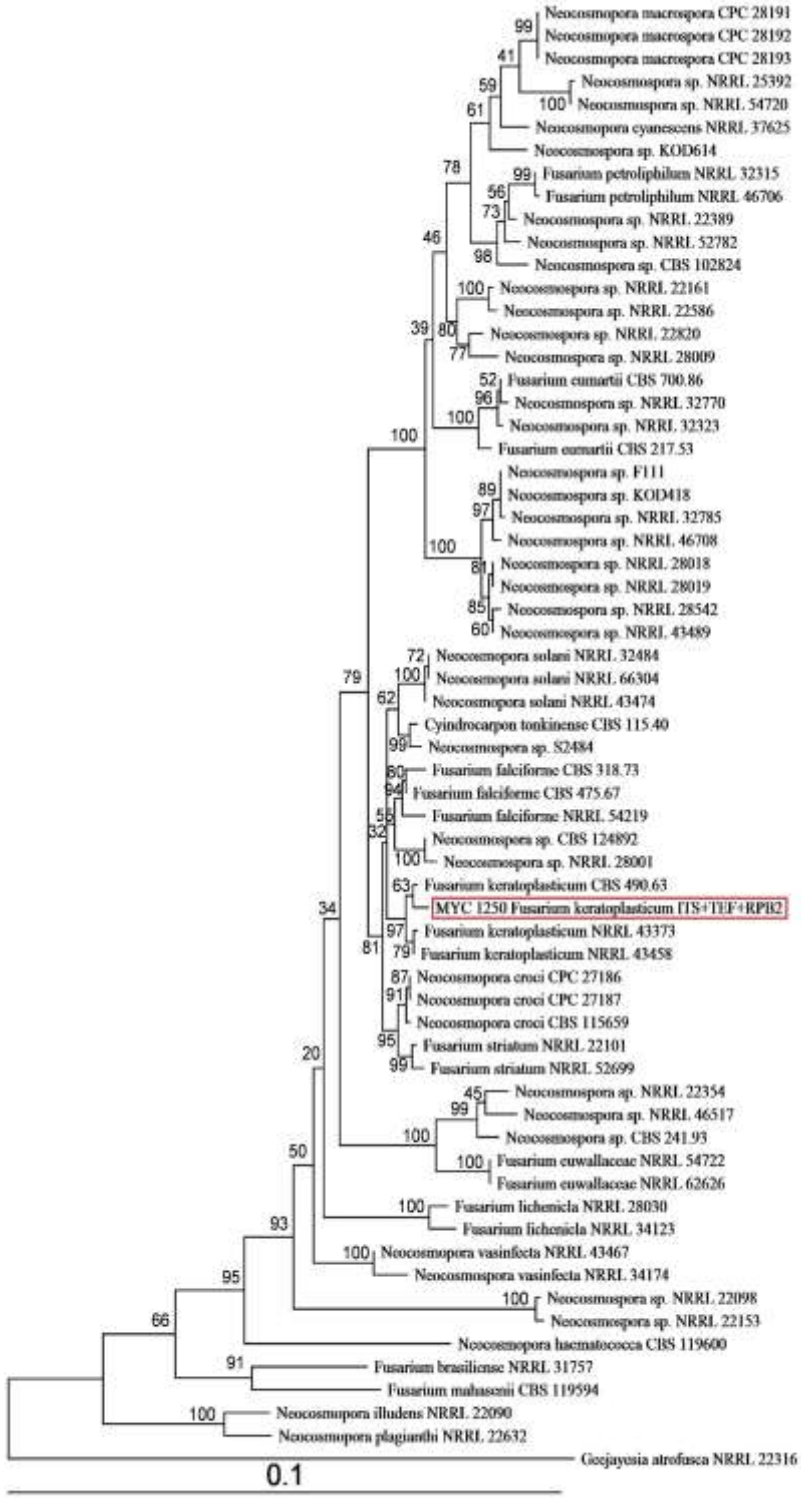


Fig. 2 *Neocosmospora keratoplastica* isolate MYC-1250. **a:** colony in PDA; **b:** sporodochia formed in PDA; **c** and **d:** chlamydospores; **e-g:** aerial microconidia; **h-j:** sporodochial macroconidia. Bars=10 µm.

Molecular characterization of the mentioned FSSC isolates was also performed. Thus, after DNA extraction, sequencing of the internal transcribed spacer (ITS) region, a fragment of the translation elongation factor-1α (TEF-1α) and a fragment of RNA polymerase II (RPB2) gene, using ITS1/ITS4 (White et al. 1990), EF1 / EF2 (O'Donnell et al. 1998) and fRPB2-7cF / fRPB2-11aR (Reeb et al. 2004) primers respectively, and their comparison in BLASTn and Fusarium ID Database (http://www.westerdijkinstitute.nl/fusarium/), three isolates (Table 1) were identified as *Neocosmospora keratoplastica* (Geiser et al.) Sand.-Den. & Crous. Among these, ITS, TEF-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 (TEF-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 group obtained from Sandoval 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 97% of bootstrap value).



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169 **Fig. 3** Maximum Likelihood (ML) phylogram of FSSC taxa inferred from a combined

170 ITS/TEF1α/RPB2 sequence dataset, including isolate MYC 1250. Numbers above and

171 below braches represent bootstrap values

For pathogenicity tests, the same isolate MYC-1250 was grown in 250 ml flasks containing potato sucrose medium for 3 days at 25ºC in the dark with constant agitation. Ten 15-days-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 (25ºC; 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.

Neocosmospora keratoplastica belongs to the so-called *Fusarium solani* species complex (FSSC), 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 form 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 group 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 mycotoxins, 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 demonstrate that *Neocosmospora* taxa like *N. falciforme* (O'Donnell et al. 2008), *N.*

197 *petroliphila* (O'Donnell 2000) or *N. keratoplastica* (Short et al. 2013) and other
198 phylospecies that actually remain unnamed (Zhang et al. 2006), are commonly
199 associated with infections in human and other animals. Interestingly, those prevalent
200 human fusarial pathogens of *Neocosmospora* are also known to be the causal agents of
201 certain plant diseases. In this sense, *N. petroliphila*, a pathogen responsible for human
202 infections, was largely known as *F. solani* f. sp. *cucurbitae* race 1, and is also usually
203 associated with root, stem and fruits of cucurbits, having been recently reported from
204 *Cucurbita* in Spain (González et al. 2018). In the case of *N. falciformis* there have been
205 some reports of the presence of decay and root rot of this fungi on several plant species
206 like chickpea (Cabral et al. 2016), lima bean (Sousa et al. 2017), pistachio (Crespo et al.
207 2019), onion (Tirado-Ramírez et al. 2018) or even watermelon (Rentería-Martínez et al.
208 2018) and melon (González et al. 2019 in press).
209 The taxon here reported, *Neocosmospora keratoplastica*, is associated mostly with
210 infected animals or certain human-related environments like plumbing systems and has
211 been also occasionally isolated from soil (Chehri et al. 2015) or plant seeds (Shaffer et
212 al. 2017). Our study describes for the first time the presence in Europe of *N.*
213 *keratoplastica* causing decay and root rot in cucurbits (melon, watermelon, and squash),
214 associating this important human pathogen from the FSSC group with some plant
215 mycoses that were usually assigned to *F. solani* s. lato. As some studies have pointed
216 recently (Sandoval-Denis and Crous 2018; Sandoval-Denis et al. 2019), there is a need
217 for the precise identification of these FSSC taxa based on the integration of molecular
218 data in phylogenetic analyses coming from combined, multi-*locus* datasets, since we
219 now have more evidences about the broad spectrum of habitats and hosts that some of
220 the most clinically important species possess, some of which are associated with certain
221 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 extensive FSSC group. This especially true in the case of cucurbit species, where whilst soil diseases caused by members of other common complex of the genus 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* races 1 and 2). The control 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 to Fusarium wilt of melon and watermelon. The fact that members of the FSSC group, such as *N. keratoplastica*, are being 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 of the FSSC group.

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