

1 **SHORT COMMUNICATION**

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

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27 **Abstract**

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

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40 **Keywords:** *cucurbits, epidemiology, Fusarium solani species complex, pathogenicity,*
41 *molecular phylogeny, ITS, TEF-1 α , RPB2*

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51 The so-called *Fusarium solani* species complex (FSSC) is actually considered as a
52 heterogeneous assemblage of closely related species, pathogenic races or mating groups,
53 barely distinguishable from each other morphologically and with highly variable host
54 preferences, ranging from plant species to large vertebrates including humans (Zhang et
55 al. 2006; Coleman 2016). In this sense, it has been demonstrated that certain FSSC taxa
56 usually reported as opportunistic animal pathogens are practically indistinguishable at
57 the morphological and molecular level from some soil-borne isolates of the group
58 causing certain pathologies in agricultural crops. Concerning *Fusarium* species
59 associated with wilt and root rot in cucurbits, the effects and symptoms caused by
60 species and pathogenic variants of this important and ubiquitous soil-borne genus such
61 as *Fusarium oxysporum* f. sp. *niveum* and *F. oxysporum* f. sp. *melonis* are well known,
62 being both considered as some of the most important threats to these crop worldwide
63 (Martyn 1996 and 2014). On the other hand, one species of the above mentioned FSSC
64 group, *Fusarium solani* f. sp. *cucurbitae* (*Neocosmospora cucurbitae*) has also been
65 associated with cucurbits. There are described two pathogenic races (1 and 2) for this
66 taxon, being race 1 (also known as *Nectria haematococca* MPI) associated with fruit rot
67 in cucurbits, whilst isolates of race 2 (largely named as *N. haematococca* MPV) are
68 actually included under the epithet of *Neocosmospora petroliphila* and considered as an
69 important human pathogen, but also associated with several diseases in plants including
70 cucurbits (Short et al. 2013).

71 The present work describes for the first time the presence and precise characterization
72 of *Neocosmospora keratoplastica*, one of these FSSC taxa usually associated with
73 clinical events, causing also wilt and root rot symptoms in watermelon and melon, two
74 of the main cucurbit species cultivated in Spain. For this and other taxon from the FSSC
75 group, *Neocosmospora falciforme*, can be hypothesized that their isolates could be

76 much more common in agronomic environments than previously thought. In this sense,
77 *Neocosmospora keratoplastica* is commonly considered as an ubiquitous, cosmopolite
78 species mostly reported from infected animals (including humans) and as a component
79 of microbial biofilms on plumbing systems, although it has been recently cited
80 associated with several plants or inhabiting soils, suggesting a wider ecological range
81 for this species.

82 Many FSSC taxa have morphological diagnostic characters that overlap each other,
83 making discrimination between species very difficult without the help of molecular
84 techniques (mainly through the comparison of sequences from certain genomic regions).
85 This difficulty is also present with the taxonomic determination of non-clinical isolates
86 coming from environmental samples (plant, soil, etc.). In recent years, efforts have been
87 made to clarify the systematics of the group, based mainly on the recognition of
88 lineages and phylopecies from phylogenetic reconstructions based on different
89 genomic regions. This type of work has allowed establishing more accurately the
90 evolutive relationships between taxa of the complex, the recognition and redefinition of
91 certain genera or the link between isolates of clinical origin and those coming from
92 environmental samples.

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

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

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109 Among these, three diseased plants were processed, coming from different sampling
110 sites, culture types and representing different rootstock / variety combinations (Table 1).
111 Isolations were made from both severely decayed and died plants. Small pieces (0.5-1
112 cm) from the cortical lesions of both lower stems and upper roots were surface
113 disinfected for 1 min in 1.5% NaOCl, washed four times with sterilized bi-distilled
114 water and plated onto potato dextrose agar (PDA) amended with streptomycin sulphate
115 (0.5 g L^{-1}) to avoid bacterial contamination. Plates were incubated at 25°C in the dark
116 for 3-5 days.

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Isolate	Variety	Rootstock	Geographical origin City (province)	Phytosanitary status	Crop type
MYC-1168	Watermelon (local variety)	<i>Cucurbita</i>	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

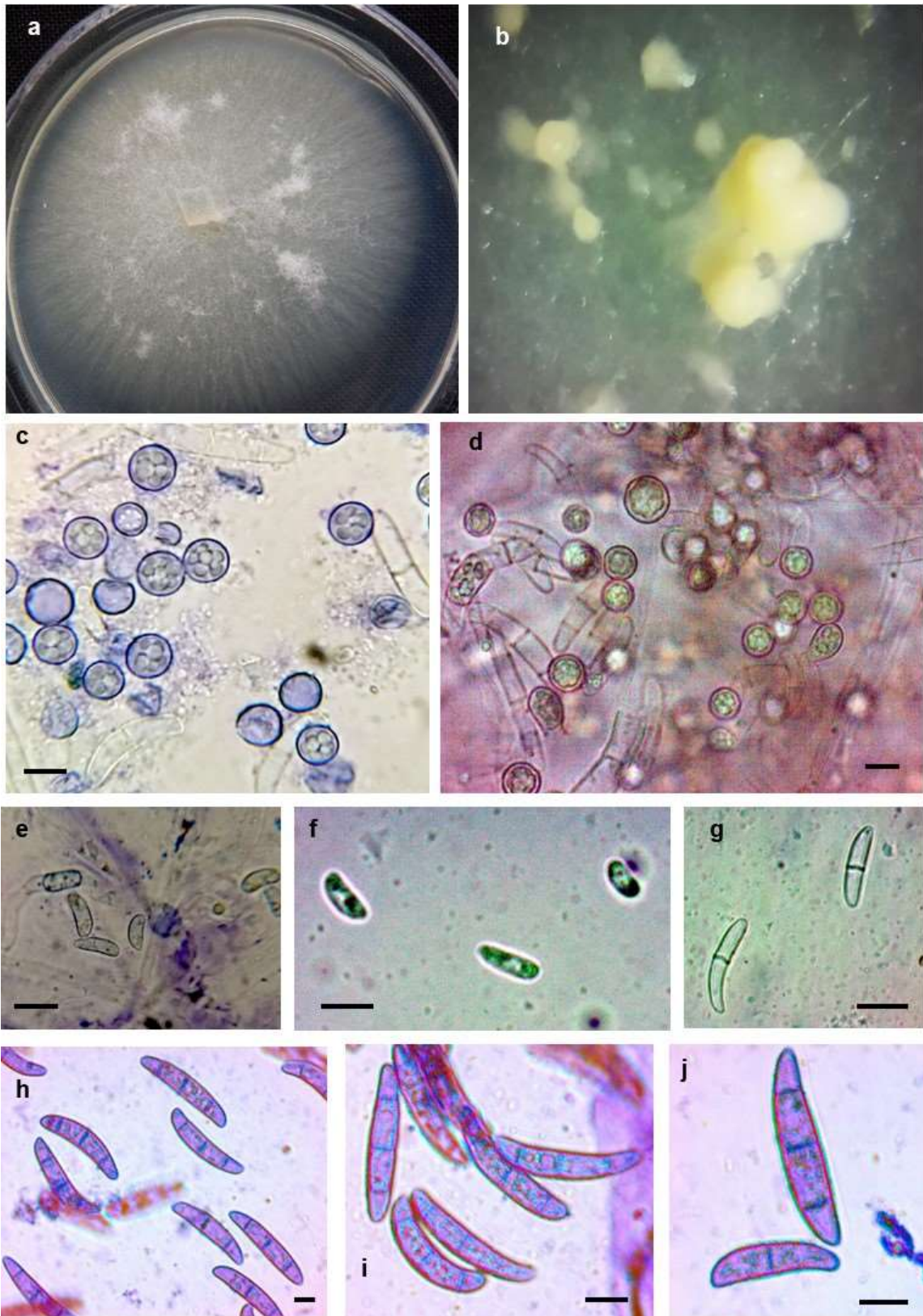
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123 **Table 1** Variety/rootstock combinations, geographical origin, symptomatology and crop
124 type of the three plants from which *N. keratoplastica* isolates were obtained.

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126 Then, some mycelia resembling those of typical *Fusarium* colonies were isolated and
127 characterized by morphological and molecular methods. Subcultured pure colonies
128 growing in PDA were firstly identified as belonging to the *Fusarium solani* species
129 complex (FSSC) on the basis of their macroscopical features. On PDA, colonies were
130 white-greyish to pale peach or pale yellow, reverse with pale yellow to pale salmon-
131 yellow tones after 4 days, sometimes growing in concentric rings with mostly
132 appressed??? mycelia with scarce aerial tufts. Irregular, rounded sporodochia of cream
133 to pale yellow tones were usually (not in all isolates) formed after 6-8 days of culture.
134 Sporodochial macroconidia were sometimes abundant, narrowly cylindrical to falcate
135 with acute, curved apexes, usually with a wider basal cell, hyaline, 3-5 septate of 40.2
136 (27.2-56.5) x 5.2 (3.5-7.8) μm ; aerial microconidia were abundant, borne on short,
137 sometimes branched, undifferentiated monophialides, oval to cylindrical, pyriform,
138 straight, 0-2 septate of 12.5 (3.5-28.5) x 3.8 (2.2-6) μm . Chlamydospores were usually
139 present, rounded to globose, either single or in pairs, mostly intercalary, thin to thick-
140 walled, sometimes warted (Fig. 2).

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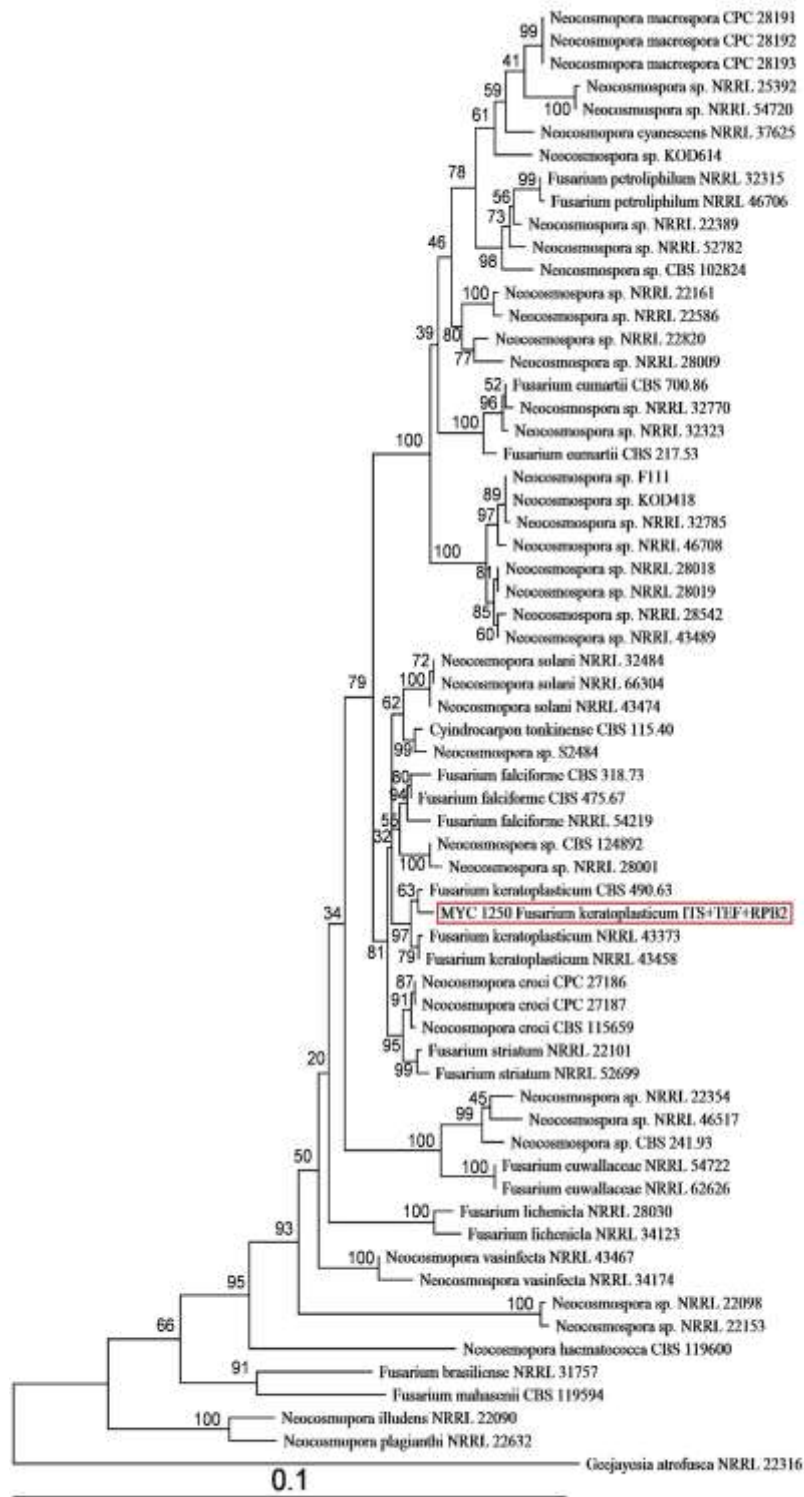
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143 **Fig. 2** *Neocosmospora keratoplastica* isolate MYC-1250. **a:** colony in PDA; **b:**

144 sporodochia formed in PDA; **c** and **d:** chlamydospores; **e-g:** aerial microconidia; **h-j:**

145 sporodochial macroconidia. Bars=10 µm.

146 Molecular characterization of the mentioned FSSC isolates was also performed. Thus,
147 after DNA extraction, sequencing of the internal transcribed spacer (ITS) region, a
148 fragment of the translation elongation factor-1 α (TEF-1 α) and a fragment of RNA
149 polymerase II (RPB2) gene, using ITS1/ITS4 (White et al. 1990), EF1 / EF2
150 (O'Donnell et al. 1998) and fRPB2-7cF / fRPB2-11aR (Reeb et al. 2004) primers
151 respectively, and their comparison in BLASTn and Fusarium ID Database
152 (<http://www.westerdijkinstituut.nl/fusarium/>), three isolates (Table 1) were identified as
153 *Neocosmospora keratoplastica* (Geiser et al.) Sand.-Den. & Crous. Among these, ITS,
154 TEF-1 α and RPB2 sequences of isolate MYC-1250 showed a 99-100% homology with
155 the mentioned taxon: e.g. MF411133 (ITS), DQ790473 (EF-1 α) and JN235886 (RPB2),
156 and were deposited in GenBank with accession numbers MN535800 (ITS), MN629918
157 (TEF-1 α), and MN648896 (RPB2). In addition, a phylogenetic reconstruction was
158 carried out employing ITS, EF-1 α and RPB2 sequences in a combined dataset that
159 included sequence fragments of isolate MYC-1250 and a selection of combined
160 reference sequences of the FSSC group obtained from Sandoval and Crous (2018). A
161 multi-locus alignment with Clustal W was subjected to a Maximum Likelihood (ML)
162 analysis in MEGA6 interface (Tamura et al. 2013), assessing branch confidence with
163 1000 non-parametric bootstraps. The phylogram obtained (Fig. 3) supported the
164 previous taxonomic assignation of our isolate to *N. keratoplastica*, placing the
165 combined ITS/TEF-1 α /RPB2 sequence of isolate MYC-1250 in a monophyletic clade
166 with the rest of *N. keratoplastica* sequences included in the analysis (supported with a
167 97% of bootstrap value).



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169 **Fig. 3** Maximum Likelihood (ML) phylogram of FSSC taxa inferred from a combined
 170 ITS/TEF1 α /RPB2 sequence dataset, including isolate MYC 1250. Numbers above and
 171 below braches represent bootstrap values

172 For pathogenicity tests, the same isolate MYC-1250 was grown in 250 ml flasks
173 containing potato sucrose medium for 3 days at 25°C in the dark with constant agitation.
174 Ten 15-days-old 'Piel de Sapo' melon seedlings were removed from the trays where they
175 were grown with sterilized substrate (Projar Professional, Projar, Spain), and then,
176 dipped into a suspension of 10⁶ conidia/ml for 2 min, and transferred to plastic pots
177 (Teku-tainer, Pöppelmann) with sterilized substrate. Three non-inoculated plants
178 submerged in sterile water were used as controls. Plants were incubated in a growth
179 chamber (25°C; 16/8 h photoperiod). Scarce development, wilting and yellowing
180 accompanied by dry necrosis of the central veins of some leaves as well as necrosis and
181 thinning of the roots, followed by plant death were observed 15-20 days post-
182 inoculation. Non-inoculated controls remained asymptomatic. The fungus was re-
183 isolated and identified using ITS, TEF-1 α and RPB2 sequences from all the inoculated
184 plants fulfilling Koch's postulates.

185 *Neocosmospora keratoplastica* belongs to the so-called *Fusarium solani* species
186 complex (FSSC), whose taxa are actually included under the concept of genus
187 *Neocosmospora* (Sandoval-Denis et al. 2019). Members of the genus are ubiquitous
188 soil-borne fungi frequently isolated from plant debris, soil, water, living plants or air
189 and constitute one large and important group of plant pathogens. In addition, some taxa
190 of the FSSC group have been associated with human and animal mycoses (Zhang et al.
191 2006; Sarmiento-Ramírez et al. 2014; O'Donnell et al. 2016) through the production of
192 a huge range of mycotoxins, and an increase in this type of clinical problems is
193 currently being experienced (Sutton and Brandt 2011). Although most of these plant
194 diseases or clinical infections were classically reported to be associated with *F. solani* s.
195 *lato*, recent molecular systematic studies (Sandoval-Denis and Crous 2018) have
196 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

222 confusion existing among both clinical mycologists and plant pathologists, regarding
223 the stability of the nomenclature of the group, has led, in the case of plant diseases, to a
224 deficit in the knowledge of the incidence, extent and etiology of fusarioses caused by
225 members of the extensive FSSC group. This especially true in the case of cucurbit
226 species, where whilst soil diseases caused by members of other common complex of the
227 genus like *F. oxysporum* are well-known, little attention has been paid to FSSC-related
228 diseases, with the exception of *N. cucurbitae* (formerly *F. solani* f. sp. *cucurbitae* races
229 1 and 2). The control of Fusarium wilt in cucurbits is mainly based on the use of
230 *Cucurbita* rootstocks known to be resistant to *Fusarium oxysporum* f sp. *melonis* and f
231 sp. *niveum*, the two main pathogens associated to Fusarium wilt of melon and
232 watermelon. The fact that members of the FSSC group, such as *N. keratoplastica*, are
233 being found causing wilting in cucurbit plants grafted onto *Cucurbita* rootstocks,
234 suggest that there is a need to check the resistance of current rootstocks and to develop
235 new ones with resistance to pathogens of the FSSC group.

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