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Characterization of *Coniella granati* isolates causing pomegranate decline in Italy

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Abstract

Coniella granati, the causal agent of pomegranate crown rot, twig blight and fruit decay, is an emerging worldwide pathogen with heavy impact on pomegranate cultivation. With this study, we report the rapid spread of the fungus in Italian pomegranate orchards associated to crown rot symptoms, and provide new results on fungal development, baseline sensitivity to different fungicides and intraspecific variability by analyzing eleven isolates, representative of populations of the pathogen from comparable pomegranate orchards in different regions of Italy. *In vitro* assays showed that 25-30°C was the optimal range for both colony growth and conidia germination, corroborating the results previously obtained for Californian and Greek isolates. According to the baseline sensitivity assay on colony growth and conidial germination to ten fungicides, fludioxonil, thiophanate methyl, tebuconazole and cyprodinil were the most effective. Random Amplified Polymorphic Analysis (RAPD), carried out using 14 10-mer primers, showed a very low intraspecific variability (similarity coefficient >0.95), probably as a result of the recent spread of the pathogen in Italy and the uncommon occurrence of the sexual process as source of genetic variability. In summary, this study provides new knowledge on *C. granati* that will be helpful for improving pomegranate crown rot management.

Key words: *Pilidiella granati*, crown rot, fungicides, RAPD, variability.

Introduction

Pomegranate (*Punica granatum* L.) is one of the most ancient fruit crops cultivated in the Mediterranean region (Kahramanoglu and Usanmaz, 2016). The human health benefits associated with fruit consumption have rapidly increased the off-season demand for pomegranate fruits and juice. Consequently, pomegranate cultivation and storage facilities expanded all over the world as well as in Italy (Tinebra et al., 2021).

In the pomegranate growing regions, the spread of new cultivars (*e.g.*, Wonderful and Mollar de Elche), also facilitated by the adoption of new agronomic techniques allowing improved quality and productivity, resulted in the replacing of local ecotypes (Tinebra et al., 2021).

Several pathogens can affect pomegranate trees and fruits (Munhuweyi et al., 2016), including *Coniella granati* (Sacc.) Petr. & Syd. causing crown rot and fruit rot in both pre- and

postharvest conditions (Pollastro et al., 2016; Mincuzzi et al., 2016). First reported in Turkey (Yildiz and Karaca, 1973), *C. granati* has spread in recent years everywhere in pomegranate growing areas, like Spain (Palou et al., 2010), Greece (Thomidis and Exadaktylou, 2011), Israel (Levy et al., 2011), Iran (Mirabolfathy et al., 2012), China (Chen et al., 2014), Florida (Kc and Vallad, 2016), Italy (Pollastro et al., 2016), Mexico (Cintora-Martínez et al., 2017), South Africa (Lennox et al., 2018), Tunisia (Jabnoun-Khiareddine et al., 2018), India (Mahadevakumar et al., 2019) and Hungary (Szendrei et al., 2022).

Crown rot-affected pomegranates show reduced growth, yellowish leaves, wilting and dieback of branches, decline and ultimately death (Pollastro et al., 2016). During the winter, the fungus can survive in dead shoots and fruit tissues, and on symptomatic tissues can develop globose black pycnidia containing hyaline, one-celled, elliptical spores. Pycnidiospores from overwintered pycnidia can spread by irrigation water and rain, causing new infections under favorable environmental conditions. The control of the disease is essential to prevent heavy yield losses (Sharma et al., 2020).

In this context, the availability of new protection tools against *C. granati* is required. So far, only few fungicides have proved to be effective against the fungus and few studies have focused on biological control and on disease resistance in pomegranate genotypes (Thomidis, 2015; Uysal, 2018). Additionally, the genetic diversity in *C. granati* populations needs to be explored, since it may help in understanding the disease epidemiology and the reasons underlaying the rapid spreading of the fungus in different countries (Milgroom and Fry, 1997).

With this study *C. granati* isolates obtained from different Italian pomegranate orchards were identified by morphological and molecular analyses and used to evaluate the response of conidial germination and colony growth to different temperatures, as well their baseline sensitivity to fungicides representative of different modes of action. Finally, their diversity was explored by Random Amplified Polymorphic DNA (RAPD) analysis.

Material and methods

Obtainment and identification of Coniella granati *isolates*

The prevalence (P, %) of pomegranate symptomatic plants was recorded in 10 orchards located in five regions in Italy [Apulia (1), Calabria (1), Lazio (4), Sicily (1) and Tuscany (3)], during the years 2017 – 2019.

Isolations from sampled symptomatic plant tissues were performed on potato dextrose agar (PDA, 200 g L^{-1} of peeled and sliced potatoes, 20 g L^{-1} of dextrose, 20 g L^{-1} of agar) medium. Plates were incubated 7-10 days at 25°C and pure cultures obtained by transferring hyphal tips on fresh PDA.

Ten isolates (Table 1) were first identified based on morphological characteristics, using the description key reported by Alvarez et al. (2016) and by comparison with the reference strain CG1 (CBS144846). Molecular assay was also performed to confirm their identity. Briefly, DNA was extracted by using the procedure described by De Miccolis Angelini et al. (2010) and amplified with the primer pairs ITS5/ITS4 (White et al., 1990) and EF1-728F/EF1-986R (Carbone and Kohn, 1999), as described by Gerin et al. (2020). Amplicons were sequenced (Macrogen, Seoul, South Korea), and the partial sequences of ITS1-5.8S-ITS2 of rDNA (*ITS*) and translation elongation factor 1-α (*TEF1*) were obtained. All sequences were analyzed by BLASTn in the entire nucleotide collection database of GenBank

(https://www.ncbi.nlm.nih.gov). Finally, for the pathogenicity test, all isolates were singly inoculated artificially on one-year old pomegranate plants cv. Wonderful, followed by the re-isolation on PDA medium, as described by Pollastro et al. (2016).

The reference strain CG1 and the ten selected *C. granati* isolates (CG2-CG11) were stored at -80°C in 10% glycerol and used for the subsequent assays. *Phoma betae* IV27, an Apulian (south Italy) isolate, available in the culture collection of Department of Plant Soil and Food Sciences, section Plant Pathology, was used as outgroup in the RAPD analysis.

Water agar (WA, 20 g L^{-1} of agar), malt extract agar (MEA, 20 g L^{-1} of malt extract, 20 g L^{-1} of agar) and PDA were the media used for *in vitro* assays.

Conidial germination and colony growth under different temperatures

For the conidial germination assay, conidia were harvested from pycnidia produced on PDA, under darkness at 25°C, 10 days after inoculation (dai) by using 2 mL of sterile distilled water and a sterile spatula. Conidial suspensions, obtained by filtering through a layer of sterile miracloth (Calbiochem, CA, USA), were adjusted to 3×10^5 conidia mL^{-1}. Two replicated WA,

106 PDA and MEA plugs (7 mm diameter) were inoculated with 10 µL of conidial suspension for
107 each isolate, placed on microscope slides and maintained in moist chambers at six different
108 temperatures (5, 15, 20, 25, 30 and 35°C). After 18-20 hours, conidia were fixed and stained
109 by adding 10 µL of cotton blue-lactophenol solution and samples observed under an optical
110 microscope (DM2500, Leica, Wetzlar, Germany) equipped with a micrometer. Three random
111 samples of 100 conidia per condition was observed and the percentage of conidia germination
112 recorded. In each sample, the germ tube length (µm) of 30 germinated conidia was measured.
113 For the colony growth assay, mycelial plugs of 4 mm in diameter collected from the edges of
114 actively growing colonies on PDA of each isolate were used to inoculate three replicated Petri
115 dishes containing PDA. The orthogonal diameters of developing colonies were measured after
116 2 and 7 dai at 5, 15, 20, 25, 30 and 35°C in the darkness, and mean daily growth was
117 calculated.
118 For the different analysed conditions (media and/or temperatures), the average value of the
119 conidia germination percentage (CGP), germ tube length (GTL) and daily growth increase (DGI)
120 was obtained using the eleven isolates as biological replicates. CGP and GTL data were
121 submitted to two way-ANOVA analysis by using CoStat software (CoHort, Monterey, CA, USA).

122

123 ***Baseline sensitivity to fungicides***

124 Fungicides

125 Technical-grade Cyprodinil (Syngenta Crop Protection, Műnchwilen, Switzerland),

126 Thiophanate-methyl (Merck KGaA, Darmstadt, Germany), Phosethyl Aluminium (Merck KGaA,

127 Darmstadt, Germany), Fludioxonil (Syngenta Crop Protection) and Tebuconazole (Merck KGaA,

128 Darmstadt, Germany) were dissolved in dimethylsulfoxide. Commercial formulations of

129 Proquinazid [Talendo, 20% active ingredient (a.i.); Du Pont, Milan Italy], Fenpyrazamine

130 (Prolectus, 50% a.i.; Sumitomo Chemical Italia, Milan, Italy), Boscalid (Cantus, 50% a.i.; BASF

131 SE, Ludwigshafen am Rhein, Germany), Fenhexamid (Teldor, 50% a.i.; Bayer CropScience,

132 Leverkusen, Germany) and Fluxapyroxad (Sercadis, 26.5% a.i; BASF SE) were suspended in

133 sterile distilled water. The final concentration of the solvent was the same in all media and

134 never exceeded 1 mL L^{-1}.

135

136 Conidial germination and colony growth assays

137 The inhibition of conidia germination was evaluated using five different serial concentrations

138 of each fungicide (0.01, 0.1, 1, 10, and 100 mg L^{-1}) into WA medium. For each condition, 7-

139 mm WA-plugs, placed on microscope slides, were inoculated with 10 µL of conidial suspension
140 obtained as described above. WA non-amended with fungicides was used as control. The
141 percentage of germinated conidia on fungicide-amended media, after 16–20 h of incubation at
142 25±1°C, was assessed observing 100 conidia under an optical microscope (×20 magnification).
143 The same fungicides concentrations (0.01, 0.1, 1, 10, and 100 mg L^{-1}) were used for colony
144 growth assay. Each PDA plate was inoculated with 4-mm plugs collected from actively growing
145 colonies of *C. granati* on PDA. PDA non-amended with fungicides was used as control. The
146 orthogonal diameters of the *C. granati* colonies on fungicide-amended media, after 4 and 7
147 days of incubation at 25±1°C in the darkness, were measured.
148 In all cases, the inhibition of conidial germination or colony growth was calculated considering
149 the number of conidia germinated or the colony growth on control medium and on fungicide-
150 amended medium, according to the formula:

151 $$[(C-T)/C] \times 100$$

where C and T are conidial germination or the colony growth on the control medium and on fungicide-amended (treated) medium, respectively. For each condition, three replicated random counts of 100 conidia (conidial germination) or PDA plates (colony growth) were used.

C. granati *diversity*

The ITS sequences of our *C. granati* isolates and those of other 26 isolates of *C. granati* and 30 of *Coniella* species available in GenBank (Table 1; www.ncbi.nlm.nih.gov/) were aligned using MUSCLE in MEGA version 6 (Tamura et al., 2013). The same alignment was performed with *TEF-1* sequences of our *C. granati* isolates, and those of other 11 isolates of *C. granati* and 18 of *Coniella* species available in GenBank (Table 1). *ITS* and *TEF-1* sequences were trimmed and phylogenetic analyses were conducted with the same software using the maximum likelihood (ML) method based on the Tamura-Nei model. In detail, MEGA was used to infer the best model of nucleotide substitution for the dataset via the Tamura-Nei model and the nearest neighbour interchange heuristic search method. Branch stability was determined by 1,000 bootstrap replicates.

Fourteen random 10-mer primers (OPA-2, OPA-3, OPA-9, OPA-12, OPA-13, OPA-16, OPA-17, OPA-20, OPB-15, OPB-20, OPD-5, OPD-8, OPD-15, OPD-20; Operon Technology, Alameda, CA, USA) were used to amplify genomic DNA of the eleven investigated isolates.

In detail, 20 ng of total DNA, extracted as described above, were amplified in a reaction mixture of 25 µL containing 1× Green GoTaq Flexi Buffer, 2 mM $MgCl_2$, 0.3 mM dNTPs, 0.5 µM each primer and 0.75 U of GoTaq DNA polymerase (all PCR reagents were from Promega Corp., Madison, WI). Amplification conditions were 94°C for 3 min, 40 cycles of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C followed by final extension at 72°C for 10 min.

PCR products were separated by electrophoresis in a 1.5 % agarose gel with 1× TAE buffer and amplicons size estimated by comparing the molecular standard 50-2,000 bp DNA ladder (Bio-Rad Laboratories, Hercules, CA, USA). Gel images were recorded and analyzed under UV light into System Gel Doc 1000 (Bio-Rad Laboratories), using Quantity One software version 4 (Bio-Rad Laboratories). Each isolate was scored for the presence or absence of each amplicon and results were transformed in a binary matrix (1 presence and 0 absence of a specific amplicon). Genetic similarity between all pairs of isolates was calculated based on Jaccard's coefficient

182 with the SIMQUAL program of NTSYS pc software (version 2.02, Rohlf, 1998). Similarity
183 coefficients were used to generate the dendrogram, using the UPGMA (unweighted pair group
184 method with arithmetic average) and the SHAN (Sequential agglomerative hierarchical nested
185 cluster analysis) methods of the NTSYS-pc software (Rohlf, 1998).
186
187
188 **Results**
189 *Isolation and identification of C. granati*
190 Pomegranate plants with wilting and dieback of the branches, crown rot, and wood browning
191 confirmed by internal inspection (Figure 1a) were found in all the 10 surveyed orchards with a
192 prevalence over 60% (data not shown). Based on fungal isolations, colonies prevalently
193 referred to *C. granati* were obtained, with few other fungi identified as *Alternaria* spp.,
194 *Cladosporium* spp., and *Penicillium* spp..
195 Ten putative *C. granati* isolates were selected as representative of the different fungal
196 populations. These showed yellowish cream-colored colonies with abundant black pycnidia

producing ellipsoid to fusiform single-celled hyaline conidia (average 11-16×3.5–5 µm) (Figure 1b). According to their morpho-taxonomic characters (Alvarez et al. 2016), the correctness of the preliminary identification as *C. granati* was confirmed.

Moreover, the isolates were submitted to molecular identification through BLASTn analysis of *ITS* and *TEF1* sequences whose accession numbers are reported in Table 1. For all isolates, *ITS* sequences of 524-554 bp and *TEF1* sequences of 278-301 bp were obtained. The multiple alignment of *ITS* sequences of ten isolates and the reference strain CG1 resulted in 100% identity (coverage 100%, e-value 0.0) among them and with available sequences from several *C. granati* isolates, such as CBS 155.71 (MH860043), CBS 152.33 (MH855389.1), A1 (KX507098), STE-U7688 (KU666470), KM61 (KT279814), UACH-156 (KX369239), P2 (KT852449), Akh602 (OK384636). Identity was ≥99% (coverage 100%, e-value 0.0) with other *Coniella* species, such as *C. pseudogranati* (strain CBS 137980; NR_154793.1), *C. castaneicola* (strain LGZ3; MW856809), *C. quercicola* (strain CBS 904.69; MH859478) and *C. diplodiella* (strain CBS 111022; KX833512). The multiple alignment of *TEF1* sequences resulted in identity >99% among the selected isolates and the reference CG1 and ranged from 99.65 to 100% (coverage

212 100% e-value >1e-148) with the other 13 available *TEF1* sequences of *C. granati* isolates (*e.g.*
213 CBS 814.71; KX833682 and CBS252.38; KX833681). Identity values were in the range 77.9 to
214 79.7% (coverage 66 to 93%, e-value 1e-29 to 3e-44) with the *TEF1* sequences of other
215 *Coniella* species, such as *C. africana* (strain CBS 114133; KX833600.1), *C. quercicola* (strain STE-
216 U 3829; AY339365), *C. vitis* (MFLUCC 18-0094; MH366485), *C. koreana* (CFCC 52986;
217 MK578112), and *C. diplodiella* (CBS 166.84; KX833623).
218 All the investigated isolates were artificially inoculated on pomegranate plants and Koch's
219 postulates were proved (Figure 1c).
220
221 *Conidial germination and colony growth under different temperatures*
222 According to two-way ANOVA, a highly significant effect ($p<0.0001$) of temperature ($F= 792.21$
223 and 836.53), medium ($F= 22.53$ and 390.12) and their interaction (4.65 and 87.55) was
224 observed for CGP and GTL, respectively. For the different culture conditions, the data referred
225 to each isolate and the mean values are showed in Figure 2.

226 On the average, CGP was 85.1, 84.9 and 88.9% at 15°C on WA, PDA and MEA (Figure 2a).
227 Conidia germination was always inhibited at 5°C, while increased at 20°C, ranging from 88.6
228 (WA) to 94.5% (MEA), and at 25°C, from 90.2 (WA) to 98.9% (MEA). A slight reduction of CGP
229 was observed at 30°C, ranging from 86.9 (WA) to 96.7% (MEA), while a strong reduction was
230 recorded at 35°C on all the media (15.7%, WA; 43.3%, PDA; 37.6% MEA). At this temperature,
231 on WA, the isolates CG1, CG5, CG6, CG7 and CG11 showed a CGP ≤1%, while the isolates CG2,
232 CG4 and CG9 showed the highest CGP (39.7-52.7%). On PDA and MEA at 35°C, CGP values
233 were generally higher and ranged from 9.0% (CG6) to 69.3% (CG2), and from 17.0% (CG7) to
234 65.3% (CG5), respectively.
235 GTL data at different temperature are shown in Figure 2b. GTL mean values were 19.3 µm on
236 WA, 21.3 µm on PDA, and 22.6 µm on MEA at 15°C; the values increased at 20°C (76.1 µm on
237 WA, 344.9 on PDA, 325.5 on MEA), and even more at 25°C (169.0 µm on WA, 541.3 on PDA,
238 545.0 on MEA). Then, germ tube growth decreased at 30°C (119.6 µm on WA, 441.8 on PDA,
239 472.2 on MEA), and was very restricted a 35°C (6.2 µm on WA, 10.4 on MEA, 12.2 on PDA).

240 The daily increase of colony growth on PDA at different temperature is reported in Table 2. No
241 growth was observed at 5°C. The average daily increase was 6.3 mm at 15°C, 7.6 at 20°C, 9.0
242 mm at 25°C, and 7.9 mm at 30°C. The daily growth was strongly reduced at 35°C, showing an
243 average value of 1.0 mm, with the isolates CG3 and CG5 that were totally inhibited and the
244 isolate CG10 that showed the highest value (3.0 mm).

245

246 *Response to fungicides*
247 The inhibition of conidial germination on WA amended with different fungicide concentrations
248 is shown in Figure 3. Fludioxonil and cyprodinil fully inhibited the conidia germination at 0.1-1
249 μg mL^{-1}, while the EC_{50} was in the range 0.01-0.1 μg mL^{-1}. For all isolates, EC_{50} values <0.01 μg
250 mL^{-1} were observed for fenpyrazamine and proquinazid, while MIC for both fungicides was
251 higher than 100 μg mL^{-1}. For fluxapyroxad, differences in EC_{50} were observed among the
252 isolates. In detail, EC_{50} was <0.01 μg mL^{-1} for the isolates CG2, CG4, CG7, CG10, 0.01-0.1 μg
253 mL^{-1} for CG5 and CG10, 0.1-1 μg mL^{-1} for CG9, 1-10 μg mL^{-1} for CG1, CG8 and CG11 and >100
254 μg mL^{-1} for CG3. MIC was always >100 μg mL^{-1}. For tiophanate-methyl, MIC was 100 μg mL^{-1}
255 while EC_{50} was <0.01 μg mL^{-1} for CG2, CG6, CG7, and CG9, 0.01-0.1 μg mL^{-1} for CG5 and CG11,

256 and 1-10 μg mL^{-1} for the remaining isolates. A MIC of 100 μg mL^{-1} was also observed for
257 tebuconazole for all the assayed isolates, while EC_{50} was in the range 0.01-0.1 μg mL^{-1} for GC6
258 and CG7, 1-10 μg mL^{-1} for CG4, CG5, CG9, CG10 and CG11, and 10-100 μg mL^{-1} for CG1, CG2,
259 CG3 and CG8. Isolates showed a broad variability in response to boscalid, EC_{50} was 1-10 μg
260 mL^{-1} for CG6 and CG7, 10-100 μg mL^{-1} for CG2 and CG9 and >100 μg mL^{-1} for the remaining
261 isolates. A broad variability was also observed in response to phosetyl aluminium: EC_{50} was
262 <0.1 for CG7 and CG9; 0.1-1 μg mL^{-1} for CG2 and CG11, 1-10 μg mL^{-1} for CG4, CG5, CG6 and
263 >100 μg mL^{-1} for CG1, CG3, CG8 CG10. However, the MIC was always >100 μg mL^{-1}. For
264 fenhexamid, the EC_{50} values were in the range 1-10 μg mL^{-1} for CG6, CG9 and CG11 and 10-
265 100 μg mL^{-1} for the remaining isolates, while MIC was always >100 μg mL^{-1}.
266 Data on colony growth inhibition were more uniform among the isolates as compared to those
267 referring to conidial germination (Figure 4a). The colony growth of the reference strain CG1 is
268 represented in Figure 4b. Phosethyl aluminium and proquinazid were ineffective even at the
269 highest tested concentration (100 μg mL^{-1}). The SDHI fungicides boscalid and fluxapyroxad
270 poorly reduced the colony growth, never exceeding 20.6 and 39.9% at 100 μg mL^{-1},

271 respectively. The SBI-group III fungicides fenhexamid and fenpyrazamine showed EC_{50} around
272 100 µg mL^{1}. Tebuconazole and tiophanate-methyl showed EC_{50} values at 0.1-1 µg mL^{-1}, while
273 MIC values were 10 µg mL^{-1} and 10-100 µg mL^{-1}, respectively. The colony growth was almost
274 totally inhibited (>90%) at 100 µg mL^{-1} by cyprodinil, while the EC_{50} ranged between 1 and 10
275 µg mL^{-1} for the CG4 and CG8 isolates and between 10 and 100 µg mL^{-1} for the others.
276 Fludioxonil showed EC_{50} between 0.01 and 0.1 µg mL^{-1}, and MIC was 1 µg mL^{-1} for all the
277 isolates.
278
279 *ITS- and TEF-1-phylogenetic analysis and RAPD polymorfism*
280 The preliminary phylogenetic analyses using the ML based on the Tamura-Nei model resulted
281 in trees with the highest log likelihood of - 1968.84 and -4264.46 using *ITS* and *TEF-1*
282 sequences, respectively. The ITS sequences of the CG1-CG11 isolates, characterized in this
283 study, clustered with all the other *C. granati* isolates (100% support with 1,000 bootstrap
284 replicates) from different localities. All *C. granati* isolates clustered together in a group
285 phylogenetically closed to *C. pseudogranati* and *C. fici* (87% support, branch length 0.03 and

286 0.08) and less *to C. lanneae*, and well separated from the other *Coniella* species (Figure 5a).
287 Also for the *TEF-1* locus, considering the sequences available in GenBank, the CG-CG11 isolates
288 clusterized with all the other *C. granati* isolates (100% support). Considering the available
289 sequences of other *Coniella* species, *C. erumpens* was the species most phylogenetically close
290 to *C. granati* group (96% support, branch length 0.324), that were well separated by the other
291 *Coniella* species (Figure 5b).
292 RAPD PCR generated a total of No. 98 recognizable bands corresponding to molecular sizes
293 ranging from ~100 bp to ~1,500 bp. The highest number of bands (No. 6) was obtained by
294 using the primer OPD-20, while the lowest number of bands (No. 3) with the primer OPA-20
295 (Figure 6a). Sixty markers were exclusive for all *C. granati* isolates, while 27 markers were
296 common to *C. granati* isolates and the outgroup *P. betae* IV27. Bands present only in the *C.*
297 *granati* isolates CG1-CG6 were observed by the examination of OPA-2, OPA-17 and OPD-20
298 profiles, while No. 34 bands were exclusive to *P. betae*. The Jaccard's similarity index was over
299 0.95 among *C. granati* isolates and 0.30 among those and *P. betae* (Figure 6b). The small

300 differences observed allowed to identify three major clusters into *C. granati*: i) CG1, CG7,
301 CG10, CG4, CG6; ii) CG2, CG3, CG8, CG5, CG9; and iii) CG11.

302

303

304 **Discussion**

305 The fungal pathogen *Coniella granati*, inducing crown rot and fruit rot, is one of the major
306 threats for pomegranate cultivation causing heavy economic losses worldwide (Jabnoun-
307 Khiareddine et al., 2018). Knowledge on the biology, epidemiology and genetic variability of
308 the fungus is scant and deeper insights are required for improving disease management in the
309 field as well as in post-harvest (Munhuweyi et al., 2016).

310 With this study, we report the rapid spread of *C. granati* in several Italian regions where is
311 causing a severe decline in pomegranate orchards. Eleven isolates were selected as
312 representatives of fungal populations from different areas and characterized for the response
313 of colony growth and conidial germination to different media and temperatures as well as for
314 their sensitivity to ten fungicides representative of 8 modes of action. Moreover, preliminary
315 observations on genetic variability in *C. granati* were carried out through the analysis of RAPD
316 profiles.

317 Knowledge on environmental conditions inducive to disease development can be of help in
318 explaining the pathogen's spreading in a new area and in setting sustainable crop protection
319 strategies. A first study on two Californian isolates of *C. granati* reported that temperatures of
320 25-30°C are favorable to colony growth (Michailides et al., 2011). More recently, observations
321 on three Greek isolates confirmed 25-30°C as the optimal temperature range for mycelial
322 growth, conidia germination and germ tube elongation; the mycelial growth was reduced up
323 to 40-50% at 15°C, while conidial germination was fully inhibited at 4 and 35°C media
324 (Thomidis, 2015). Our results with Italian isolates showed that the highest conidial
325 germination percentages and germ tube lengths occurred, as expected, on PDA, MEA and WA,
326 in the order. On average, 95% conidia germinated at 25-30°C in the three compared conidial
327 germination was slightly reduced at 15°C (average 86%), while it was strongly restricted at
328 35°C, observing no germination for several isolates on the WA medium. Conidial germination
329 was fully inhibited at 5°C. PDA and MEA promoted germ tube elongation 1.5-2 times more
330 than WA. The maximum germ tube length was recorded at 25°C followed by 30, 20, 15 and
331 35°C, in the order. The highest values of daily colony growth (average 9 mm day^{-1}) was also

noticed at 25°C, followed by both 30°C and 20°C (~8 mm day^{-1}) and 15°C (~6 mm day^{-1}). Four isolates, CG1, CG6, CG8, and CG10, irrespective of their origin, were more thermotolerant than the others showing a daily growth rate >1 mm day^{-1} at 35°C, while 5°C fully prevented mycelial growth. These findings confirmed the results from previous studies on conidial germination and colony growth (Thomidis, 2015) and on artificially inoculated pomegranate fruits (Palou et al., 2013;), and are a step forward in the knowledge on *C. granati* adaptation to environmental conditions, particularly temperature, useful to foresee the possible evolutionary trajectories of the pathogen's populations.

Plant protection products allowed in Europe against *C. granati* on pomegranate are very few. Although data regarding the chemical control of the pathogen are very scant, the effectiveness of benzimidazoles (e.g., thiophanate methyl) and triazoles (e.g., tebuconazole), against other pycnidia-forming fungi, such as several species of the genera *Botryosphaeria*, *Diaporthe* and *Phoma*, was well demonstrated in numerous studies (e.g., Ma et al., 2002; Schmitz et al., 2006). The mixture boscalid/pyraclostrobin, tebuconazole and tiophanate methyl have been proposed for the management of fruit rot caused by *C. granati* (Uysal et al., 2018).

Additionally, Munhuweyi et al. (2016) evaluated eight fungicides (captan, mancozeb, ziram, carbendazim, copper oxychloride, tiophanate-methyl, bordeaux mixture and chlorothalonil) against *C. granati* on pomegranate cv. Ganesh and found that carbendazim, mancozeb and bordeaux mixture were the most effective to control the pathogen and its spread in the field. In Italy, according to European and national regulation, in addition to copper oxychloride and sulphate, sulphur, the mixture of terpenes geraniol+tymol+eugenol and *Bacillus amyloliquefaciens*, the only synthetic fungicides allowed on pomegranate are boscalid and fosetyl aluminium. Therefore, 10 fungicides representative of eight different modes of action, allowed on different crops and pathogens, were tested through *in vitro* assays to evaluate their inhibitory effects on conidial germination and colony growth of *C. granati*. The phenylpyrrole fludioxonil was the most effective fungicide, causing full inhibition of both conidial germination and colony growth at concentration as low as 1 mg L^{-1}. The anilinopyrimidine cyprodinil displayed the same activity against conidial germination but was much less effective against colony growth. The triazole tebuconazole and the benzimidazole thiophanate-methyl proved more effective against colony growth, whereas the activity against

conidial germination was very variable depending on isolates. Concerning the SBI-group III fungicides, fenpyrazamine was more effective than fenhexamid in inhibiting conidial germination but both fungicides showed poor inhibition of colony growth. Notably, the two fungicides allowed on pomegranate, the SDHI boscalid (as well as fluxapyroxad) and fosetyl aluminium showed *in vitro* a very poor biological activity against *C. granati*. It is then obvious that further research, field trials and consequent normative updates are urgently needed to provide pomegranate growers with effective tools to manage both crown rot and fruit rot caused by *C. granati* that can threaten the investments for planting orchards and cause heavy yield losses.

Knowledge about the diversity within pathogen populations is essential to understand its population biology, and the interactions established with host plants, beneficial microorganisms, and associated microbiome, as well as to explore the reasons underlying different response of cultivars to the disease (Jabnoun-Khiareddine et al., 2018). This is especially required when a sudden and rapid spread of a pathogen occurs, as in the pathosystem pomegranate - *C. granati*. The eleven *C. granati* isolates investigated in this study showed >99% identity in *ITS* and *TEF1* sequences, and the partial *ITS* sequences

analyzed were identical to all *C. granati* isolates isolated from different countries. Therefore, intraspecific variation was investigated by using RAPD markers. Such markers were indeed used, for instance, to discriminate defoliating and non-defoliating pathotypes of *Verticillium dahliae* (Nigro et al., 2005), as well as for developing specific molecular diagnostic tools, as SCARs (Sequence Characterized Amplified Region) for pathogens associated to grapevine trunk diseases (Pollastro et al., 2000; 2001). A total of 98 RAPD markers were generated by using 14 random 10-mer primers. A very limited diversity was observed among the eleven *C. granati* isolates (similarity coefficient >0.95). This finding, although based on the analysis of few isolates, suggests that single o few *C. granati* clones were recently introduced in Italy, likely as a result of the very common adoption of international cultivars (ISTAT, 2023), such as 'Wonderful', mainly through plant propagation materials that are usually originated in few big nursery centers. The scant, if any, diversity might also depend on the uncommon occurrence of the sexual stage in *C. granati*, as reported for other *Coniella*-like fungi (Aveskamp et al., 2008). Further in-depth research is needed to better clarify the role of plant propagation materials in the spreading of *C. granati* and to put in place actions aimed to prevent or

393 manage the phytosanitary risks due to further introductions of the pathogen and/or new
394 highly virulent genotypes.

395 In conclusion, the present paper reports the rapid spread of crown rot caused by *C. granati*
396 occurred in recent years in Italian orchards of pomegranate cv. Wonderful, following the first
397 detection in Italy (Pollastro et al., 2016), and provide new insights on thermal requirements
398 and baseline sensitivity to common fungicides of *C. granati*, as well as on the diversity among
399 isolates of the fungus from different regions of Italy. The improved knowledge will contribute
400 to improve the sustainable management of the disease and then the quality of pomegranate
401 growing and produce.

402

403

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413

414 **Conflict of interest**

415 The authors have no conflict of interest to declare.

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417

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516 **Table 1** Isolates of *Coniella* spp. used in this study.

Species	Isolate	Host	Location	Accession No.[b]	
				ITS	*TEF-1*
C. granati	CG1[a]	*Punica granatum*	Apulia, Italy	KU147239.1	OQ812112
	CG2	*Punica granatum*	Tuscany, Italy	MH660274.1	OQ812113
	CG3	*Punica granatum*	Sicily, Italy	OQ793774	OQ812114
	CG4	*Punica granatum*	Tuscany, Italy	OQ793775	OQ812115
	CG5	*Punica granatum*	Lazio, Italy	OQ793776	OQ812116
	CG6	*Punica granatum*	Lazio, Italy	OQ793777	OQ812117
	CG7	*Punica granatum*	Tuscany, Italy	OQ793778	OQ812118
	CG8	*Punica granatum*	Calabria, Italy	OQ793779	OQ812119
	CG9	*Punica granatum*	Lazio, Italy	OQ793780	OQ812120
	CG10	*Punica granatum*	Lazio, Italy	OQ793781	OQ812121
	CG11	*Punica granatum*	Apulia, Italy	OQ793782	OQ812122
	CBS 252.38	*Vitis vinifera*	Italy	KX833581.1	KX833681.1
	MEL2	*Punica granatum*	Italy	MT611223.1	n.a.
	UACH-156	*Punica granatum*	Mexico	KX369239.1	n.a.
	CBS 152.33	*Punica granatum*	Cyprus	MH855389.1	KX833678.1
	Hungary01	*Punica granatum*	Hungary	MW581953.1	OM908764.1
	P2	*Punica granatum*	Florida	KT852449.1	n.a.
	CPC 19626	*Punica granatum*	Iran	JN815313.1	n.a.

	Anhui	*Punica granatum*	China	KF560320.1	n.a.
	A1	*Punica granatum*	China	KX507098.1	n.a.
	11B	*Punica granatum*	Hatay, Turkey	KX962562.1	n.a.
	C26	*Punica granatum*	Turkey	MH890480.1	n.a.
	CPC 19625	*Punica granatum*	Iran	JN815312.1	n.a.
	CBS 814.71	*Punica granatum*	Turkey	MH860368.1	KX833682.1
	ET 85	*Punica granatum*	Turkey	MH992151.1	n.a.
	CBS 132860	*Punica granatum*	Turkey	KX833577.1	KX833677.1
	CBS 155.71	*Citrus* sp.	Turkey	KX833579.1	KX833679.1
	NLD-2021-1	*Rosa* sp.	India	MW898436.1	MW916275.1
	NLD-2021-2	*Rosa* sp.	India	MW898437.1	MW916276.1
	NLD-2021-3	*Punica granatum*	-	MW898438.1	MW916277.1
	NLD-2021-4	*Punica granatum*	-	MW898439.1	MW916278.1
	RoseMKS320	*Rosa* sp.	India	MF067429.1	n.a.
	PomeMKS321	*Punica granatum*	India	MF067428.1	n.a.
	PomeMKS322	*Punica granatum*	India	MF067427.1	n.a.
	KM61	*Punica granatum*	South Africa	KT279814.1	n.a.
	STE-U7687	*Punica granatum*	South Africa	KU666469.1	MG255164.1
	STE-U7688	*Punica granatum*	South Africa	KU666470.1	n.a.
C. pseudogranati	CBS 137980	*Terminalia stuhlmannii*	Zambia	KJ869132.1	n.a.
C. fici	MFLU 18-2578	*Ficus septica*	Taiwan	NR_174842.1	n.a.
C. pseudokoreana	MFLUCC 12-0427	-	-	NR_154860.1	n.a.

C. diplodiopsis	CBS 590.84	*Vitis vinifera*	Sardinia, Italy	MH861786.1	n.a.
C. diplodiella	CBS 111858	*Vitis vinifera*	France	MH862886.1	n.a.
C. fusiformis	CBS 141596	*Eucalyptus* sp.	Indonesia	NR_154815.1	KX833674.1
C. hibisci	CBS 109757	*Hibiscus* sp.	Africa	KX833589.1	KX833689.1
C. javanica	CBS 455.68	*Hibiscus sabdariffa*	Bogor, Indonesia	MH859180.1	n.a.
C. nicotianae	CBS 875.72	*Nicotiana tabacum*	Jamaica	KX833590.1	KX833690.1
C. straninea	CBS 149.22	-	USA	MH854728.1	KX833704.1
C. pseudostraminea	CBS 112624	*Fragaria* sp.	Pretoria, South Africa	KX833593.1	KX833696.1
C. quercicola	CBS 904.69	*Quercus robur*	Vorden, Netherlands	MH859478.1	n.a.
C. koreana	CBS 143.97	-	Taejon, South Korea	KX833584.1	KX833684.1
C. tibouchinae	CPC 18512	*Tibouchina granulosa*	Minas Gerais, Brazil	NR_111706.1	JQ281779.1
C. lannae	CBS 141597	*Lannea* sp.	Zambia	NR_154818.1	KX833685.1
C. malaysiana	CBS 141598	*Corymbia torelliana*	Kuala Lumpur, Malaysia	NR_154820.1	KX833688.1
C. eucalyptigena	CPC 24793	*Eucalyptus brassiana*	Sabah, Malaysia	KR476725.1	n.a.
C. limoniformis	CBS 111021	*Fragaria* sp.	South Africa	KX833586.1	n.a.
C. pesudodiospyri	CBS 145541	*Corymbia hybrid*	Malaysia	MK876382.1	n.a.
C. diospyri	CBS 145071	*Diospyros mespiliformis*	Limpopo, South Africa	MK047439.1	MK047562.1
C. duckerae	CBS 142045	*Lepidosperma concavum*	Victoria, Australia	KY924929.1	n.a.
C. macrospora	CBS 524.73	*Terminalia ivoriensis*	Man, Ivory Coast	KX833587.1	KX833687.1
C.a lustricola	ILLS 80714	-	Pennsylvania, USA	NR_156349.1	n.a.
C. erumpens	CBS 523.78	-	Valdivia, Chile	KX833535.1	KX833630.1
C. wangiensis	CBS 132530	*Eucalyptus* sp.	Northen territory, Australia	JX069873.1	KX833705.1

C. peruensis	CBS 110394	Forest soil	Iquitos, Peru	KJ710463.1	KX833695.1
C. paracastaneicola	CBS 141292	*Eucalyptus sp.*	Victoria, Australia	KX833591.1	KX833693.1
C. fragrariae	CBS 198.82	Vineyard soil	Chançay, Francia	MH861493.1	KX833666.1
C. solicola	CBS 766.71	Soil	Potchefstroom, South Africa	MH860343.1	KX833701.1
C. nigra	CBS 165.60	Soil	Maharashtra, India	MH857944.1	KX833691.1

a: The reference strain CG1 is available at the CBS culture collection under code CBS 144846.

b: Accession number on NCBI GeneBank (www.ncbi.nlm.nih.gov) for *ITS* (ITS1-5.8S-ITS2 of rDNA) and *TEF-1* (translation elongation factor 1-α) partial sequences. n.a.: sequence not available in GenBank.

521 **Table 2.** Daily growth increase (mm) of *Coniella granati* colonies on

522 PDA at different temperature.

Isolate	5°C	15°C	20°C	25°C	30°C	35°C
CG1[a]	0.0±0.0	4.9±0.4	7.5±0.2	9.5±0.2	8.8±0.1	1.7±0.1
CG2	0.0±0.0	6.1±0.1	7.0±0.0	8.6±0.3	7.0±0.0	0.3±0.1
CG3	0.0±0.0	5.9±0.1	7.3±0.1	9.5±0.7	7.4±0.2	0.0±0.0
CG4	0.0±0.0	6.1±0.1	7.1±0.3	8.6±0.2	7.4±0.5	0.8±0.3
CG5	0.0±0.0	6.8±0.2	8.3±0.3	9.4±0.5	8.6±0.7	0.0±0.0
CG6	0.0±0.0	6.9±0.4	7.9±0.4	9.7±0.2	8.4±0.5	1.9±0.4
CG7	0.0±0.0	5.9±0.7	7.2±0.6	7.8±0.8	7.3±0.5	0.3±0.1
CG8	0.0±0.0	6.0±0.1	6.9±0.1	8.5±0.6	7.0±0.2	1.2±0.5
CG9	0.0±0.0	6.5±0.1	7.6±0.0	8.5±0.2	7.6±0.1	0.9±0.2
CG10	0.0±0.0	7.7±0.5	9.2±0.3	9.8±0.5	9.4±0.1	3.0±0.2
CG11	0.0±0.0	6.3±0.1	7.7±0.0	9.1±0.2	7.9±0.1	0.8±0.2
Average	0.0±0.0	6.3±0.2	7.6±0.2	9.0±0.2	7.9±0.2	1.0±0.3

523 Data represent mean value ± standard error. a: the CBS culture

524 collection code of the isolate CG1 is CBS 14484.

525 **Figure captions**

526 **Figure 1.** Isolation, identification, and pathogenicity assay of *Coniella granati* from
527 pomegranate: a) crown rot symptoms; b) 15-day-old PDA colonies of *C. granati* and, from left
528 to right, pycnidia under stereomicroscope and optical microscope and conidia (bar=10 µm); c)
529 external and internal symptoms in plants inoculated and non-inoculated with *C. granati*.

530

531 **Figure 2.** Percentages of conidial germination (a) and germ tube length (b) of *Coniella granati*
532 isolates on different media and temperature. WA: water agar; PDA: potato dextrose agar;
533 MEA: malt extract agar.

534

535 **Figure 3.** Inhibition of germination of *Coniella granati* conidia by fungicides.

536

537 **Figure 4.** Inhibition of colony growth of *Coniella granati* by fungicides a); and b) response to
538 fungicides of the reference strain CG1 of *Coniella granati*.

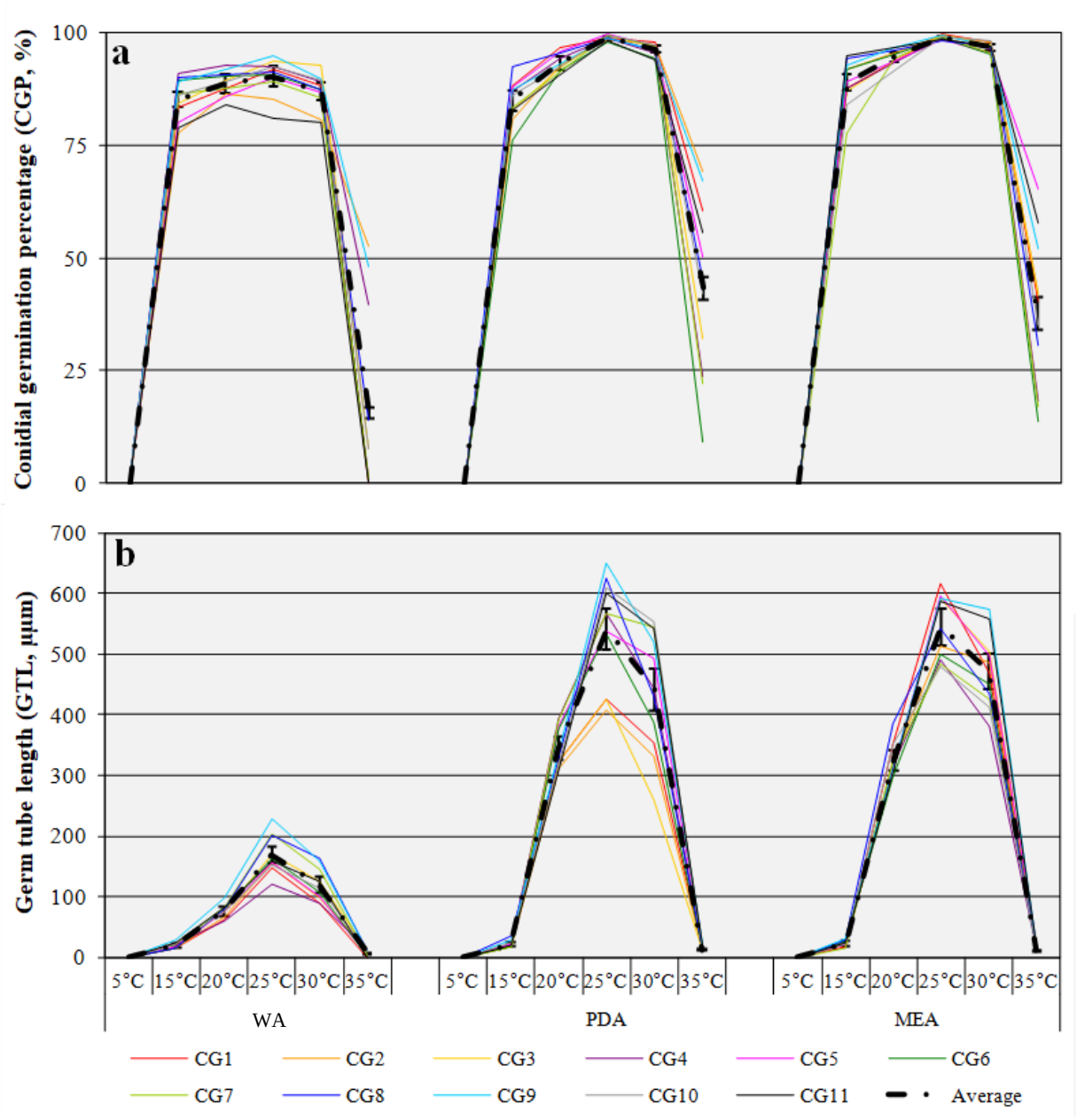
539

Figure 5. Phylogenetic analysis using the maximum likelihood (ML) method based on the Tamura-Nei model withing the genus *Coniella* based on *ITS* (a) and *TEF-1* (b) sequences available in GenBank (www.ncbi.nlm.nih.gov). Values on the nodes are the results of 1,000 bootstrap replicates.

Figure 6. RAPD profiles (a) and UPGMA/SHAN dendrogram (b) of *Coniella granati* isolates sampled in different regions of Italy. The *Phoma betae* isolate IV27 was used as outgroup.

547

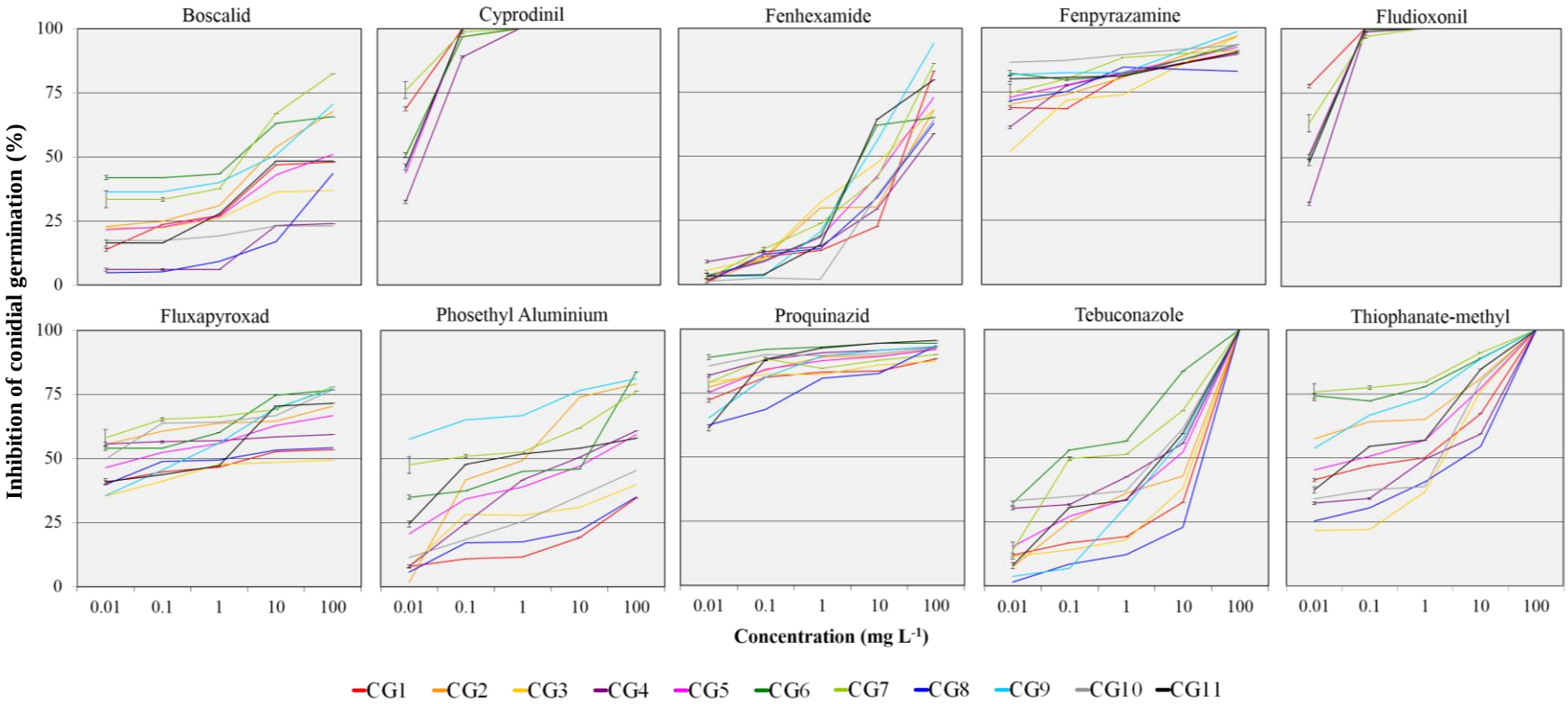
548 Figure 1.



549

550 Figure 2.

Donato Gerin
Plant Disease



551

552 Figure 3.

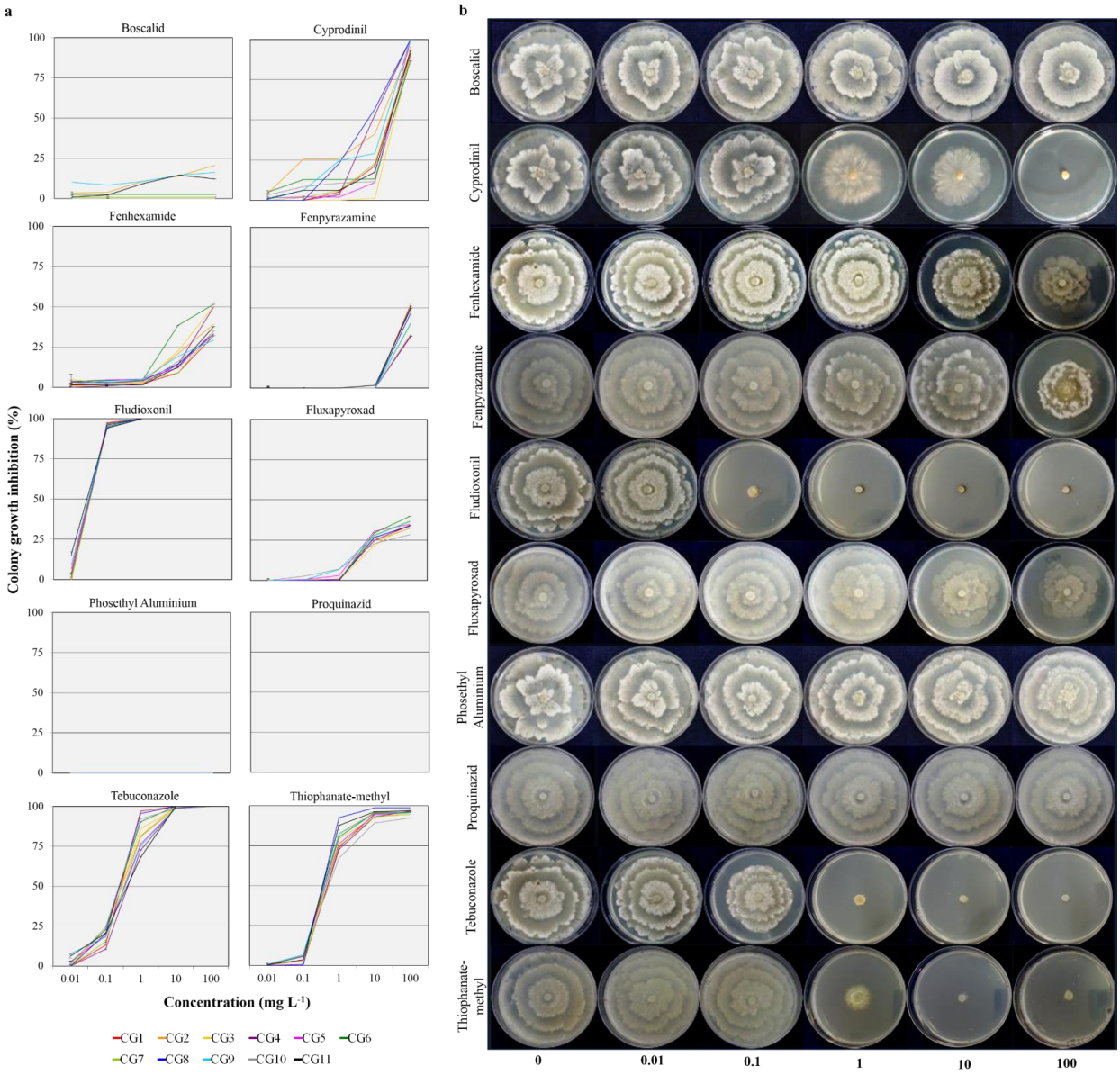

a
Boscalid
Cyprodinil
Fenhexamide
Fenpyrazamine
Fludioxonil
Fluxapyroxad
Phosethyl Aluminium
Proquinazid
Tebuconazole
Thiophanate-methyl
Colony growth inhibition (%)
Concentration (mg L-1)
CG1 CG2 CG3 CG4 CG5 CG6
CG7 CG8 CG9 CG10 CG11
b
Boscalid
Cyprodinil
Fenhexamide
Fenpyrazamnie
Fludioxonil
Fluxapyroxad
Phosethyl Aluminium
Proquinazid
Tebuconazole
Thiophanate-methyl
0
0.01
0.1
1
10
100

Figure 4.

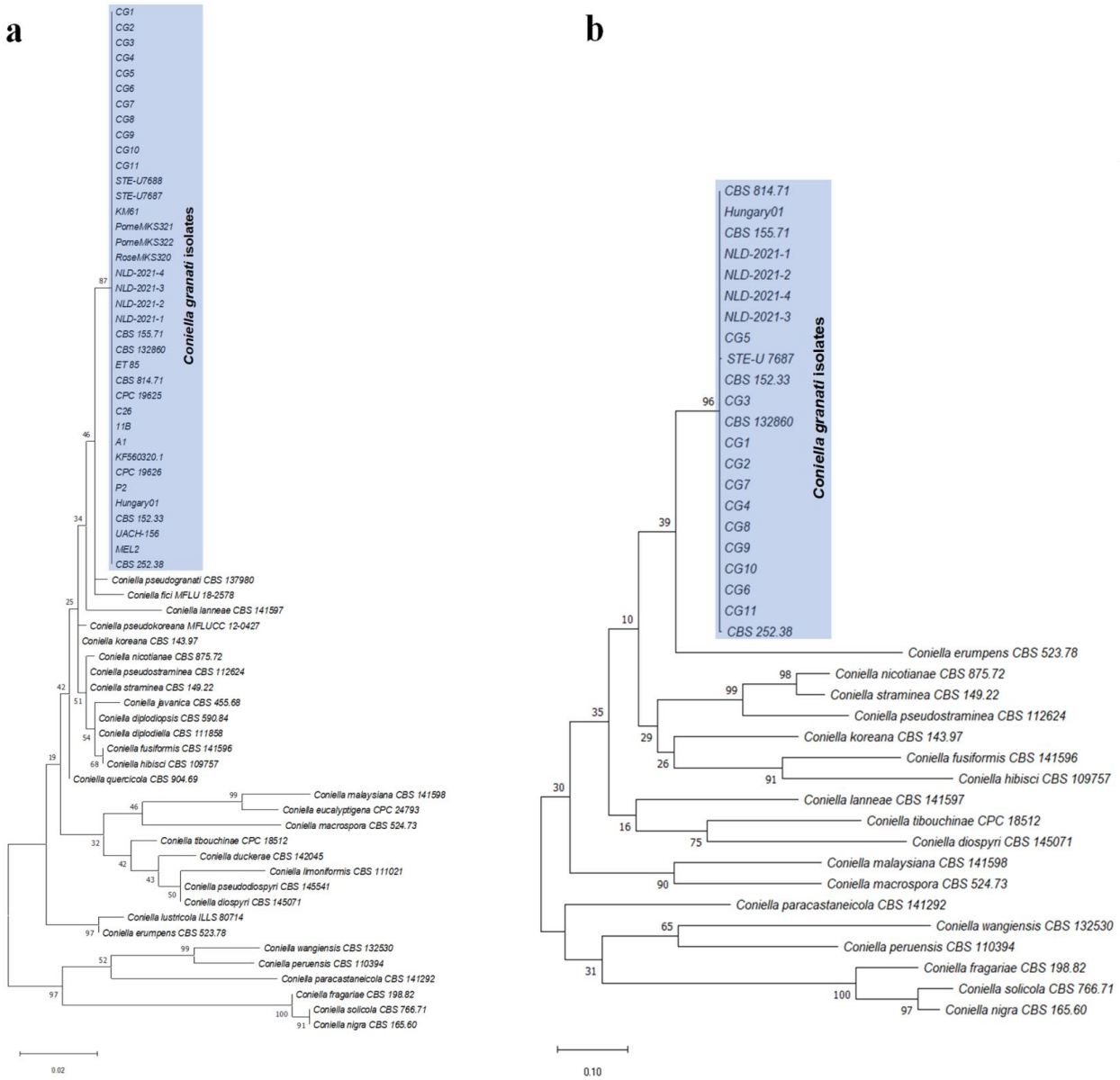

a
CG1
CG2
CG3
CG4
CG5
CG6
CG7
CG8
CG9
CG10
CG11
STE-U7688
STE-U7687
KM61
PomeMKS321
PomeMKS322
RoseMKS320
NLD-2021-4
NLD-2021-3
NLD-2021-2
NLD-2021-1
CBS 155.71
CBS 132860
ET 85
CBS 814.71
CPC 19625
C26
11B
A1
KF560320.1
CPC 19626
P2
Hungary01
CBS 152.33
UACH-156
MEL2
CBS 252.38
Coniella granati isolates
Coniella pseudogranati CBS 137980
Coniella fici MFLU 18-2578
Coniella lanneae CBS 141597
Coniella pseudokoreana MFLUCC 12-0427
Coniella koreana CBS 143.97
Coniella nicotianae CBS 875.72
Coniella pseudostraminea CBS 112624
Coniella straminea CBS 149.22
Coniella javanica CBS 455.68
Coniella diplodiopsis CBS 590.84
Coniella diplodiella CBS 111858
Coniella fusiformis CBS 141596
Coniella hibisci CBS 109757
Coniella quercicola CBS 904.69
Coniella malaysiana CBS 141598
Coniella eucalyptigena CPC 24793
Coniella macrospora CBS 524.73
Coniella tibouchinae CPC 18512
Coniella duckerae CBS 142045
Coniella limoniformis CBS 111021
Coniella pseudodiospyri CBS 145541
Coniella diospyri CBS 145071
Coniella lustricola ILLS 80714
Coniella erumpens CBS 523.78
Coniella wangiensis CBS 132530
Coniella peruensis CBS 110394
Coniella paracastaneicola CBS 141292
Coniella fragariae CBS 198.82
Coniella solicola CBS 766.71
Coniella nigra CBS 165.60
0.02
b
CBS 814.71
Hungary01
CBS 155.71
NLD-2021-1
NLD-2021-2
NLD-2021-4
NLD-2021-3
CG5
STE-U 7687
CBS 152.33
CG3
CBS 132860
CG1
CG2
CG7
CG4
CG8
CG9
CG10
CG6
CG11
CBS 252.38
Coniella granati isolates
Coniella erumpens CBS 523.78
Coniella nicotianae CBS 875.72
Coniella straminea CBS 149.22
Coniella pseudostraminea CBS 112624
Coniella koreana CBS 143.97
Coniella fusiformis CBS 141596
Coniella hibisci CBS 109757
Coniella lanneae CBS 141597
Coniella tibouchinae CPC 18512
Coniella diospyri CBS 145071
Coniella malaysiana CBS 141598
Coniella macrospora CBS 524.73
Coniella paracastaneicola CBS 141292
Coniella wangiensis CBS 132530
Coniella peruensis CBS 110394
Coniella fragariae CBS 198.82
Coniella solicola CBS 766.71
Coniella nigra CBS 165.60
0.10

Figure 5.