Running Head: *Detecting olive anthracnose pathogens*

Quantitative detection of *Colletotrichum godetiae* and *C. acutatum sensu stricto* in the phyllosphere and carposphere of olive during four phenological phases

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ABSTRACT

A duplex qPCR detection method was developed to detect and quantify *Colletotrichum godetiae* and *C. acutatum sensu stricto (s.s.)* in olive tissues. The method proved highly specific and sensitive with a detection limit of 10 pg for both pathogens. The analysis of green and senescent leaves, fertilized fruitlets with floral residues, green fruit and symptomatic and asymptomatic fruit collected in May, June, October and December revealed a high incidence of both *C. godetiae* and *C. acutatum s.s.* in Calabria, southern Italy. In comparison with previous reports, these results highlighted an ongoing population shift from *C. godetiae* to *C. acutatum s.s.* Interestingly, *C. godetiae* was slightly more abundant in terms of number of infected samples, yet the quantity of *C. acutatum* in infected samples was always higher, suggesting greater aggressiveness and/or sporulation ability of the latter pathogen. The populations of both *C. godetiae* and *C. acutatum s.s.* increased sharply in December even though both pathogens were detected widely in asymptomatic samples in May, June and October, confirming an important role of latent infections in the disease cycle. A large quantity of both *C. godetiae* (1.7×10^8 cells/mg of tissue) and *C. acutatum s.s.* (7.5×10^8 cells/mg of tissue) was estimated in symptomatic fruit, presenting an enormous inoculum potential for secondary infections. Two other important observations were a high incidence and quantity of both pathogens in senescent leaves and in fertilized fruitlets with floral residues as compared to green leaves.

Keywords: Olive anthracnose, *Colletotrichum godetiae*, *Colletotrichum acutatum* sensu stricto, Quantitative PCR, Duplex PCR

INTRODUCTION

Olive anthracnose is caused by different species of the genus *Colletotrichum* and can have a profound effect reducing both fruit yield and quality of oil in olive growing regions worldwide (Cacciola et al. 2012). Symptoms typically occur on mature fruit as dark necrosis with abundant orange conidial masses, leading to premature fruit drop or mummification. Under favorable conditions, symptoms can occur on branches and leaves, leading to necrosis, defoliation and eventual death of branches (Talhinhas et al. 2009; Cacciola et al. 2012).

Significant advances have been made in understanding diversity within the genus *Colletotrichum* through analysis of a comprehensive worldwide collection of isolates based on phylogenetic, morphological and pathological features (Damm et al. 2012; Weir et al. 2012). These studies have redefined the taxonomy of the genus and have helped determine the etiology of a number of diseases,

including olive anthracnose. The causal agent was originally described in Portugal as *Gloeosporium olivarum* and subsequently reclassified as *Colletotrichum gloeosporioides*. Later, two complexes of species showing high phenotypic and genotypic diversity, *C. gloeosporioides sensu lato* (*s.l.*) and *C. acutatum s.l.*, were found to be associated with the disease (Talhinhas et al. 2005; Moral et al. 2008; Talhinhas et al. 2011, Cacciola et al. 2011). In addition, *Colletotrichum karstii*, a species belonging to the *Colletotrichum boninense* complex was isolated from olives (Schena et al. 2014). Despite the large number of species associated with olive anthracnose, only six, formerly identified as species groups within *C. acutatum s.l.*, are currently considered important as causing losses to olive production. They include *C. simmondsii*, *C. fioriniae*, *C. godetiae* (syn. *C. clavatum*), *C. nymphaeae*, *C. acutatum sensu stricto* (*s.s.*), and *C. rhombiforme* (Sreenivasaprasad & Talhinhas, 2005, Faedda et al. 2011; Damm et al. 2012, Cacciola et al. 2012). *Colletotrichum nymphaeae* is the predominant species in Portugal, followed by *C. godetiae* and *C. acutatum s.s.*, although one study from a different region in Portugal found only four species (*C. nymphaeae, C. fioriniae, C. godetiae* and *C. acutatum s.s.*), with no single sub-population being dominant (Talhinhas et al. 2009). Other data indicate *C. godetiae* is the prevalent species in Greece, Montenegro, Andalusia (Spain) and some restricted areas of Portugal (Moral et al. 2008; Moral et al. 2009; Moral & Trapero, 2009; Moral et al. 2014). *Colletotrichum acutatum s.s.* is the prevalent species affecting olive in Australia, South Africa and Tunisia, but until a few years ago, it was not reported on olives in Italy, where *C. godetiae* and, to a lesser extent *C. gloeosporioides s.s.*, were considered the causal agents of olive anthracnose (Sergeeva et al. 2010; Faedda et al. 2011; Cacciola et al. 2012). However, recent amplicon-based metagenomic analyses revealed wide distribution of both *C. acutatum s.s.* and *C. godetiae* in olive orchards located in Calabria, southern Italy (Mosca et al. 2014; Abdelfattah et al. 2015). The same investigation highlighted the presence of other phylotypes associated with *C. gloeosporioides s.s., Colletotrichum karstii, C. kahawae* and non-well-defined species of *C. acutatum s.l.* and *C. gloeosporioides s.l.*; however, they were very rare and do not to appear to play an important role in causing olive anthracnose (Mosca et al. 2014).

Currently available data on etiology, biology and epidemiology of *Colletotrichum* species associated with olive anthracnose are based on conventional culturing of isolates and subsequent morphological and molecular identification (Sreenivasaprasad & Talhinhas, 2005; Moral et al. 2014). These methods are time-consuming and costly. Furthermore, the lack of selective media for the genus *Colletotrichum* exacerbates problems with isolation of the pathogen and may cause a serious underestimation of populations as fast-growing fungal species are likely to conceal its presence. This is particularly true for thin organs like leaves, flowers and fertilized fruitlets, since contaminant

110 microorganisms cannot be physically avoided by aseptically removing the outer parts of the tissues
111 (Sanzani et al. 2014). As a consequence, the role of leaves, flowers and fertilized fruitlets in the
112 epidemiology of olive anthracnose is not completely understood, although the vegetative organs are
113 assumed to be important for pathogen survival, multiplication and dissemination (Moral et al. 2014).
114 Recent metagenomic analyses based on *Colletotrichum* specific primers have proved to be a powerful
115 tool to characterize those *Colletotrichum* species associated with different olive organs and have provided
116 further valuable information on the epidemiological role of flowers and leaf infections (Mosca et al.
117 2014; Abdelfattah et al. 2015). However, these analyses only provided information on the relative
118 abundance of one taxon compared to others, rather than quantifying a specific microorganism. Although
119 metagenomic analyses are a powerful tool for investigating the genetic diversity in a given environment,
120 they are less useful for quantifying a specific target species as they require complicated bioinformatics
121 analyses and may not be sensitive enough to detect low abundance taxa, especially when based on
122 universal primers.

123 Quantitative real-time PCR (qPCR) detection methods enable the rapid, accurate, sensitive and
124 reliable detection and quantification of one or more (multiplex PCR) target species (Schena et al. 2004;
125 Sanzani et al. 2014). The high sensitivity of qPCR techniques enable the quantification of very low titers
126 of target DNA, which might correspond to the amount present at the initiation of infection or during
127 latent, non-symptomatic infections. As a consequence, qPCR may be used to quantify the build-up of the
128 pathogen throughout the entire disease cycle, and to examine in fine detail all stages of the infection and
129 colonization process in plant material (Sanzani et al. 2014). The ITS regions of the nuclear-encoded
130 ribosomal RNA genes (rDNA) are the most widely used target genes for detection of fungal species
131 (Schena et al. 2004; Schena et al. 2013). However, the ITS regions are not variable enough to differentiate
132 closely related species within *C. acutatum s.l.* due to the many very closely related taxa that have identical
133 or very similar ITS sequences (Damm et al. 2012).

134 Among genes used to redefine the taxonomy of the genus *Colletotrichum*, the β-tubulin gene
135 contains more phylogenetic information compared to the ITS region, and thus has potential to be an ideal
136 marker to differentiate closely related species with a PCR-based detection method (Huang et al. 2009;
137 Damm et al. 2012; Schena et al. 2014). Similarly, the histone H3 gene has well conserved exons
138 sequences, but also contains variable introns that enhance its value in taxonomic and phylogenetic studies
139 of closely related organisms (Damm et al. 2012). Both β-tubulin and histone H3 are single copy genes,
140 which may represent an advantage in developing accurate qPCR methods since the quantification of the

target organisms is not affected by the variable number of copies, which is an issue with multicopy genes (Schena et al. 2006; Schena et al. 2013).

The aim of the present study was to develop a duplex qPCR method based on hydrolysis probes (also known as TaqMan), to simultaneously detect and quantify *C. godetiae* and *C. acutatum s.s.*, using the β-tubulin and histone H3 as target genes, respectively. Subsequent to validation, the method was used to analyze naturally infected samples of flowers, leaves, fertilized fruitlets and green and mature fruit to explore aspects of the epidemiology of olive anthracnose.

MATERIALS AND METHODS

Development of a duplex qPCR method

A duplex qPCR assay was developed for the simultaneous identification, detection and quantification of *C. godetiae* and *C. acutatum s.s.* Specific primers and TaqMan® MGB (minor groove binder) probes (Thermo Fischer) were designed based on highly variable regions of the β-tubulin and histone H3 genes for *C. godetiae* and *C. acutatum s.s.*, respectively. To design the primers and probes, comprehensive sets of validated sequences of β-tubulin and histone H3 genes from *C. acutatum s.l.* were analyzed (Damm et al. 2012). For both genes, the software ELIMDUPES (http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html) was utilized to delete multiple identical sequences within each species. Selected sequences were aligned using MUSCLE as implemented in MEGA (Kumar et al. 2016) and manually checked to identify the most specific target regions for *C. godetiae* and *C. acutatum s.s.* Particular attention was paid to avoid eventual intraspecific polymorphic bases. Primers and probes were designed on the selected regions using Primer Express Software 3.0 (Applied Biosystems, Foster City, CA). Probes were labeled with FAM (*C. godetiae*) or VIC (*C. acutatum s.s.*) at the 5' end, and with Black Hole Quenchers (BHQ1) at the 3'end (Table 1). Primers and TaqMan® probe were synthesized by Applied Biosystems (Life Technologies Inc., Monza, Italy).

Optimization of reaction conditions and specificity assays

Experiments were conducted to optimize amplification conditions (primer and probe concentration) and to determine the best annealing extension temperature for duplex qPCR reactions (data not shown). All amplifications were conducted using DNA from isolate CBS 193.32 of *C. godetiae* and isolate UWS 14 of *C. acutatum s.s.* (Faedda et al. 2011). Total DNA was extracted as previously described (Schena & Cooke, 2006). Optimized qPCR reactions were conducted in a total volume of 20

173 µl containing 1X Universal TaqMan Master Mix with passive reference dye ROX (Applied Biosystems),
174 200 nM of *C. godetiae* primers, 300 nM of *C. acutatum* primers, 100 nM of both *C. godetiae* and *C.*
175 *acutatum s.s.* probes and 1 µl of template DNA (approximately 50 ng for DNA from pure cultures).
176 Amplifications were incubated in a StepOne plus thermal cycler (Applied Biosystems, Monza, Italy)
177 using an initial denaturation step of 10 min at 95°C and 40 cycles of 15 s at 95°C and 20 s at 61°C. In all
178 reactions data were analyzed using the StepOne Plus software according to the manufacturer's
179 instructions. The quantification cycle (Cq) values for each reaction were calculated automatically by the
180 StepOne Plus software by determining the PCR cycle number at which the reporter fluorescence
181 exceeded the background.
182 Specificity tests were conducted using target DNA of 15 different isolates of both *C. godetiae*
183 and *C. acutatum s.s.* from different geographical areas and hosts. Other *Colletotrichum* species utilized
184 in specificity tests comprised the closely related species *C. fioriniae* (4 isolates) and *C. nymphaeae* (8
185 isolates) and 31 isolates of 11 different species recently associated with olives (Schena et al. 2014) (Table
186 2). All experiments were repeated twice, and all reactions were performed in triplicate. In negative
187 control reactions water replaced template DNA.
188
189 **Construction of standard curves**
190 Sensitivity of duplex qPCR reactions and standard curves for quantitative analyses were
191 constructed using total DNA of *C. godetiae* and *C. acutatum s.s.* DNA of both pathogens was quantified
192 using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Monza, Italy), mixed and serially
193 diluted with sterile water to yield final DNA concentrations ranging from 10 ng/µl to 100 fg/µl for each
194 pathogen. In order to evaluate the possible inhibitory effect of DNA extracts from olive tissues, reaction
195 mixtures containing different concentrations of *C. godetiae* and *C. acutatum s.s.* were spiked with 1 µl
196 of DNA from non-infected olive leaves and fruit collected from a *Colletotrichum*-free olive growing
197 area. All amplifications were performed in triplicate and water replaced template DNA for negative
198 control reactions.
199 Amplification mixtures, incubation conditions and data analyses were performed as described
200 above. A standard curve was generated by plotting the DNA quantities against the corresponding Cq
201 value. For both pathogens, correlation coefficients, mathematical functions, and PCR efficiencies were
202 calculated and visualized using StepOne software.
203
204

205 **Analysis of naturally infected olive samples**

206 *Olive sampling and total DNA extraction*

207 The optimized duplex qPCR method was used to test olive samples (cv Ottobratica) of green and
208 senescent leaves, fertilized fruitlets with floral residues, green immature fruit , and symptomatic and
209 asymptomatic mature fruit for presence and quantity of *C. godetiae* and *C. acutatum s.s.* (Table 3).
210 Samples were collected in 2014 at four phenological phases (May, June, October and December) from
211 three Italian olive orchards (F1, F2 and F3) located in the Gioia Tauro plain (38°22'45.0"N 15°56'35.7"E,
212 38°22'16.7"N 15°55'38.7"E and 38°24'48.7"N 15°56'20.1"E, respectively), a locality characterized by
213 annual epidemics of anthracnose (Graniti et al. 1993). Trees were approximately 20 years-old. All
214 samples were from a total of 6 groups of olive trees identified in orchard F1 (3 groups), F2 (2 groups),
215 and F3 (1 group). Each group consisted of three olive trees. Individual samples were cumulatively
216 collected from each group (three plants) and randomly divided to have 3 sub-samples (biological
217 replicates) consisting of 30–200 g of tissues (depending on organ type). Samples were collected from the
218 same groups of plants at all assessment times. At each assessment time, additional samples of all olive
219 tree organ types were collected from a *Colletotrichum*-free olive growing area in Monopoli, southern
220 Italy (40°53'11.6"N 17°18'17.1"E); these samples were used as negative controls. Senescent leaves were
221 characterized by general symptoms of decline (discoloration and local necrosis) not necessarily
222 attributable to anthracnose. Symptoms on fruit collected from the canopy in October and December were
223 considered more typical of *Colletotrichum* infection, even though it was not always possible to exclude
224 a role of other fungal pathogens.

225 Soon after collection, all sub-samples were lyophilized and processed to extract total DNA using
226 a previously described protocol (Mosca et al. 2014). For all sub-samples, DNA was extracted from 50
227 mg (fresh weight) of tissue. Extracted DNA was analyzed using gel electrophoresis (1.5% agarose gel)
228 stained with GelRed (Biotium, Fremont, USA) in TBE buffer, and visualized under UV light using a
229 GelDoc imaging system (BioRad, Segrate, Milano, Italy). DNA concentration and quality was
230 determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Monza, Italy).

231

232 *Detection and quantification of target pathogens*

233 DNA samples from all collected olive sub-samples were analyzed by duplex qPCR to detect both
234 *C. godetiae* and *C. acutatum s.s.* using the amplification conditions described above. The Cq value
235 obtained for each sub-sample (biological replicate) was an average of the three technical replicates. Only

samples that yielded Cq values lower or equal to those determined in standard curves for 10 pg of DNA (detection limit) were considered infected.

To ensure an accurate comparison between collected samples and to take into account small variations in i) sample size (weight of tissue for the DNA extraction), and ii) efficiency of extraction and amplification (that can be influenced by inhibitors), the data were normalized using a qPCR method based on two universal primers and a probe (Schena et al. 2006). Specifically, for each sub-sample Cq values obtained for *C. godetiae* and *C. acutatum s.s.* were corrected against Cq values of the universal method (Cq-u) by multiplying the uncorrected Cq values by the ratio of the average of Cq-u and the Cq-u of each specific sub-sample. The concentration of DNA of *C. godetiae* and *C. acutatum s.s.* in unknown positive sub-samples was extrapolated using corrected Cq values based on the specific mathematical functions (regression solutions) for the standard curves. The concentration of DNA in each sample was the average of the concentrations in its specific sub-samples.

In order to estimate the number of pathogen cells in the samples, DNA quantities expressed in pg were divided by 0.051, i.e. the weight of the genome of *C. acutatum* (http://www.zbi.ee/fungal-genomesize/index.php). The values were assumed to be number of cells/mg of fresh tissue considering that the DNA contained in 1 µl was quantified in the qPCR reactions and that total DNA was extracted from 50 mg of fresh tissues and diluted in 50 µl of water.

RESULTS

Duplex qPCR specificity, sensitivity, standard curves and mathematical functions

Specificity tests using optimized amplification condition revealed high specificity using the duplex qPCR method. Indeed, a specific increase in fluorescence was obtained exclusively for all isolates of *C. godetiae* (FAM) and *C. acutatum s.s.* (VIC) with average Cq values of 24.7 and 25.1, respectively (data not shown).

According to the standard curves the detection limit of both *C. godetiae* and *C. acutatum s.s.* was 10 pg with average Cq values of 35.5 and 37.3, respectively (Fig. 1). For *C. godetiae* some positive amplifications were also obtained with 1 pg of target DNA, but this limit of detection was not considered reliable due to the very high Cq values (≥39). The incorporation of olive DNA did not affect the Cq value or the intensity of the fluorescence (data not shown). The efficiency of the amplification reactions was 103% and 99.8% for *C. godetiae* and *C. acutatum s.s.*, respectively. For the same pathogens the correlation coefficients (R^2) were 0.9941 and 0.9937, respectively (Fig. 1). Mathematical functions for the standard curves were determined for both pathogens (Table 1).

Analysis of naturally infected olive samples

Colletotrichum godetiae or *C. acutatum s.s.* were detected in all sample types but the incidence of the pathogens (% of infected samples) and the estimated quantity of pathogen cells varied according to sampling time and sample types (Table 3). The lowest incidence for both pathogens was in green leaves in May and June followed by green fruit in June (Table 3). Both pathogens were detected in most sample types: the single exception was green leaves in May, in which only *C. godetiae* was detected. Interestingly, a higher incidence was revealed in senescent leaves compared to green leaves at all sample times (Table 3). The incidence of the pathogens increased progressively with time in the calendar year, being higher in October and December compared to May and June. In May the highest incidence of both pathogens was found in fertilized fruitlets with floral residues; *C. godetiae* and *C. acutatum s.s.* were detected in 66.7 and 50% of the samples, respectively. *Colletotrichum godetiae* or *C. acutatum s.s.* was detected in all mature fruit showing symptoms of anthracnose collected in October and December (Table 3). Similarly, all samples of asymptomatic fruit collected in December were found to be infected with the pathogens, while a lower incidence of infection was detected in October. In May and June, the incidence of *C. godetiae* was higher compared to *C. acutatum s.s.* in most samples, but there was no clear different in frequency of detection in October or December (Table 3). Neither *C. godetiae* nor *C. acutatum* were detected in any of the samples collected in the *Colletotrichum*-free olive growing area.

The estimated number of cells of *C. godetiae* and *C. acutatum s.s.* in positive samples showed a sharp increase in population in December in all samples types (Fig. 2). The highest quantity was revealed in symptomatic fruit in December with 1.7×10^8 and 7.6×10^8 cells/mg of fresh tissue for *C. godetiae* and *C. acutatum s.s.*, respectively. A lower quantity of cells was determined for symptomatic fruit in October. In December, a very high quantity of cells was detected in asymptomatic fruit with 1.2×10^8 and 1.3×10^8 cells/mg of fresh tissue for *C. godetiae* and *C. acutatum s.s.*, respectively (Fig. 2). Furthermore, both pathogens were invariably more abundant in senescent leaves compared to green leaves. A moderately high quantity of both species was detected in fertilized fruitlets with floral residues. Overall, lower quantities ranging from 124 and 320 cells/mg of fresh tissues were determined in May, June and October in green leaves and in green and asymptomatic fruit. Interestingly, the quantity of *C. acutatum s.s.* was always greater compared to *C. godetiae* in all positive samples and at all sample time (Fig. 2).

DISCUSSION

A duplex qPCR detection method was developed to detect and quantify *C. godetiae* and *C. acutatum s.s.* in the carposphere and phyllosphere of olive. The method proved highly specific since it

300 did not cross-react with other fungal species of the genus *Colletotrichum*. The number of *Colletotrichum*
301 species included in the specificity test was limited (Damm et al. 2012), but the availability of a
302 comprehensive dataset of validated sequences enabled very accurate *in silico* analysis to identify the
303 regions for designing highly specific primers and probes. According to *in silico* analyses the best regions
304 were identified within the β-tubulin and the histone H3 gene for *C. godetiae* and *C. acutatum s.s.*,
305 respectively. The choice of different genes for the two species was useful as it avoided any similarity in
306 the target region and thus facilitated their use in duplex reactions. Although a cross reaction with very
307 closely related species not included in laboratory tests (such as *C. johnstonii* and *C. pyricola*) cannot be
308 completely excluded, the panel of species utilized in the present study is representative of most
309 *Colletotrichum* species currently known to be associated with olive anthracnose (Sreenivasaprasad &
310 Talhinhas, 2005; Talhinhas et al. 2009; Talhinhas et al. 2011; Cacciola et al. 2011; Cacciola et al. 2012;
311 Moral et al. 2014; Schena et al. 2014). In particular, all *Colletotrichum* species detected in the olive
312 carposphere using metagenomic analyses based on both *Colletotrichum* spp. and fungal specific primers
313 were included in specificity tests (Mosca et al. 2014; Abdelfattah et al. 2015). This consideration in
314 combination with the absence of amplification from healthy olive samples and with the accurate *in silico*
315 analyses represent a guarantee of high specificity for the detection of *C. godetiae* and *C. acutatum s.s.* in
316 olive.

317 The duplex qPCR method was not influenced by olive DNA and enabled detection of as little as
318 10 pg of DNA of both species despite an average ΔCq of 1.8 between *C. godetiae* and *C. acutatum s.s.*
319 using equal DNA concentrations. Corrected DNA concentrations were utilized to estimate the number
320 of cells of both pathogens taking into consideration their assumed genome weight (assumed to be 0.051
321 pg). This also assumes that the genome size of *C. godetiae* and *C. acutatum s.s.* are equal. These
322 assumptions are based on currently available data on the genome weight of *C. acutatum* syn. *C. fiorinae*
323 (http://www.zbi.ee/fungal-genomesize/index.php), although the precise affinity of the fungus is not
324 confirmed. Considering the genetic affinity between *C. godetiae* and *C. acutatum s.s.* and the similarity
325 in genome weight of other genetically distant *Colletotrichum* species including *C. gloeosporioides* (0.060
326 pg), *C. graminicola* (0.053 pg) and *C. higginsianum* (0.051) we are confident that any error, although
327 possible, is very limited. Furthermore, the estimated DNA concentrations assume that all fungal DNA
328 was extracted from the samples, which is unlikely. Luo and coworkers (2009) estimated that the
329 efficiency of a commercial kit used to extract DNA from soil was approximately 60–71%. Unfortunately,
330 no information is available on the efficiency of DNA extractions from plant tissue. The loss of some
331 target DNA during extraction may have resulted in an underestimate of the number of cells in samples,

332 although we contend the potential error seem is of secondary importance. The most important point
333 epidemiologically is that there are huge differences in the number of cells present in different olive tissues
334 at different sample times, which has ramifications for the reproduction of *C. acutatum s.s.* and *C.*
335 *godetiae*.

336 Both *C. godetiae* and *C. acutatum s.s.* were detected in all samples. The results indicate that *C.*
337 *acutatum s.s.* is widely distributed in southern Italy and support the contention of an ongoing population
338 shift from *C. godetiae* to *C. acutatum s.s.* hypothesized by Mosca and coworkers (2014). *Colletotrichum*
339 *godetiae* has been traditionally considered the major pathogen causing anthracnose of olive in Italy and
340 other central Mediterranean countries (Talhinhas et al. 2015). On the contrary, until a few years ago, *C.*
341 *acutatum s.s.* was reported only from the Algarve region of Portugal, and was the prevalent species
342 reported on olive in Australia and South Africa (Sreenivasaprasad & Talhinhas, 2005; Talhinhas et al.
343 2005; Sergeeva et al. 2010; Talhinhas et al. 2011; Damm et al. 2012). Also, a recent study demonstrated
344 the prevalence of *C. acutatum s.s.* on olive in Tunisia, with only a small population component of *C.*
345 *gloeosporioides* (Chattaoui et al. 2016).

346 It is important to emphasize that the samples were collected in 2014, and the population shift
347 from *C. godetiae* to *C. acutatum s.s.* appears to proceed rapidly. In 2015, conventional isolation of
348 *Colletotrichum* spp. from symptomatic fruit collected in the same area detected a greater prevalence of
349 *C. acutatum s.s.* compared to *C. godetiae* (L Schena and GE Agosteo, unpublished data). Many factors
350 including climate change, introduction of new pathogen species or strains from other countries, or
351 outbreaks of endemic strains from other hosts may play a role in the emergence of *C. acutatum s.s.* on
352 olive in Calabria, southern Italy. Furthermore, variation in sporulation or virulence may have contributed
353 to the emergence of this species. Indeed, *C. acutatum s.s.* produces more abundant masses of conidia
354 compared to *C. godetiae* and recent inoculation studies indicate a higher virulence of *C. acutatum s.s.*
355 compared to *C. godetiae* (Talhinhas et al. 2015). However, in another study a Tunisian isolate of *C.*
356 *acutatum s.s.* was found to be significantly less virulent compared to a Spanish isolate of *C. godetiae*
357 (Chattaoui et al. 2016). In the present study *C. godetiae* was detected slightly more frequently (at least
358 in the May and June samples) but the quantity of *C. acutatum s.s.* in infected samples was invariably
359 greater compared to *C. godetiae*. The greater quantity of *C. acutatum s.s.* in infected samples agrees with
360 observations of greater sporulation and aggressiveness of this species as determined with the Portuguese
361 isolates (Talhinhas et al. 2015). Recent findings that *C. acutatum s.s.* is associated with the olive fruit fly
362 *Bactrocera oleae* in the same area as the present study (Malacrinò et al. 2015; 2016) may suggest a
363 possible role of this key pest of olive in the ongoing population shift in *Colletotrichum* spp..

As expected, the population of both *C. godetiae* and *C. acutatum s.s.* increased sharply in December. Both species were detected widely in asymptomatic samples in May, June and October. Green leaves in May were an exception as only *C. godetiae* was detected. The detection of *C. godetiae* in small quantities in asymptomatic green leaves and fruit in May, June and October confirms the important role of latent infections, which favor survival of the pathogen during the hot, dry summer and represent an inoculum source for anthracnose epidemics on ripening fruit (Moral et al. 2009; Talhinhas et al. 2011). In a previous study using a metagenomic approach, a lower incidence of both pathogens was revealed in green leaves and fruit in May and October (Mosca et al. 2014). Differences between studies are likely to be due to the lower sensitivity of the conventional PCR used in metagenomic analyses compared to the qPCR utilized in the present study. Furthermore, the different sampling years (2012 and 2014, respectively) and consequently different environmental conditions may have resulted in a different incidence of latent infection.

Additional research is needed to understand the relevance of spring and summer latent infections and to determine the efficacy of control strategies. The method developed in the present study will be valuable as it is specific and sensitive and enables the detection of the target pathogens during latent infection (Sanzani et al. 2014). *Colletotrichum* species can produce a large numbers of conidia and epidemic-conducive conditions can result in sufficient inoculum from one diseased olive per tree producing sufficient spores to infect 100% of the remaining fruit (Moral et al. 2014). These observations are supported by our results: a very large number of cells were detected in symptomatic fruit in December for both *C. godetiae* (1.7×10^8 cells/mg of tissues) and *C. acutatum s.s.* (7.5×10^8 cells/mg of tissues). In symptomatic fruit a large proportion of these cells are likely to represent conidia.

The greater quantity of *C. godetiae* and *C. acutatum s.s.* in senescent leaves compared to green leaves in all samples confirms an important role of these pathogens in defoliation of olive trees. However, their overall contribution to defoliation is confounded due to the presence of other defoliating pathogens including *Spilocaea oleagina* and *Mycocentrospora cladosporioides*. In particular, *M. cladosporioides* has been reported as a very common species in the olive phyllosphere in this region of Italy (Abdelfattah et al. 2015).

The incidence and, more remarkably, the quantity of both pathogens sharply increased in December. This result is in agreement with previous reports and indicate that infection of olive fruit and foliage occurs mainly in the late fall as fruit ripen and environmental conditions are conducive to anthracnose (Moral & Trapero, 2012). It is well documented that the susceptibility of fruit to anthracnose

increases with the maturation process and that diseased fruit are subsequently essential as a source of inoculum to infect leaves (Moral et al. 2008, Moral et al. 2009).

We detected *C. godetiae* and *C. acutatum s.s.* in May on fertilized fruitlets with floral residues and the quantity of pathogen was higher compared to either green or senescent leaves. This observation provides further evidence for saprophytic behavior of both species on senescent floral parts and suggests that fruit can be infected during the spring, which can become a source of inoculum for infection of fruit later in the autumn (Moral et al. 2009; Mosca et al. 2014). Colonization of floral residues may increase pathogen populations and favor the establishment of latent infections in fruitlets as commonly occurs with necrotrophic fungi (Sanzani et al. 2012).

The duplex qPCR method we developed is a valuable tool to study both *C. godetiae* and *C. acutatum s.s.* populations in olive. The present study has provided some valuable information on the epidemiology of the two species causing olive anthracnose in Calabria, southern Italy. The use of this and other recently developed molecular tools (Mosca et al. 2014) in specific well-defined experiments will contribute to our understanding and knowledge of the etiology and epidemiology (as well as other aspects) of olive anthracnose.

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Table 1. Primers and minor groove binder (MGB) probes developed for duplex qPCR detection of *C. godetiae* and *C. acutatum*, causal agents of olive anthracnose.

Target species	Primer name	Primer and probes sequence (5′–3′)[a]	Amplicon length	Mathematical function (linear regression model)
C. acutatum	Acu-his-F	TCCAGCGTCTGGTAAGTTGAGAA	62	y=-3.2477x + 28.786
	Acu-his-R	AGAAGTGTTAGCCGATGCGATT		
	Acu-his-Probe	VIC-CACAGCGCGTCACC-BHQ1		
C. godetiae	God-tub-2F	CGACAGCAATGGCGTGTATG	60	y=-3.4424x + 30.087
	God-tub-3R	CTGCTGGGTCCACGAGGTA		
	God-tub-Probe	FAM-TGCACTGGAGGACAAGAG-BHQ1		

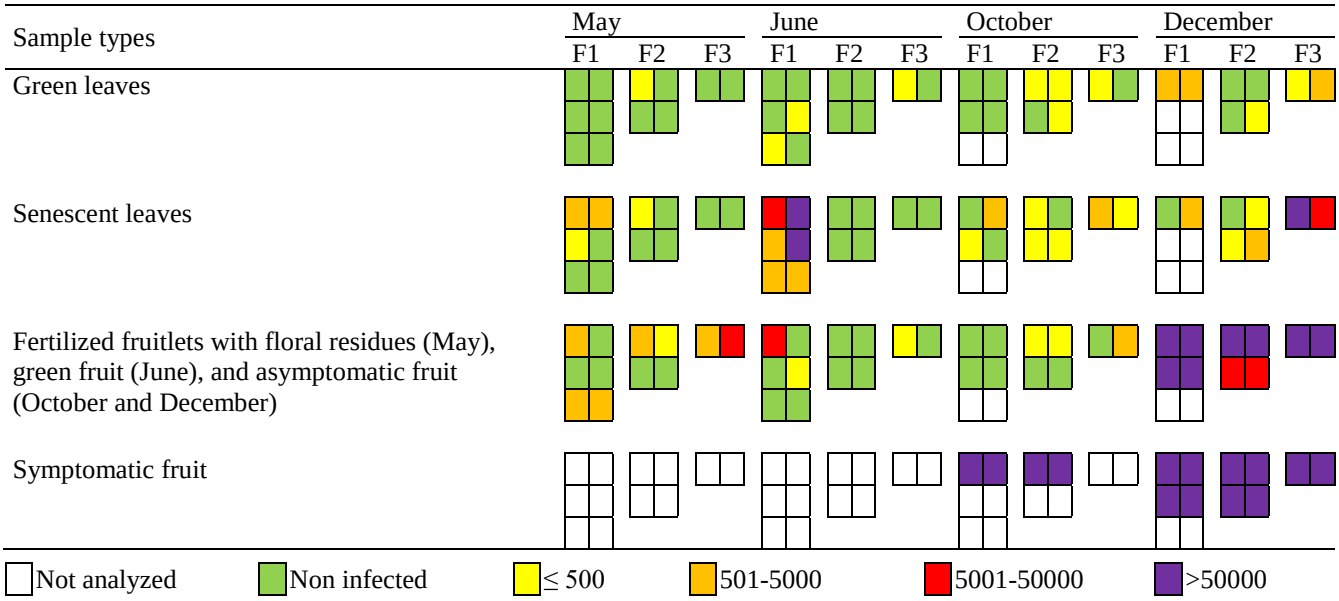
[a] FAM: 6-carboxyfluorescein; VIC: 6-carboxyrhodamine; BHQ1: black hole quencher.

508 **Table 2.** Isolates of *Colletotrichum* spp. used to assess specificity of the qPCR method developed for
509 detection of *C. godetiae* and *C. acutatum sensu stricto*, causal agents of olive anthracnose.
510

Species	No. of isolates	Host genus	Geographic origin of sample
C. acutatum s.s.	15	*Olea*	Italy (10), Australia (5)
C. aenigma	3	*Olea* (2), *Pyrus* (1)	Italy
C. aenigma	3	*Olea (2), Pyrus (1)*	Italy
C. alienum	1	*Nerium*	Australia
C. fioriniae	4	*Vitis* (1)*, Olea* (1)*, Carica* (1)*, Vaccinium* (1)	Italy (2), Turkey (1), Hawaii (1)
C. gleosporioides s.s	6	*Olea* (5), *Citrus* (3), *Capsicum* (1)	Italy (5), Australia (3)
C. godetiae	15	*Olea*	Italy (14), Greece (1)
C. kahawae subsp. ciggaro	2	*Olea*	Italy
C. karstii	6	*Olea* (3), *Citrus* (1), *Camelia* (1), *Pistacia* (1)	Italy
C. musae	1	*Musa*	Unknown
C. nymphaeae	8	*Olea* (5), *Fragaria* (3)	Portugal (5), Italy (3)
C. queenslandicum	1	*Olea*	Former Yugoslavia
C. siamense	3	*Olea* (2), *Hibiscus* (1)	Italy (2), Australia (1)
C. theobromicola	4	*Olea*	Australia
Colletotrichum sp.	1	*Olea*	Italy

511
512

Table 3. Summary of field surveys sampling different olive tissues to detect and quantify *C. godetiae* and *C. acutatum s.s.* by duplex qPCR. Samples were from 6 groups of olive trees identified in orchard F1 (3 groups), F2 (2 groups), and F3 (1 group) and were collected in four different months (May, June, October and December). They comprised green and senescent leaves collected at each sample time, fertilized fruitlets with floral residues collected in May, green fruits collected in June, and asymptomatic and symptomatic fruits collected in October and December. For each combination of sample type, phenological phase and orchard, specific boxes indicated the infection status of the sample with *C. godetiae* (left panel) and *C. acutatum s.s.* (right panel). The color scale indicates the number of pathogen cells estimated per mg of fresh tissue.



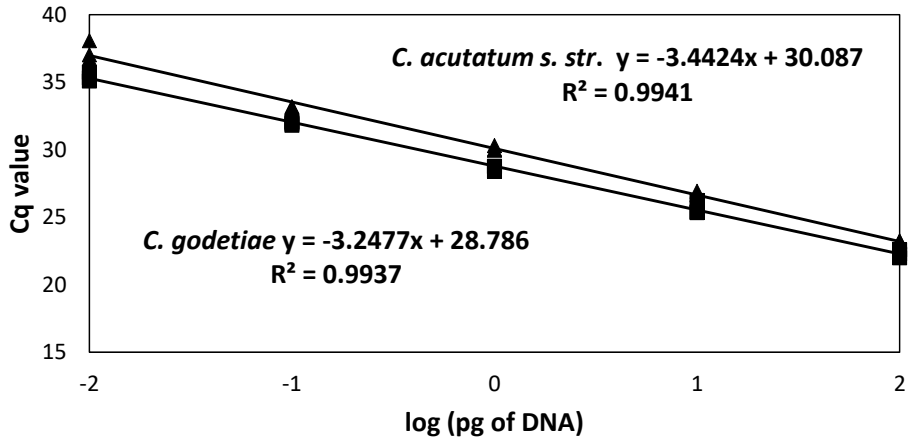


Figure 1. Standard curves, mathematical functions and coefficients of determination for the duplex quantitative PCR (qPCR) used to detect *Colletotrichum acutatum s.s.* and *C. godetiae* using serial dilution of target DNA from pure cultures.

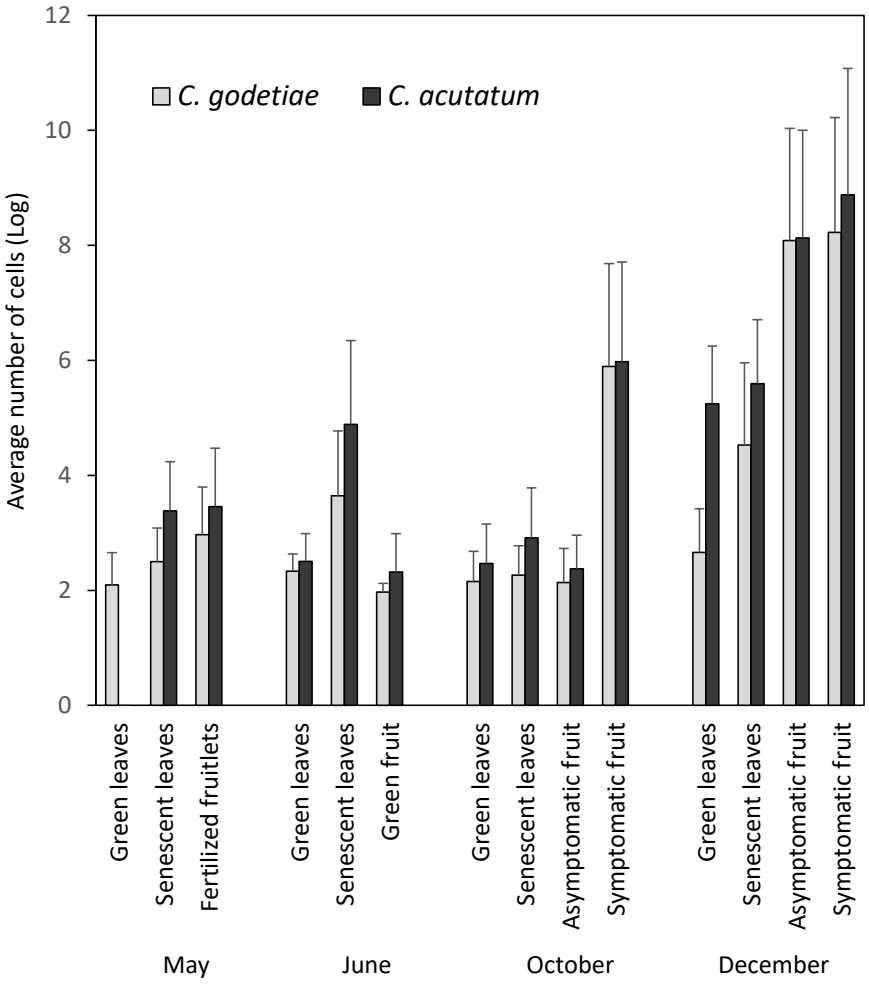


Figure 2. Estimated number of cells of *C. godetiae* and *C. acutatum s.s.* (cells/mg of fresh tissue) in infected samples of olive collected at four different phenological phases (May, June, October and December). Bars indicate standard errors of the means.