Characterization and epidemiology of *Colletotrichum acutatum sensu lato* (*C. chrysanthemi*) causing *Carthamus tinctorius* anthracnose

R. Baroncelli†, S. Sarrocco*, A. Zapparata, S. Tavarini, L. G. Angelini and G. Vannacci

Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124, Pisa, Italy

*E-mail: sabrina.sarrocco@unipi.it

†Present address: Centro Hispano-Luso de Investigaciones Agrarias (CIALE), Departamento de Microbiología y Genética, Universidad de Salamanca, C/Río Duero 12, Villamayor, 37185 Salamanca, Spain

Abstract

In 2012, *Colletotrichum* isolates were collected from field-grown safflower (*Carthamus tinctorius*) crops in central Italy from plants exhibiting typical anthracnose symptoms. Colletotrichum isolates were also collected from seed surfaces and from within seeds. The genetic variability of these isolates was assessed by a multilocus sequencing approach and compared with those from *Colletotrichum chrysanthemi* and *Colletotrichum carthami* isolates from different geo- graphic areas and other *Colletotrichum acutatum sensu lato*-related isolates. Phylogenetic analysis revealed that all of the strains isolated from *C. tinctorius* belonged to the species described as *C. chrysanthemi*, whereas all of the strains belonging to *C. carthami* had been isolated from Calendula officinalis. Phenotypic characterization of isolates was performed by assessing growth rates at different temperatures, morphology of colonies on potato dextrose agar (PDA) and the size of conidia. All *C. chrysanthemi* isolates from safflower had similar growth rates at different temperatures, comparable colony morphologies when grown on PDA and conidial sizes consistent with previously described *C. chrysanthemi* isolates. Pathogenicity tests were performed by artificially inoculating both seeds and plants and confirmed the seedborne nature of this pathogen. When inoculated on plants, *C. chrysanthemi* caused the typical symptoms of anthracnose on leaves. This is the first record of this pathogen on C. tinctorius in Italy, and it presents an updated characterization of *Colletotrichum* isolates pathogenic to safflowers in Europe.

Keywords: anthracnose, *Colletotrichum acutatum*, *Colletotrichum chrysanthemi*, safflower

Introduction

Safflower (*Carthamus tinctorius*), an asteraceous plant species, is an important annual industrial crop that can be grown in arid and rainfed agricultural areas thanks to its high tolerance to heat and cold. It can also grow on irrigated agricultural land because of its tolerance to salinity and weeds (Beyyavas et al., 2011). This plant has recently received increased attention in Mediterranean environments, such as Italy, as a new oilseed crop, its seeds contain up to 46% oil. Safflower oil contains an average of 75% linoleic acid, and it also contains tocopherols, which are known for their antioxidant effects, in addition to vitamin E. Given these interesting characteristics, safflower oil is used in the diets of cardio- vascular disease patients and is very important for its anti-cholesterol effects (Beyyavas et al., 2011). *Colletotrichum acutatum* is one of the most pathogenic species within the *Colletotrichum* genus, causing anthracnose and blight in many agriculturally important hosts, including safflower and other asteraceous species, such as chrysanthemum (*Chrysanthemum* sp.) and marigold (Calendula officinalis) (Vıchova et al., 2011; Uematsu et al., 2012). *Gloeosporium carthami* was first described as the causal agent of safflower leaf blight in Japan (Tanaka, 1917). It was assigned to the genus *Glo- eosporium* in 1919 (Hemmi, 1919) and was then treated as a synonym of *Colletotrichum*

gloeosporioides based on a description by Hemmi (Arx, 1957). *Gloeosporium chrysanthemi* was described as a pathogen of garland chrysanthemum (Takimoto, 1924) and was transferred to *Colletotrichum* by Sawada (1943). In 1958, the anthracnose of pot marigold was found to be caused by a *Gloeosporium* species (Numata et al., 1958). Cross- inoculation tests demonstrated that several *Colletotrichum* strains that were isolated from garland chrysanthemum, safflower and marigold were pathogenic to all three species, indicating that all of the strains belonged *to G. carthami* (Ueda & Kajiwara, 1968). Kwon et al. (1998) identified a pathogen that caused anthracnose in safflower, in addition to pot marigold and garland chry- santhemum; the pathogen was assigned to C. acutatum. In 2012, *G. carthami* from safflower, *Colletotrichum chrysanthemi* from chrysanthemum *and C. acutatum* from marigold isolates were morphologically and mole- cularly examined and assigned to the *Colletotrichum carthami* species (Uematsu *et al.*, 2012).

Identifying *Colletotrichum* within the genus is compli- cated because the species have few distinguishing mor- phological characteristics and because the teleomorphic stage is rarely formed (Baroncelli, 2012; Schena *et al.*, 2014). Although the limits of the genus seem to be well established, the concept of species (such as *C. acutatum*) within this genus is not universally defined and accepted (Sutton, 1992). In the case of *C. acutatum*, there has been some discussion about further subdividing the com- plex into distinct species (Vinnere *et al.*, 2002). The recent release of a whole genome sequence of *Colletotri- chum fioriniae* (Baroncelli *et al.*, 2014) represents a new resource that will be useful for further research into the genetic basis of *C. acutatum* species complex–host inter- actions. Several research groups have shown that global populations of *C. acutatum* potentially comprise nine distinct genetic groups, namely A1 to A9, based on the ITS and *TUB* sequences (Sreenivasaprasad & Talhinhas, 2005; Whitelaw-Weckert *et al.*, 2007). This finding indicates that *C. acutatum sensu lato* is a typical species complex of which *C. carthami* is considered a member (Uematsu *et al.*, 2012). A preliminary multilocus analysis (Cannon *et al.*, 2012) suggested that *C. chrysanthemi* might not be a synonym of *C. carthami*, as stated by Uematsu *et al.* (2012). Recently, 21 strains that were once identified as *C. carthami* on the basis of *TUB2* were analysed (Uematsu *et al.*, 2012; Sato & Moriwaki, 2013). The strains were divided into three clades: 14 of the strains that clustered with the reference strains of *C. chrysanthemi* (that were accepted by Damm *et al.*, 2012), four strains that were isolated from pot marigold, and three strains from strawberry. The first two clades were recognized as *C. carthami sensu stricto*, because they were combined with the *C. carthami*-type specimen based on *TUB2* sequences (Uematsu *et al.*, 2012; Sato & Moriwaki, 2013). The latter clade was tentatively classi- fied, and newly designated, as *C. acutatum* (group) A2-S in Sato & Moriwaki (2013) because it did not correspond to any species listed by Damm *et al.* (2012). In this paper, genetic and phenotypic characterizations were performed on *Colletotrichum* isolates collected from plants and seeds of field-grown safflower crops cultivated in 2012 at two sites in central Italy. Comparison was made with data from *C. acutatum sensu lato* isolates collected from safflower plants in different geographic areas and from other *C. acutatum* isolates from different hosts. This report is the first description of *C. chrysanth- emi* on *C. tinctorius* in Italy and the first complete characterization of *Colletotrichum* isolates that are pathogenic to safflower in Europe. This work also presents new information on the epidemiology of an emerging disease that can be exploited for its control.

Materials and methods

Fungal isolates

Two safflower (cv. Pieve) fields were sown in the Pisa country- side (San Piero a Grado and Santa Luce) at the beginning of March 2012. Both sites were located in the province of Pisa, northern Tuscany (Table 1). The climate is typically Mediterra- nean and is characterized by two primary rainy periods in the autumn (from September to December) and spring (from March to May) and a total annual rainfall of *c.* 900 mm. During the summer (from July through the first half of August), a dry period generally occurs, with low rainfall and high air temperatures (Table 1). The San Piero site was flat, and the soil was an alluvial deep clay loam, which is typical of the lower Arno River plain. The Santa Luce site was within an inland hilly area, and the soil was characterized by a clay loam texture. In 2012, plants in both fields that were heavily affected by anthracnose symptoms (Fig. 1) were sampled before and after flowering. The seeds were also sampled at harvest (first week of August).

Six Italian *Colletotrichum* sp. isolates and three isolates from the Czech Republic, all collected from safflower seeds and plants, were used in this work. The genetic information for two *C. carthami* isolates from *C. officinalis* and three *C. chrysanthemi* isolates from *Chrysanthemum coronarium* and *C. tinctorius* in Japan (Sato & Moriwaki, 2013) and from *Chry- santhemum* sp. in the Netherlands (Damm *et al.*, 2012) were used. *Colletotrichum lupini*, *Colletotrichum nymphaeae*, *Collet- otrichum scovillei*, *Colletotrichum laticiphilum*, *Colletotrichum brisbaniense* and *Colletotrichum simmondsii* isolates, representa- tive of the *C. acutatum* species complex (Damm *et al.*, 2012) and closely related to *C. carthami* and *C. chrysanthemi*, were included in the phenotypic and genetic analyses. All isolates are shown in Table 2. Stock cultures, with the exception of isolates from Japan, were maintained on potato dextrose agar (PDA; Difco) under mineral oil at 4°C, whereas routine cultures were maintained on PDA at 25°C, 12 h light/12 h darkness, for up to 10 days.

Seed health test

To estimate the contamination level of *C. tinctorius* seeds by *Colletotrichum* sp., a seed health test was performed on seeds sown in the spring of 2012 and on seeds harvested in August 2012 from field crops in both Santa Luce and San Piero (Pisa, Italy). The health test was performed on both treated (seeds immersed in sodium hypochlorite solution with 1% available chlorine for 10 min, then drained) and untreated seeds. The seeds were aseptically placed onto the agar surface of PDA plates, with 10 seeds on each plate. Four independent replicates, each consisting of 100 seeds, were analysed for both treated and untreated seeds. The plates were incubated for 7 days at 24°C, 12 h light/12 h darkness, and the developing colonies were then observed; those colonies resembling *Colletotrichum* sp. were transferred to new PDA plates for confirmation. Representative Italian isolates were then selected for further molecular, phenotypic and pathogenic analyses in the present work (Table 2). Single spore cultures were performed for all of the *Colletotrichum* sp. isolates, and the isolates were stored as described in the previous paragraph.

Genetic characterization

DNA of all isolates included in Table 2, except those from Japan (MAFF239358, MAFF239361, MAFF239362 and MAFF239370), was extracted according to the Chelex 100 protocol with some modifications (Baroncelli, 2012). Microfuge tubes (1 5 mL) containing 10% Chelex 100 (0.1 g Chelex 100 + 900 μL water) were autoclaved. A small amount of myce- lium (c. 2-mm diameter clump) from a culture growing actively on PDA was collected with a sterile toothpick. The samples were vortexed for 15 s and centrifuged at 17 530 g for 15 s. The tubes were incubated at 90°C for

20 min. The samples were again vortexed for 15 s, centrifuged at 17 530 g for 1 min and the supernatant (c. 500 μL) was transferred into a new sterile microfuge tube to be used for further amplification.

To analyse the genetic variability within *Colletotrichum* isolates, a region including the ITS1, 5 8S and ITS2 (ITS), exons 3 to 6 (introns 2–4), a partial sequence of the b-tubulin 2 gene (*TUB*) and a partial sequence of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were amplified. PCR reaction mixes (20 μL) contained 1 μL DNA, 1 μL of each primer (20 μM), 7 μL H_2O and 10 μL ReadyMix REDTaq (Sigma- Aldrich). Primers ITS1Ext (5′-GTAACAAGGTTTCCGTAGGTG-3′) and ITS4Ext (5′-TTCTTTTCCTCCGCTTATTGATATGC-3′) (Talhinhas *et al.*, 2002) were used for ITS. The amplification programme consisted of 2 min of initial denaturation (95°C), 30 cycle of amplification (1 min at 94°C, 1 min at 55°C and 1 min at 72°C) and a final extension at 72°C for 5 min. Primers TB5 (5′-GGTAACCAGATTGGTGCTGCCTT-3′) and TB6 (5′-GCAGTCGCAGCCCTCAGCCT-3′) (Talhinhas *et al.*, 2002) were used for TUB. The amplification programme consisted of 2 min of initial denaturation (95°C), 30 cycles of amplification (1 min at 94°C, 1 min at 65°C and 1 min at 72°C) and a final extension at 72°C for 5 min. Primers GDF1 (50-GCCGTCAAC GACCCCTTCATTGA-30) and GDR1 (50-GGGTGGAGTCGTACTTGAGCATGT-30) (Guerber et al., 2003) were used for GAPDH. The amplification programme consisted of 2 min of initial denaturation at 95°C, 35 cycles of amplification (1 min at 94°C, 1 min at 60°C and 30 s at 72°C) and a final extension at 72°C for 3 min. All amplifications were performed in a Gene- Amp PCR System 2400 (Perkin Elmer). The PCR products were separated by gel electrophoresis and purified using a QIAquick PCR purification kit (QIAGEN). PCR product sequencing was performed by the Bio Molecular Research (BMR) Servizio Sequenziamento-CRIBI of the University of Padova, Italy, using an Applied Biosystems Prism 3100 DNA Sequencer (Perkin Elmer/ABI) and a BigDye Terminator v. 3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems). All of the sequences were aligned using MAFFT (Katoh & Standley, 2013). Multiple sequence alignments were exported to MEGA5 (Tamura et al., 2011), where best-fit substitution models were calculated for each separate sequence data set. When necessary, tree topologies with 75% neighbour joining bootstrap and maximum parsimony analyses (100 000 replicates) were separately performed on single genes and visually compared to evaluate whether the four sequenced loci were congruent and suitable for concatenation (Mason-Gamer & Kellogg, 1996). A multilocus concatenation alignment (ITS, TUB2 and GAPDH) was performed with GENEIOUS v. 6.2 (Biomatters, available from http://www.geneious.com/). A Markov chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities in MRBAYES v. 3.2.1 (Ronquist & Huelsenbeck, 2003) for each single set of data in addition to combined sequence data sets. Nucleotide substitution models for each gene determined by MEGA5 were included for each locus. The analysis in MRBAYES ran for 5 000 000 generations with two parallel searches using three heated and one cold Markov chain sampled every 100 generations; 25% of generations were discarded as burn-in.

Cultural and phenotypic characterizations

All isolates, except those from Japan (Table 2), were used for cultural growth studies and for phenotypic characterizations on PDA. In both tests, five PDA plates were inoculated with a 7-mm-diameter mycelial plug excised from the margins of actively growing cultures on PDA. In the growth experiment, the colony radii were assessed daily on plates that were incubated at seven different temperatures (10, 15, 20, 25 30, 35 and 40°C) in the dark, and the data were

subjected to regression analysis to create growth curves and to calculate slope values (mm h^{-1}) for each isolate. Regression analysis was performed using SIGMAPLOT v. 10 (SigmaPlot Software). In the second experiment, colony characteristics, such as the colours of the recto and verso, the transparency and the presence of conidial masses or differentiating structures, were recorded after 15 days of incubation at 25°C, 12 h light/12 h darkness. Both experiments were replicated three times.

To measure the conidial size produced by each of these isolates, a conidial suspension was prepared by flooding 10-day-old PDA cultures with sterile deionized water. The lengths and widths of the conidia were measured by microscopic observation and are expressed as the average of three replicates of 30 conidia each.

Greenhouse pathogenicity tests

Colletotrichum chrysanthemi 10190, 10372 and Coll408, *C. simmondsii* CBS122122 and *C. lupini* HY09 (these last two as out-groups) were used as safflower seed inoculants for a greenhouse pathogenicity test. In addition, *C. chrysanthemi* 10190 was used as an inoculant of 24-day-old safflower plants in an additional test.

The aim of the first experiment was to assess the seed transmission capacity of *C. chrysanthemi*. *Carthamus tinctorius* seeds were surface-sterilized in a sodium hypochlorite solution with 1% available chlorine in 50% ethanol for 10 min, followed by three rinses in sterile distilled water. Then a conidial suspension of each *Colletotrichum* isolate was prepared by flooding 10-day-old PDA cultures with a 1% sodium alginate aqueous solution. The spore concentration was adjusted to 1×10^5 spores mL^{-1}, and 10 mL were used to treat the sterilized *C. tinctorius* seeds. Inoculated seeds were sown in sterilized river sand, with 40 seeds per replicate and three replicates for each isolate. Uninoculated seeds were used as the control, with three replicates of 40 seeds each. The capacity of *C. chrysanthemi*, particularly that of Italian origin, to produce the typical symptoms of anthracnose on safflower seedlings (no emergence, crown rot, leaf spot and seedling wilt or collapse) was evaluated at regular intervals and expressed as a percentage of seedling emergence and mortality at the end of the experimental period (17 days). To compare the behaviour of the different isolates, the emergence and mortality percentages were subjected to ANOVA after angular transformation, and the means were compared by Tukey's multiple range test in SYSTAT v. 11 (Systat Software). *Colletotrichum* infection was evaluated by reisolating the pathogen from plant parts with symptoms to confirm Koch's postulates. Reisolated colonies were submitted to molecular identification by amplifying and sequencing the GAPDH gene, as previously described, and sequences were compared with those from the original isolates.

For the second pathogenicity test, 24-day-old seedlings were inoculated with *C. chrysanthemi* isolate 10372. The strain was cultured on PDA at 25°C for 10 days to prepare an aqueous conidial suspension containing $1x10^6$ spores mL^{-1}. The suspension was sprayed onto plants until they were saturated, and the plants were then covered with plastic bags and kept at 27–28°C for 4 days. Plants sprayed only with water were used as a control (Uematsu *et al.*, 2012). Each experiment consisted of three independent replicates, each of which included three plants. After 1 week, the plants were evaluated for anthracnose symptoms on the leaves, and the pathogen was reisolated from affected plants and identified as described in the previous experiment.

Results

Seed health test

Natural (untreated) seeds exhibited different levels of *Colletotrichum* infection, depending on the samples. Specifically, the seeds used for sowing in the spring of 2012 and those harvested in August 2012 in Santa Luce exhibited very low percentages of infection (0 25 and 4 5%, respectively), whereas seeds harvested in August 2012 in San Piero exhibited a higher level of infection (23 25%). When submitted to sterilization, no *Colletotrichum* sp. colonies resulted from seeds sown in the spring of 2012 and harvested in August 2012 in Santa Luce, whereas 8 0% of the seeds harvested in August 2012 in San Piero developed pathogen colonies. Isolate 10194 (from steril- ized seeds harvested in San Piero in 2012), isolates 10250 and 10252 (from untreated seeds harvested in 2012 in Santa Luce) and isolate 10372 (from untreated seeds sown in spring 2012) were chosen for further investigation. They were studied along with isolates from the stems (10190) and inflorescences (10191) of plants affected by anthracnose that were collected during the 2012 growing season in San Piero (Table 2).

Genetic characterization

The ITS, *TUB* and GAPDH sequences were used in this work to correctly separate and group isolates belonging to different species of *C. acutatum sensu lato*. Analysis based on ITS sequences alone suggested that this locus should not be used alone for species recognition of isolates belonging to this taxon. Based on this locus, no nucleotide differences were identified among strains belonging to *C. carthami*, *C. chrysanthemi*, *C. brisbaniense* and *C. laticiphilum.* The sequence data used in this work indicate that the ITS locus had lower variability (1 4%) when compared with TUB (8 0%) and GAPDH (10 6%). Nine strains isolated from *C. tinctorius* in Italy and the Czech Republic were identified on the basis of the three loci sequences, with no nucleotide differences between the reference strains CBS126519 (isolated from *Chrysanthemum* sp. in the Netherlands) and MAFF 239362 (isolated from *C. coronarium* in Japan), both assigned to *C. chrysanthemi* by Damm *et al.* (2012) and Sato & Moriwaki (2013). Phylogenetic analysis (Fig. 2) confirmed this observation, grouping the strains in the same cluster with a high posterior probabilities value (100%). Strains described as *C. carthami* formed a separate and unique cluster, including only strains associated with *C. officinalis* disease and isolated in Japan.

Phenotypic characterization

When radial growth data were submitted to regression analysis, it was possible to calculate the growth rates (mm h^{-1}), corresponding to the slope of the linear phase of each growth curve, for all *Colletotrichum* isolates at each temperature (Table 3). Almost all isolates, except *C. lupini* HY09 and *C. nymphaeae* CBS125973, had an optimum growth temperature of 25°C. These two isolates had the highest growth rates at 20 and 30°C, respectively. At 35°C, the growth rates were very slow for all isolates except *C. lupini*, C. nymphaeae and *C. brisbaniense*, in which no growth was observed. *Colletotrichum* isolates were not able to grow at 40°C; plates incubated at this temperature were transferred to 25°C after 1 week, but no growth was observed for these colonies. Interestingly, all *C. chrysanthemi* isolates, both from the Czech Republic and Italy, had the same behaviour at all temperatures, with an optimum level at 25°C, slow growth at 10°C and 30°C and no growth at 40°C.

Colony morphology analysis was used to assign *Colletotrichum* isolates to seven different phenotypic groups (Fig. 3). Group A includes all three isolates of *C. lupini* and is characterized by an orange/pale grey colony with light orange conidial masses, with a pale orange to light brown reverse. Group B includes all three *C. nymphaeae* isolates and is characterized by a non-sporulating white/ pale grey cottony mycelium and a dark brown reverse. Group C includes the *C. scovillei* isolate, and it is characterized by a white/grey colony without an especially evident sporulation zone and an orange reverse. Group D includes the *C. simmondsii* isolate and is

characterized by grey colonies, with differentiated and abundant spore masses and is dark brown/black in reverse. Group E includes *C. laticiphilum* and is characterized by a grey colony with a non-evident sporulation zone, and it is dark orange/light brown in reverse. Group F includes *C. brisbaniense* and is characterized by cottony white/ pale yellow colonies with evident sporulating masses, and it is light yellow in reverse. Finally, all *C. chrysanthemi* isolates from the Netherlands, the Czech Republic and Italy belong to group G, which is characterized by grey/pale brown colonies with abundant orange conidial masses (with the exception of the non-sporulating isolate CBS126519 from the Netherlands) and is dark brown/ black in reverse (Fig. 3).

The conidial size (Table 3) of *C. lupin*i was 10·4– 11·5 x 3·1–3·7 μm. No sporulation occurred in the three *C. nymphaeae* isolates. Conidia from *C. scovillei, C. simmondsii* and *C. laticiphilum* exhibited the smallest values among all tested isolates (9·7 x 2·9, 8·7 x 2·5, and 8·1 x 2.8 μm, respectively), whereas the conidial size of *C. brisbaniense* was 10·6 x 2·5 μm. All of the *C. chrysanthemi* isolates from the Czech Republic and Italy had similar average sizes, ranging from 10· 2 to 11·4 x 3·2 to 3·4 μm.

Greenhouse pathogenicity tests

The three *C. chrysanthemi* isolates reduced the percent- age of safflower emergence significantly compared with both an uninoculated control and seeds that were inoculated with the two out-groups (Table 4). For these last two out-groups, the emergence values were not significantly different from the control. *All C. chrysanthemi* isolates also caused high levels of mortality, which were significantly different from that of the control, with *C. chrysanthemi* 10190 causing the highest percentage (97 5%) of dead plants. Percentages of seedling mortality of *C. simmondsii* and *C. lupini* showed that these two isolates did not affect plant vitality as there were no differences from the uninoculated control.

This test confirms the pathogenicity of both Italian isolates (10190 and 10372) when inoculated onto seeds, despite isolate 10190 originating from stems with symptoms. All the diseased plants showed typical symptoms of anthracnose, distinguishable as crown rot, which caused the wilting and collapse of the seedlings, or cotyledon necrosis. Pathogens were consistently reisolated from plants that exhibited these symptoms.

When the *C. chrysanthemi* 10372 isolate was used as an inoculant of 24-day-old safflower plants, all inoculated plants showed typical symptoms of anthracnose on their leaves. The pathogen was reisolated from plants exhibiting these symptoms. This test confirms the pathogenicity of isolate 10372 that was originally isolated from seeds.

Discussion

This study identified the agent of *C. tinctorius* anthracnose in Italy by phenotypically and genotypically comparing the Italian isolates with *C. acutatum sensu lato* isolates and defined the important epidemiological steps of the disease. This is the first record of this pathogen on C. tinctorius in Italy, and it presents an updated charac- terization of Colletotrichum isolates that are pathogenic to safflowers in Europe.

During the 2012 growing season, plants in two safflower fields near Pisa (Tuscany) exhibited typical anthracnose symptoms. Some recent papers indicate *C. chrysanthemi*, *C. carthami* and *C. simmondsii*, or more generally *C. acutatum sensu lato*, as the cause of this disease in *C. tinctorius*. The pathogen infects both seedlings and fully grown safflower plants, and the dis- ease symptoms occur on the leaves, stems and flower parts, as observed in fields.

The results of seed health tests and artificial seed inoculation tests confirmed the seedborne nature of this pathogen, a feature known in other *Colletotrichum* species (Richardson, 1979), and its ability

to infect internal seed tissues. This information allowed the life cycle of *C. chrysanthemi* in safflower to be determined (Fig. 4). The very low infection percentage of the seed lot sown in 2012, with a plant density at emergence of nearly 40 per m^2, yielded approximately 2000 randomly distributed foci per hectare. This type of primary inoculum most probably initiated the epidemics in both San Piero a Grado and Santa Luce, demonstrating the pathogen's high capacity for spreading from infected plants within the field and suggesting the need for adoption of very low threshold levels for seed infection percentages in certification schemes. The higher post- emergence rainfall in San Piero a Grado relative to Santa Luce and the flat soil disposition in San Piero a Grado (favouring rain water stagnation when compared with the sloping field in Santa Luce) could have favoured pathogen dispersal at this site, which can be inferred from the higher percentage of infected seeds collected from San Piero a Grado. No attempts have been made to define the role of the infected plant residues in the epidemiology, but these residues are known as the most important source of *Colletotrichum* infection in some pathogen/host systems (Kang et al., 2009). In the present case, the two fields had never before hosted safflower crops, and the previous crop in both fields was wheat.

According to recent literature, identification of isolates of *Colletotrichum* from *Carthamus* has been made by a molecular approach (Whitelaw-Weckert et al., 2007; Peres et al., 2008; Damm et al., 2010). The set of nucleotide sequences used for the genetic characterization (ITS, TUB and GAPDH) appears to be appropriate for the species determination of strains belonging to *C. acutatum sensu lato*. ITS alone does not have suffi- cient resolution for this purpose and therefore should not be used for species recognition in this taxon. Phyloge- netic analyses revealed that all of the strains isolated from C. tinctorius worldwide (Japan, Czech Republic and Italy) belong to the species described as *C. chrysanthemi*; however, all of the strains belonging to *C. carthami* have been isolated from *C. officinalis*. These results suggest a revision in the taxonomic designation to avoid further confusion.

The simultaneous use of both molecular and morpho- logical techniques represents a valid and appropriate approach for studying the Colletotrichum species complex (Cannon et al., 2000). For this reason, a poly- phasic approach was taken in the present work to compare *C. chrysanthemi* strains, which were first isolated in Italy from C. tinctorius, to *C. chrysanthemi* isolates from the Czech Republic and the Netherlands and to isolates belonging to other species within the *C. acutatum* species complex. All *C. chrysanthemi* isolates from safflower had similar growth rates at different temperatures and comparable colony morphologies when grown on PDA, which was independent of their geographic origin. The Dutch isolate from chrysanthemum that was unable to sporulate in vitro also belongs to this group. For all of these sporulating isolates, the conidial sizes, 10·2– 11·4 x 3·2–3·4 μm, with a mean of 10·8 x 3·2 μm, are consistent with those described by Sato & Moriwaki (2013) in which the *C. chrysanthemi* conidia on PDA were 7·3–15·8 x 2·6–5·3 μm, with a mean of 10·8 x 4.0 μm.

In conclusion, the data presented here enabled the confirmation of *C. chrysanthemi* as the global causal agent of anthracnose in *C. tinctorius*. This pathogen has a seedborne aetiology and is able to contaminate seed sur- faces and to colonize their internal tissues. Additionally, it has high host specificity, as demonstrated for all of the isolates characterized here. The capacity to induce epidemics starting from a barely infected seed lot and the sharp increase of infected seeds in the second generation suggests seed transmission as an important route for pathogen diffusion. From this point of view, there is a need for tools for the rapid and early detection of the pathogen and for methods to control and prevent disease spread.

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460 # Tables

461 **Table 1**. Details about cropping sites, crop management and climatic conditions during the 2012 experimental period.

	San Piero a Grado, Pisa, Italy		Santa Luce, Pisa, Italy	
Geographic coordinates	43°40'48″N, 10°20'55″E		43°28'0″N, 10°34'00″E	
Height a.s.l. (m)	5 (flat land)		200 (inner hill)	
Previous crop	Durum wheat		Durum wheat	
Tillage	Deep ploughing		Minimum tillage	
Climatic conditions	Mean temperature (°C)	Total rainfall (mm)	Mean temperature (°C)	Total rainfall (mm)
March	10.7	23.4	12.2	17.0
April	13.1	159.6	13.2	115.1
May	17.1	56.8	16.1	67.1
June	21.4	17.6	21.9	9.1
July	23.5	2.8	24.0	0.0
Mean temperature	17.1		17.5	
Total rainfall		260.2		208 3

462 The average temperature and total rainfall refer to the period between seedling emergence (the end of March 2012)
463 and crop harvest (the first week of August 2012).

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Table 2. *Colletotrichum* spp. strains used in this study, with isolation details and GenBank accession numbers.

Strain	Species	Origin	Location	Host	Matrix	ITS	TUB	GAPDH
CBS109225[a]	*C. lupini*	Ukraine		*Lupinus albus*		JQ948155	JQ949806	JQ948485
CBS109221[a]	*C. lupini*	Germany		*L. albus*		JQ948169	JQ949820	JQ948499
HY09	*C. lupini*	Canada		*L. albus*		KJ018635	KJ018647	KJ018659
CBS125973[a]	*C. nymphaeae*	UK	Harpenden Herts	*Fragaria* 9 *ananassa*		JQ948232	JQ949883	JQ948562
IMI335544	*C. nymphaeae*	Italy	Trento	*Fragaria* 9 *ananassa*		KJ018636	KJ018648	KJ018660
CBS526.77[a]	*C. nymphaeae*	Netherlands	Kortenhoefse Plassen	*Nymphaea alba*		JQ948199	JQ949850	JQ948529
Coll-25	*C. scovillei*	Taiwan	Tainan	*Capsicum annuum*		KJ018637	KJ018649	KJ018661
CBS122122[a]	*C. simmondsii*	Australia	Queensland, Yandina	*Carica papaya*		JQ948276	JQ949927	JQ948606
CBS112989[a]	*C. laticiphilum*	India		*Hevea brasiliensis*		JQ948289	JQ949940	JQ948619
CBS292.67[a]	*C. brisbanense*	Australia	Queensland, Brisbane	*C. annuum*		JQ948291	JQ949942	JQ948621
CBS126519[a]	*C. chrysanthemi*	Netherlands		*Chrysanthemum* sp.		JQ948272	JQ949923	JQ948602
CollS109	*Colletotrichum* sp.	Czech Republic	Zabcice	*Carthamus tinctorius*	Seed	KJ018638	KJ018650	KJ018662
Coll408	*Colletotrichum* sp.	Czech Republic	Popuvky	*C. tinctorius*	Stem leaf	KJ018639	KJ018651	KJ018663
Coll608	*Colletotrichum* sp.	Czech Republic	Kyjov	*C. tinctorius*	Inflorescence	KJ018640	KJ018652	KJ018664
10190	*Colletotrichum* sp.	Italy	San Piero (Pisa)	*C. tinctorius*	Plant stem (2012)	KJ018641	KJ018653	KJ018665
10191	*Colletotrichum* sp.	Italy	San Piero (Pisa)	*C. tinctorius*	Inflorescence (2012)	KJ018642	KJ018654	KJ018666
10194	*Colletotrichum* sp.	Italy	San Piero (Pisa)	*C. tinctorius*	Seed (F_2, 2012)[b]	KJ018643	KJ018655	KJ018667
10252	*Colletotrichum* sp.	Italy	Santa Luce (Pisa)	*C. tinctorius*	Seed (F_2, 2012)[b]	KJ018644	KJ018656	KJ018668
10250	*Colletotrichum* sp.	Italy	Santa Luce (Pisa)	*C. tinctorius*	Seed (F_2, 2012)[b]	KJ018645	KJ018657	KJ018669
10372	*Colletotrichum* sp.	Italy	San Piero (Pisa)	*C. tinctorius*	Seed (F_1, 2012)[c]	KJ018646	KJ018658	KJ018670
MAFF 239358[d]	*C. carthami*	Japan	Chiba	*Calendula officinalis*	Stem	Sato & Moriwaki (2013)		
MAFF 239361[d]	*C. carthami*	Japan	Chiba	*C. officinalis*	Stem	Sato & Moriwaki (2013)		
MAFF 239362[d]	*C. chrysanthemi*	Japan	Chiba	*Chrysanthemum coronarium*	Stem	Sato & Moriwaki (2013)		
MAFF 239370[d]	*C. chrysanthemi*	Japan	Yamagata	*C. tinctorius*	Stem	Sato & Moriwaki (2013)		

[a]Isolate information and sequences retrieved by Damm *et al.* (2012).

[b]Seeds harvested in August 2012.

[c]Seeds used for sowing in the spring of 2012.

[d]Isolate information retrieved by Sato & Moriwaki (2013). Sequences are available from NIAS Genebank (http://www.gene.affrc.go.jp/).

Table 3. Growth rates at different temperatures and conidial sizes of *Colletotrichum* spp. isolates on potato dextrose agar at 25°C.

	Growth rate (mm h^{-1})[a]							Conidial size (μm)[b]	
Isolate	10°C	15°C	20°C	25°C	30°C	35°C	40°C	Length	Width
C. lupini CBS109225	0.06 ± 0.001	0.16 ± 0.021	0.21 ± 0.001	**0.26 ± 0.003**	0.09 ± 0.004	No growth	No growth	9.5 (11.5 ± 1.3) 14.2	2.0 (3.1 ± 0.6) 3.9
C. lupini CBS109221	0.05 ± 0.002	0.16 ± 0.003	0.28 ± 0.002	**0.31 ± 0.002**	0.15 ± 0.002	No growth	No growth	7.8 (10.4 ± 1.6) 15.8	2.8 (3.7 ± 0.4) 4.8
C. lupini HY09	0.06 ± 0.000	0.16 ± 0.001	**0.24 ± 0.002**	0.23 ± 0.003	0.14 ± 0.006	No growth	No growth	7.1 (10.9 ± 1.5) 13.1	2.5 (3.2 ± 0.3) 3.6
C. nymphaeae CBS125973	0.03 ± 0.002	0.09 ± 0.004	0.08 ± 0.004	0.13 ± 0.009	**0.18 ± 0.003**	No growth	No growth	No sporulation	No sporulation
C. nymphaeae IMI335544	0.04 ± 0.001	0.10 ± 0.005	0.16 ± 0.003	**0.27 ± 0.002**	0.21 ± 0.003	No growth	No growth	No sporulation	No sporulation
C. nymphaeae CBS526.77	0.06 ± 0.001	0.13 ± 0.001	0.18 ± 0.002	**0.20 ± 0.004**	0.04 ± 0.005	No growth	No growth	No sporulation	No sporulation
C. scovillei Coll-25	0.02 ± 0.004	0.08 ± 0.043	0.10 ± 0.004	**0.11 ± 0.005**	0.07 ± 0.005	0.01 ± 0.004	No growth	6.2 (9.7 ± 1.7) 12.5	2.3 (2.9 ± 0.4) 3.6
C. simmondsii CBS122122	0.01 ± 0.002	0.06 ± 0.002	0.09 ± 0.001	**0.12 ± 0.001**	0.09 ± 0.004	0.02 ± 0.003	No growth	6.0 (8.7 ± 1.7) 11.8	1.8 (2.5 ± 0.4) 3.2
C. laticiphilum CBS112989	0.06 ± 0.002	0.14 ± 0.001	0.23 ± 0.002	**0.28 ± 0.005**	0.21 ± 0.006	0.01 ± 0.004	No growth	5.3 (8.1 ± 1.4) 10.9	2.4 (2.8 ± 0.3) 3.3
C. brisbaniense CBS292.67	0.01 ± 0.000	0.06 ± 0.002	0.09 ± 0.002	**0.18 ± 0.003**	0.18 ± 0.004	No growth	No growth	7.3 (10.6 ± 1.5) 14.1	1.8 (2.5 ± 0.3) 3.2
C. chrysanthemi CBS126519	0.04 ±0.002	0.12 ± 0.001	0.17 ± 0.002	**0.23 ± 0.002**	0.19 ± 0.002	0.00 ± 0.001	No growth	No sporulation	No sporulation
C. chrysanthemi CollS109	0.04 ± 0.005	0.11 ± 0.022	0.23 ± 0.003	**0.30 ± 0.005**	0.22 ± 0.001	0.00 ± 0.002	No growth	7.3 (11.0 ± 1.9) 13.7	2.4 (3.4 ± 0.4) 4.0
C. chrysanthemi Coll408	0.03 ± 0.002	0.09 ± 0.016	0.23 ± 0.012	**0.30 ± 0.002**	0.22 ± 0.001	0.01 ± 0.002	No growth	8.4 (10.9 ± 1.4) 13.9	2.5 (3.3 ± 0.4) 3.9
C. chrysanthemi Coll608	0.04 ± 0.003	0.13 ± 0.027	0.21 ± 0.014	**0.26 ± 0.005**	0.21 ± 0.002	0.01 ± 0.002	No growth	7.7 (10.7 ± 1.3) 12.9	2.6 (3.2 ± 0.3) 3.9
C. chrysanthemi 10190	0.04 ± 0.007	0.14 ± 0.024	0.25 ± 0.004	**0.30 ± 0.005**	0.23 ± 0.002	0.00 ± 0.001	No growth	8.6 (10.2 ± 0.9) 12.6	2.8 (3.2 ± 0.3) 4.3
C. chrysanthemi 10191	0.03 ± 0.002	0.14 ± 0.034	0.25 ± 0.002	**0.29 ± 0.005**	0.22 ± 0.002	0.01 ± 0.002	No growth	7.9 (11.4 ± 1.3) 13.2	2.6 (3.2 ± 0.5) 4.2
C. chrysanthemi 10194	0.04 ± 0.018	0 .05 ± 0.018	0.24 ± 0.005	**0.28 ± 0.011**	0.22 ± 0.002	0.01 ± 0.002	No growth	6.9 (10.2 ± 1 7) 12.9	2.4 (3.2 ± 0.5) 4.0
C. chrysanthemi 10252	0.04 ± 0.004	0.12 ± 0.019	0.22 ± 0.015	**0.29 ± 0.006**	0.21 ± 0.001	0.01 ± 0.000	No growth	7.1 (10.9 ± 1.6) 15.3	2.4 (3.4 ± 0.4) 4.0
C. chrysanthemi 10250	0.02 ± 0.006	0.12 ± 0.013	0.22 ± 0.007	**0.28 ± 0.004**	0.15 ± 0.006	0.01 ± 0.002	No growth	9.3 (11.2 + 1.5) 13.6	2.5 (3.3 + 0.4) 4.3
C. chrysanthemi 10372	0.04 ± 0.080	0.11 ± 0.035	0.24 ± 0.013	**0.29 ± 0.010**	0.20 ± 0.003	0.01 ± 0.003	No growth	7.9 (10.8 ± 1.2) 13.5	2.5 (3.2 ± 0.4) 4.2

[a]The slope value (mm h^{-1}) for each growth curve has been calculated from three replicates for each isolate. The values represent the average ± SD of three replicates; the highest growth rate of each isolate is indicated in bold.

[b]Values represent the minimum, the maximum and, within parentheses, the mean ± SD calculated for three replicates, with 30 conidia for each replicate.

475
476
477 **Table 4**. Pathogenicity of *Colletotrichum* isolates on *Carthamus tinctorius*.

Isolate	Emergence (%)	Seedling mortality (%)
C. chrysanthemi 10190	65.83 ± 0.01 b	97.46 ± 0.11 b
C. chrysanthemi 10372	64.16 ± 0.03 b	89.63 ± 0.03 b
C. chrysanthemi Coll408	70.83 ± 0.08 b	81.77 ± 0.11 b
C. simmondsii CBS122122	80.00 ± 0.11 a	0.44 ±0.06 a
C. lupini HY09	76.66 ± 0.09 a	0.00 ±0.00 a
Control	89.17 ± 0.06 a	0.00 ±0.00 a

478 Different letters within the same column correspond to significantly different values ($_{ANOVA}$; $P < 0\ 05$). The values are the
479 averages ± SD of three replicates, with 40 seeds each (80 seeds for the control).

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Figures' caption

Figure 1. Anthracnose symptoms on safflower plants: (a) stem blight characterized by dark-coloured stem lesions bearing salmon-coloured conidial masses in acervuli; (b) death of the distal part of the plant; (c) a stem broken down at the lesion point; and (d) diseased plants in discrete patches that were randomly distributed in the field.

Figure 2. A Bayesian analysis tree constructed from an alignment based on the concatenation of the ITS, *TUB* and GAPDH partial sequences of *Colletotrichum* isolates used in this study. The numbers on the nodes are posterior probabilities values. Host and geographic origin are indicated after the isolate code. The species designations reported in the literature are shown on the right.

Figure 3. Images of colony morphology of *Colletotrichum* isolates, showing phenotypic variability. For each colony image, the picture on the left half is the recto, and the picture on the right half is the verso and the letter denotes the phenotypic group to which the isolate belongs.

Figure 4. The disease cycle of *Colletotrichum chrysanthemi* on *Carthamus tinctorius*.

FIGURE 1.

Fig 2. Anthracnose symptoms on safflower plants: (a) stem blight characterized by dark-coloured stem lesions bearing salmon-coloured conidial masses in acervuli; (b) death of the distal part of the plant; (c) a stem broken down at the lesion point; and (d) diseased plants in discrete patches that were randomly distributed in the field.

FIGURE 2.

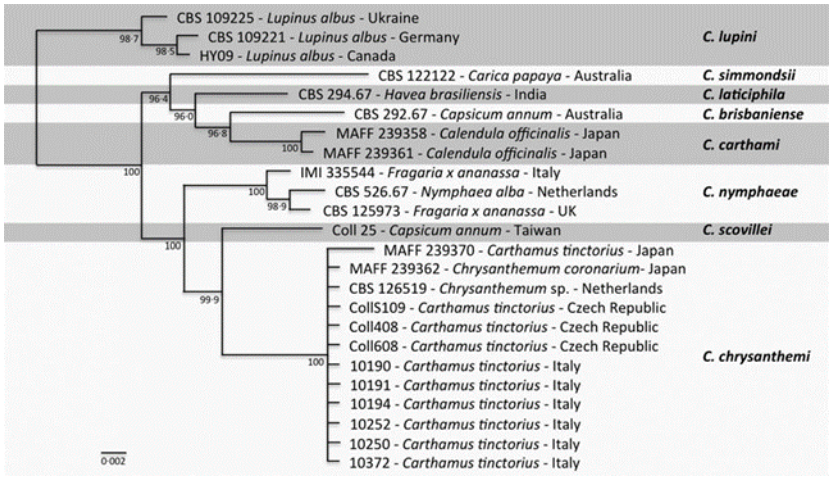

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FIGURE 3.

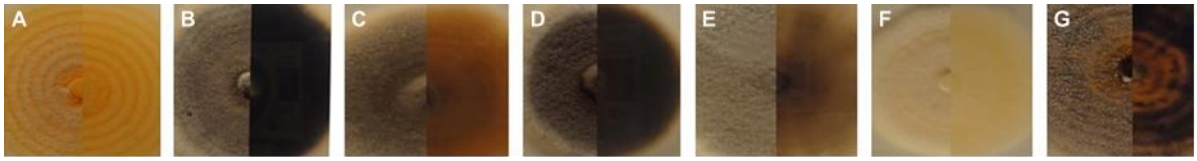

Fig. 3. Images of colony morphology of *Colletotrichum* isolates, showing phenotypic variability. For each colony image, the picture on the left half is the recto, and the picture on the right half is the verso and the letter denotes the phenotypic group to which the isolate belongs

521 FGIURE 4
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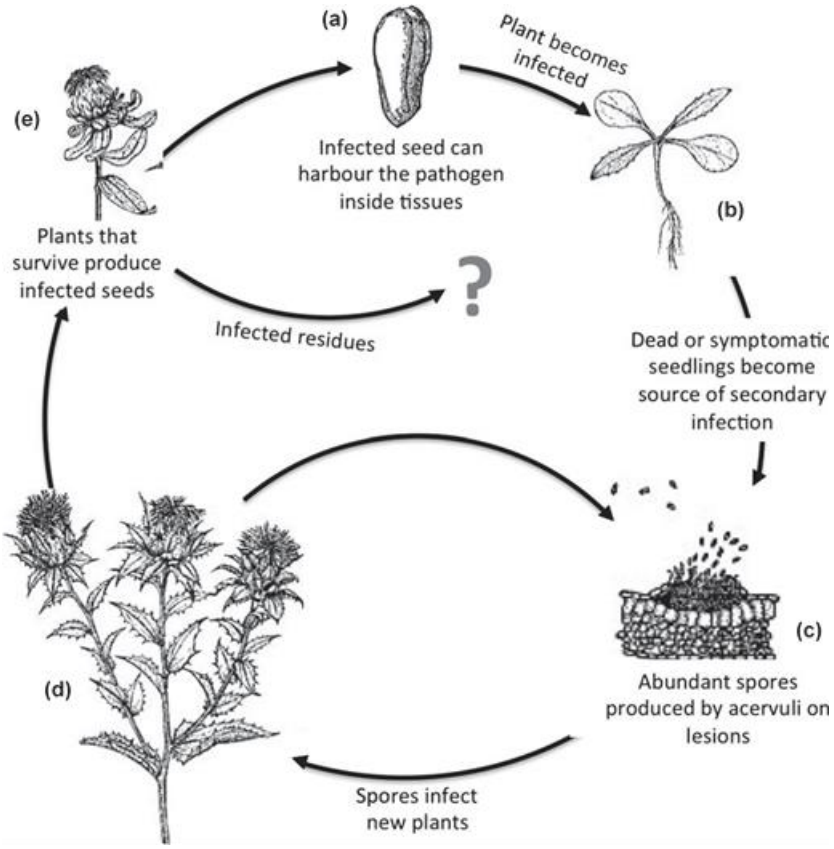

523
524 Fig 4. The disease cycle of *Colletotrichum chrysanthemi* on *Carthamus tinctorius*.
525