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SHORT COMMUNICATION

Isolation and pathogenicity of *Colletotrichum* spp. causing anthracnose of strawberry in south west Spain

Carlos Garrido · María Carbú · Francisco Javier Fernández-Acero · Giles Budge · Inmaculada Vallejo · Alison Colyer · Jesús M. Cantoral

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Abstract Anthracnose, caused by *Colletotrichum* spp., is a major disease of cultivated strawberry, *Fragaria*×*ananassa*. This study identifies the *Colletotrichum* spp. which causes strawberry anthracnose in the southwest of Spain. A survey of the region was carried out, and the strains isolated were identified as *C. acutatum* by using the polymerase chain reaction (PCR) with genus and species-specific primers, demonstrating that this species is currently the causal agent of strawberry anthracnose in the studied region. The pathogenicity of *C. acutatum* and *C. gloeosporioides* strains was evaluated on two principal strawberry cultivars (cvs Camarosa and Ventana) under field conditions, the latter being more pathogenic than the former.

Keywords Characterisation · *Colletotrichum acutatum* · *C. gloeosporioides* · Detection · *Fragaria*×*ananassa* · PCR

The genus *Colletotrichum* comprises some of the most economically important fungi causing anthracnose disease on strawberry (Buddie et al. 1999). Three species have been reported as causal agents of strawberry anthracnose, *Colletotrichum acutatum*, *Colletotrichum fragariae* and *Colletotrichum gloeosporioides* (teleomorph *Glomerella cingulata* (Denoyes-Rothan et al. 2003). All three species can be found on all parts of the plant; however crown rot and fruit rot are the most economically damaging. The late onset of fruit rot can cause serious and sudden losses both pre- and post-harvest (Denoyes-Rothan et al. 1999). *Colletotrichum acutatum* was first reported in Europe causing anthracnose on strawberry plants in France in 1981. It is thought to have been introduced to Europe from California (USA) on infected runner material and rapidly established in mainland Europe by 1993 (Denoyes-Rothan and Baudry 1995). Also it is considered a quarantine pathogen in the European Union (EU) and has been included in the list of regulated A2 pests in the European and Mediterranean Plant Protection Organization (EPPO) region since 1997 (EPPO/CABI 1997). *Colletotrichum gloeosporioides* has been reported in a large number of host species and is also widespread (Talhinhas et al. 2005), but the occurrence in strawberry in the EU is only occasional. *Colletotrichum fragariae* has never been reported in the EU. Characterisation and identification of *Colletotrichum* spp. has been traditionally based on shape and size of morphological characters such as conidia and appressoria, and differences in host range and pathogenicity, and they are still used in *Colletotrichum* studies (Kuramae-Izioka et al. 1997; Schiller et al. 2006).

C. Garrido · M. Carbú · F. J. Fernández-Acero · I. Vallejo · J. M. Cantoral (✉)
Laboratory of Microbiology, Marine and Environmental Sciences Faculty, University of Cádiz,
Puerto Real 11510 Cádiz, Spain
e-mail: jesusmanuel.cantoral@uca.es

G. Budge · A. Colyer
Central Science Laboratory,
Sand Hutton, York YO41 1LZ, UK

Since 1991, several contrasting molecular methods have been developed and widely implemented for more accurate characterisation and differentiation between *Colletotrichum* species (Denoyes-Rothan et al. 2003; Martínez-Culebras et al. 2002). The EPPO recommends two methods, based on PCR and ELISA, for the diagnosis and identification of *Colletotrichum* spp. causing anthracnose on strawberry plants (EPPO/CABI 1997). Spain is the second largest strawberry producer in the world behind the USA (Vallejo et al. 2003), accounting for half of the European strawberry production from approximately 8,000 ha mainly spread across the Huelva (90% of Spanish production with approximately 310,000 Tm) and Cádiz (5% of Spanish production with approximately 18,000 Tm) regions of the southwest of Spain (Lameyer 2006). *Colletotrichum acutatum* was first detected in Spain in 1998 (De los Santos and Muñoz 1999). To our knowledge, isolation and molecular identification to clarify which *Colletotrichum* spp. is responsible for anthracnose disease in the southwest of Spain has not been performed (De los Santos and Muñoz 1999). This work is aimed to establish which species are currently causing anthracnose disease in strawberry in southwestern Spain, and to study the pathogenicity of *Colletotrichum* spp. strains in the region.

A number of the most representative strawberry producer companies in the region were chosen for searching for severe symptoms of anthracnose disease. These were found only in (1) five companies located in three respective villages belonging to the province of Huelva: Lepe, Cartaya and Bollullos del Condado, and (2) two companies located in Conil de la Frontera, belonging to the province of Cádiz. Ninety-one samples of strawberry plant material (crowns, petioles, roots and fruits) with anthracnose-like symptoms were collected. Roots, crowns and petioles were ground using a pestle and mortar, and pieces of fruit were placed and shaken in tubes with 4 ml of 0.9% NaCl. Suspensions were filtered and spores collected by centrifugation. Serial dilutions were prepared and planted onto *Colletotrichum* isolation medium modified from Ureña-Padilla et al. (2001).

Three-hundred and nineteen monoconidial strains were isolated after 20 h of incubation (three to four strains were isolated from each fruit and plant sample). Isolated strains were compared with those of the reference strains of *C. acutatum* and *C. gloeosporioides* (Table 1) after growing on PDA, and 178 monoconidial strains were discarded since the colony and conidial morphologies were clearly different to those typical of the genus *Colletotrichum*. The remaining 141 strains (52 strains from plant material and 89 strains from fruit) were selected for molecular identification.

Identification of monoconidial cultures isolated was performed using *Colletotrichum* genus-specific primer sequences Col1 (5′-AAC CCT TTG TGA ACR TAC CTA-3′) and Col2 (5′-TTA CTA CGC AAA GGA GGC T-3′; Martínez-Culebras et al. 2003), and species-specific primer sequences CaInt2 (5′-GGG GAA GCC TCT CGC GG-3′), for *C. acutatum* (Sreenivasaprasad et al. 1996), and CgInt (5′-GGC CTC CCG CCT CCG GGC GG-3′), for *C. gloeosporioides* (Mills et al. 1992), along with the conserved ITS4 sequence (5′-TCC TCC GCT TAT TGA TAT GC-3′), based on ribosomal DNA ITS regions (White et al., 1990). DNA was extracted from approximately 80 mg of mycelium using FastDNA® kit and FastPrep Instrument (Q-BIOgene, Valencia, USA), following the manufacturer's protocols. To avoid false negative results due to possible PCR-inhibitors present in the samples, the quality of all DNA preparations from the 141 monoconidial strains was tested using universal primers ITS1–ITS4 (White et al. 1990). A product ranging in size from 400 to 700 bp was amplified using the universal primers (data not shown).

Genus-specific amplifications were carried out by PCR with cycling parameters consisted of a denaturing step of 5 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 56°C and 1 min at 72°C, ending with one cycle of 10 min at 72°C. Species-specific amplifications were carried out by PCR with a cycling parameter consisting in 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 2 min at 72°C, ending with one cycle of 10 min at 72°C. PCR products were separated on 1.5% agarose gels that were photographed on a UV transilluminator after ethidium bromide staining.

DNA from all the 141 monoconidial strains was tested by using genus-specific primers. A product of 460 bp was amplified with DNA from 84 strains, so that they were identified as belonging to the genus *Colletotrichum* (Table 1, Fig. 1a). DNA from the 84 strains was used as a template in PCRs using species-specific primers. A product of 490 bp was amplified

Table 1 *Colletotrichum* spp. strains from reference collections[a] and from the crop survey[b] used in this study, and their taxon-specific PCR identification

			PCR Identification[e]			Species
			Col1-Col2	CaInt2-ITS4	CgInt-ITS4	
Reference strain[a]	Host	Origin				
IMI 345051	Strawberry	Canada	+	−	+	*C. gloeosporioides*
IMI 345052	Strawberry	USA	+	−	+	*C. gloeosporioides*
CECT 2860	Orange	Spain	+	−	+	*C. gloeosporioides*
IMI 364856	Strawberry	France	+	+	−	*C. acutatum*
IMI 348487	Strawberry	France	+	+	−	*C. acutatum*
IMI 348489	Strawberry	France	+	+	−	*C. acutatum*
IMI 345035	Strawberry	France	+	+	−	*C. acutatum*
Strain[b]	Cultivar[c]	Location				
UCA 1001→1009	Camarosa	Lepe	+	+	−	*C. acutatum*
UCA 1010→1023	Camarosa	Bollullos	+	+	−	*C. acutatum*
UCA 1024→1043	Camarosa	Cartaya	+	+	−	*C. acutatum*
UCA 1044→1052	Camarosa	Lepe	+	+	−	*C. acutatum*
UCA 1052→1083	Camarosa	Conil	+	+	−	*C. acutatum*

[a] *IMI* International Mycological Institute, *CECT* Spanish-type Culture Collection, *UCA* University of Cádiz

[b] Strains isolated and identified in this study as *Colletotrichum* spp. (the rest of strains isolated until 319 have not been included in the table). Arrow means: from – to –.

[c] Cultivar of *Fragaria*×*ananassa*

[d] Location in the southwest of Spain. Lepe, Bollullos del Condado and Cartaya from Huelva. Conil de la Frontera from Cádiz.

[e] *Col1-Col2 Colletotrichum*-specific primers, *CaInt2-ITS4 C. acutatum*-specific primers *CgInt-ITS4 C. gloeosporioides*-specific primers

when the *C. acutatum*-specific primers were used (CaInt2-ITS4), while no products were obtained when using the *C. gloeosporioides*-specific primers (CgInt-ITS4; Table 1, Fig. 1b–c), so that all 84 strains were identified as *C. acutatum*. Two independent experiments were performed.

Morphological characterisation, including colony morphology and conidial size was performed for reference strains and randomly selected strains from those identified as *C. acutatum* from the survey for both *C. acutatum* (IMI348487, IMI348489, IMI364856, UCA1015, UCA1028, UCA1070, UCA1076) and *C. gloeosporioides* strains (IMI345051, IMI345052, CECT 2860; Table 1). *Colletotrichum acutatum* strains showed a higher variability of morphological characteristics in the mycelium (white mycelium for 4–5 days before turning to a grey-brown colour) with spore masses ranging from salmon to cream. *Colletotrichum gloeosporioides* strains showed a dense, white mycelial growth turning to a dark olive-grey colour. For each strain, the length and breadth of 300 conidia (100 conidia for each of three conidial preparations) were measured using an optical microscope. Separate analyses of variance (ANOVA) were performed on data for conidial length and breadth (GenStat® 9.1 statistical software) indicating that the interaction species×region×strain was significantly different in both cases ($P<0.001$). The majority of the variation between *C. acutatum* strains was found within the international collection group. *Colletotrichum acutatum* IMI348487 (7.12×3.30 μm) had the smallest and *C. gloeosporioides* CECT2860 the largest (12.65×4.63 μm) conidia.

The pathogenicity of *Colletotrichum* spp. causing anthracnose crown rot under field conditions was studied using ten randomly selected strains of *C. acutatum*: UCA1005, UCA1015, UCA1025 and UCA1028 isolated from Huelva in 2004, UCA1070, UCA1076, UCA1077 and UCA1078 isolated from Cádiz in 2004 and IMI348487 and IMI348489 from the International Mycological Institute. Although *C. gloeosporioides* was not isolated in this work, this species has been reported in a large number of host species and is also widespread even occasionally in strawberry in the EU. A mix of *C. acutatum* and *C.*

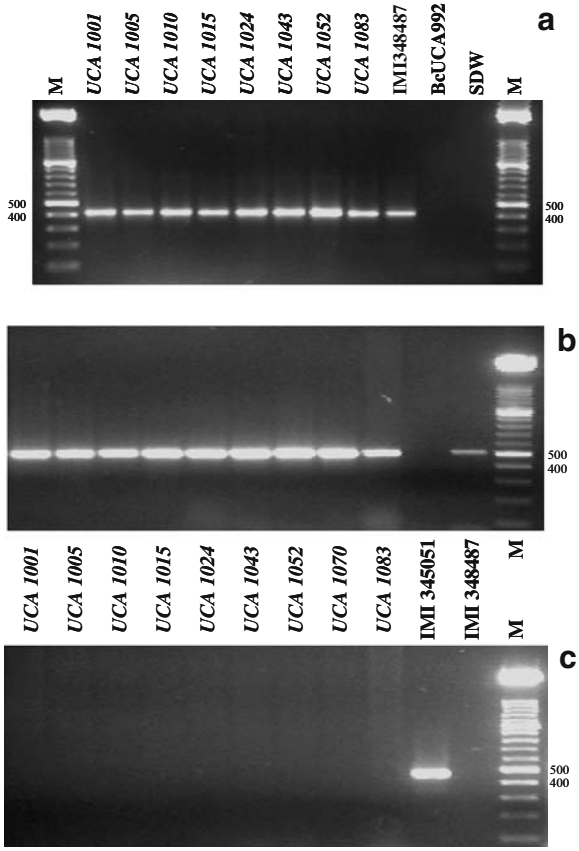

Fig. 1 Agarose gel electrophoresis of eight PCR products obtained from *Colletotrichum* spp. strains isolated from strawberry. **a** *Colletotrichum* spp.-specific products generated with primers Col1-Col2 (*Botrytis cinerea*, BcUCA992 strain, and sterile distilled water, *SDW*, were used as negative controls, and *C. acutatum* IMI348487 was used as positive ones). **b** *C. acutatum*-specific products obtained with primers CaInt2-ITS4. **c** *C. gloeosporioides*-specific products generated with primers CgInt-ITS4. **b**, **c** *C. gloeosporioides* IMI345051 and *C. acutatum* IMI348487 were used like positive and negative controls where appropriate. *Lane M*, molecular marker (100 bp DNA Molecular Weight Marker XIV; Roche Molecular Biochemicals, Mannheim, Germany)

gloeosporioides has been demonstrated as being responsible for olive anthracnose in the south of Portugal, close to Huelva (Talhinhas et al. 2005). For these reasons, two *C. gloeosporioides* strains (IMI345051 and IMI345052) were also included in the pathogenicity tests. Strawberry plants were planted at the end of October in a plastic tunnel (separated by cultivar). Ten plant crowns each of cv. Camarosa and cv. Ventana were inoculated with 0.2 ml of conidial suspensions of each strain (1×10^6 conidia ml^{-1}) respectively, using 1 ml syringe (needle gauge, 21 G×25 mm), and sterile distilled water (SDW) as a negative control. In total, 520 strawberry plants were inoculated in January and maintained until June under commercial conditions in an experimental field in Lepe (Huelva, Spain). The study was repeated during 2004–2005 and 2005–2006 seasons.

At the end of the trials, an ANOVA, performed using the size of crowns inoculated with SDW (negative controls) in both cultivars, showed that there was no significant difference between the crown size in cv. Camarosa compared to cv. Ventana (P= 0.880), and a significant difference in crown size was noted between years (P=0.004). Pathogenicity was determined by the presence/absence of a lesion inside the crown and by the ratio of lesion to crown size of *Colletotrichum* inoculated plants (Fig. 2). On the one hand, logistic and linear regression models were used to assess respective lesion presence/absence data, and lesion size was used to investigate differences in pathogenicity from crowns inoculated with *C. acutatum* strains (Table 2). The models accounted for only 5.8% and 9.8% of the variation respectively, which suggests that the measured parameters for each model are explaining little with regards to whether (1) a lesion will grow and (2) the size of the lesion relative

Fig. 2 Internal brown necrotic lesions developed in the crowns inoculated with *C. acutatum* and *C. gloeosporioides*. **a** Healthy crown; **b** lesions produced by *C. acutatum* (UCA1028); **c** lesions produced by *C. gloeosporioides* (IMI345051)

Table 2 Percentage of crowns with internal lesions, means of crown sizes and lesion sizes during 2005 and 2006 for inoculated *Colletotrichum acutatum* strains

Strain[a]	Percent Crown with lesion		Crown size[b] (mm)				Lesion size[b] (mm)			
	2005	2006	2005		2006		2005		2006	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
UCA1005	50	55	30.30	1.98	22.55	2.55	8.20	1.88	5.45	0.76
UCA1015	75	75	29.40	1.76	28.33	2.25	7.60	1.32	9.60	1.21
UCA1025	15	40	31.33	2.40	27.50	2.67	4.33	1.85	2.87	0.44
UCA1028	45	35	28.66	2.73	28.14	2.82	11.00	1.93	7.57	1.17
UCA1070	65	65	27.77	1.78	22.62	1.57	8.54	1.95	5.46	0.60
UCA1076	30	70	28.00	1.69	30.71	1.59	10.33	2.67	7.14	0.80
UCA1077	25	45	25.40	1.28	28.55	2.32	12.00	2.31	6.44	0.81
UCA1078	35	65	29.28	3.93	30.92	1.88	9.00	2.86	8.07	1.46
IMI348487	30	50	27.50	2.55	27.00	1.85	4.83	1.13	6.30	0.98
IMI348489	30	50	22.16	2.30	27.70	1.85	13.66	2.92	7.80	1.48
SDW[c]	0	0	31.15	1.43	25.05	1.28	0	0	0	0

[a] *C. acutatum* strains isolated in this study and two *C. acutatum* strains from IMI.

[b] Results are expressed as the means and standard errors of mean.

[c] *SDW* Sterile distilled water used as negative control

to the crown. On the other hand, logistic and linear regression models were used with data from crowns inoculated with *C. acutatum* and *C. gloeosporioides* strains from the IMI to investigate differences in pathogenicity between species, cultivar and year. Logistic regression models of the presence/absence of lesions accounted for only 13.4% of variation, showing only a significant interaction between species and year ($P=0.002$). For plants containing lesions, the model proved to be more robust, accounting for 65% of the variation in the data. Significant interactions were observed between species and cultivar; and between species, strain and year (Table 3).

Five randomly symptomatic crowns inoculated with *C. acutatum* and five inoculated with *C. gloeosporioides* were collected and pathogens re-isolated onto CIM medium at 25°C. DNA was extracted and used as a template for PCR amplification. All samples yielded cultures that reacted

Table 3 Accumulated analysis of deviance results from the size of the lesion relative to size of crown for *C. acutatum* and *C. gloeosporioides* species

Source of variation	*df*[a]	MD[a]	DR[a]	$P>\chi^2$ [b]	
Species	1	10.98	53.71	<0.001	**
Species × strain	2	1.88	9.22	<0.001	**
Cultivar	1	0.09	0.42	0.521	NS
Species × cultivar	1	1.01	4.96	0.030	*
Species × strain × cultivar	1	0.35	1.74	0.193	NS
Year	1	7.75	37.92	<0.001	**
Species × year	1	2.39	11.69	0.001	**
Species × strain × year	1	2.35	11.48	0.001	**
Residual	61	0.20			
Total	70	0.59			

df Degrees of freedom, *MD* mean deviance, *DR* deviance ratio, *NS* non-significant at $P>0.05$

*Significant at $P<0.05$

**Significant at $P<0.01$

positively to the respective species-specific PCR primers (data not shown). When the isolation protocol was used with asymptomatic crowns, no strains of *C. acutatum* and *C. gloeosporioides* were recovered.

The main aim of this research was to conduct a survey to identify which species of *Colletotrichum* are responsible for strawberry anthracnose in the southwest of Spain. Molecular methods were used for species identification of the monoconidial strains isolated from Cádiz and Huelva. *Colletotrichum acutatum* was isolated from fields in both regions, but *C. gloeosporioides* and *C. fragariae*, both causal agents of strawberry anthracnose, were not isolated. These results are in agreement with previous reports which state that *C. acutatum* is prevalent in Europe causing strawberry anthracnose, while the occurrence of *C. gloeosporioides* is only occasional and *C. fragariae* has never been reported (Denoyes-Rothan and Baudry 1995; Martínez-Culebras et al. 2002). The presence of *C. acutatum* in southwestern Spain, where cvs Camarosa and Ventana are the main strawberry cultivars (accounting for 98% of strawberry production in 2003/04), may be related to the fact that *C. acutatum* is the major species present in southwestern USA, which is the source of the propagation material in the EU (Freeman et al. 2001; Lameyer 2006).

The study of morphological characters, such as mycelial morphology or conidial size, was particularly variable. Although morphological differences have been used in previous researches for characterisation and discrimination of populations of *Colleotrichum* strains (Kuramae-Izioka et al. 1997; Schiller et al. 2006), our results show a high variability of *Colletotrichum* spp., with very different mophological characteristics displayed by strains belonging to the same species, and similar morphological features for strains of different species, which is in agreement with the observation reported by many other authors (Afanador-Kafuri et al. 2003; Denoyes-Rothan and Baudry 1995).

To assess the potential impact of crown infection, several strains of *C. acutatum* and *C. gloeosporioides* were inoculated into the crowns of strawberry plants. The cvs Camarosa and Ventana were selected because they are the principal cultivars in the southwest of Spain (Lameyer 2006). *Colletotrichum acutatum* was weakly pathogenic whereas strains of *C. gloeosporioides* were significantly more pathogenic causing a greater number of larger lesions than the former. Ureña-Padilla et al. (2001) have also studied this difference, reporting that *C. acutatum* isolated from diseased fruit was not able to induce symptoms when inoculated into strawberry crown tissue, and *C. gloeosporioides* strains isolated from infected plant material were not pathogenic when re-inoculated onto fruit or leaf tissue. These results suggest that the relationship between host and pathogen is dependant on the infected tissue (Denoyes-Rothan et al. 1999).

Plants inoculated in January remained symptomless until March, demonstrating a period of latency after artificial infection. Mother plants could be considered the main primary inoculum source on which the fungus proliferates and spreads from nursery to production field via transplants, as previously suggested by Freeman et al. (2001). In Spain, mother plants arrive from USA to central and northern regions, where nurseries are located. Strawberry transplants are then transported to the south for field production. Monitoring strawberry anthracnose in these fields and nurseries could be important to control the epidemiological routes for these strawberry pathogens. Such information would be crucial for any future eradication campaign. Future work could include the optimisation of faster and more sensitive protocols, using more recent molecular tools such as real-time PCR. Such tools could disminish detection thresholds and make easier the identification of *Colletotrichum* spp. from asymptomatic plant material.

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