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Colletotrichum acutatum Simmonds as Agent of Anthracnose and Stem Blight on *Nerium oleander* in Italy

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ABSTRACT

Oleander is an evergreen shrub or small tree in the genus *Nerium* and is extensively grown as an ornamental plant in parks, and along roadsides. Many diseases are reported to cause damage and death of plants, thus transforming the landscape. In the present work plants with anthracnose-like symptoms were observed and Koch's postulates were applied to determine the aetiological agent of the disease. Morphological and cultural observations indicated that *Colletotrichum acutatum* J.H. Simmonds should be the agent, but these observations could not confirm the assignment of our isolates to this species. Sequencing of ITS-rDNA fragments showed significant homology (99%) with many isolates of *Colletotrichum acutatum* present in the NCBI gene bank, solving the classification problem and indicating that molecular tools are necessary for correct classification of *C. acutatum*. In addition, the results of cluster analysis demonstrated that, according to many authors, some sub-groups may exist in *C. acutatum* species, but the common origin and/or characteristics of isolates belonging to the same sub-group are not well described yet. Data of pathogenicity tests demonstrated that oleander isolates failed to infect pepper and this finding could be useful for studying resistance mechanisms in pepper and/or host specificity.

Keywords: cluster analysis, ITS, molecular characterization, North of Italy
Abbreviations: EMBL, European Molecular Biology Laboratory; **ITS1-5.8S-ITS2**, internal transcribed spacer 1-ribosomal RNA gene-internal transcribed spacer 2; **MS**, Mathur's medium; **NCBI**, National Center for Biotechnology Information, **PDA**, potato dextrose agar

INTRODUCTION

Oleander (*Nerium oleander* L.) is a highly toxic evergreen shrub or small tree in the family Apocynaceae and is currently the only species classified in the genus *Nerium*. It is native to a broad area of Morocco and Portugal, eastward through the Mediterranean region and southern Asia to Yunnan in southern China. It grows to 2-6 m tall, with spreading to erect branches. Oleander grows well in warm subtropical regions, where it is extensively used as an ornamental plant in landscapes, parks, and along roadsides. It is drought tolerant and will tolerate occasional light frost down to -10°C (Huxley *et al.* 1992). Oleander is considered extremely toxic and it has been reported that in some cases only a small amount had lethal or near lethal effects. The most significant of these toxins are oleandrin and neriine, which are cardiac glycosides (Galey *et al*. 1996). Oleander flowers are showy and fragrant and are grown for these reasons. Over 400 cultivars have been named, with several additional flower colours not found in wild plants. New oleander cultivars have been selected, including red, purple and orange; white and a variety of pink flowers. Many cultivars also have double flowers.

Numerous diseases affect oleander and one of the most important is anthracnose; in Italy the aetiological agent of this disease is reported to be *Phoma exigua* var. *heteromorpha*, but no scientific report of such a fungal disease has been found. Alvarez *et al.* in 2005 also reported this fungus to be the causal agent of oleander anthracnose in Spain. In 2007 at Bovolone (Verona province), in the North of Italy, ornamental oleander showed symptoms similar to those already observed on almost 80% of plants. Symptoms observed were necrotic spots on stems starting on one side, which later girdled branches killing the upper part of these branches. On leaves characteristic symptoms of anthracnose began as small spots. Leaves later became completely covered by irregular necrotic spots and many of them died (**Fig. 1**). Small orange conidial masses appeared on lesions. The aims of the present work were: i) to identify the etiological agent of this disease by applying Koch's postulates and molecular tools; ii) to characterize fungal isolates collected from diseased oleander. In the present study we obtained isolates from one location, so characterization is only related to oleander in that specific environment and does not represent the oleander situation in general.

Fig. 1 Necrotic symptoms on stem and leaves of oleander plants in the North of Italy (Verona Province).

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MATERIALS AND METHODS

Isolation, morphological characterization and sensitivity to benomyl

Isolations were made from symptomatic oleander tissues of an ecotype with pink flowers. Leaves and stem pieces, from the middle part of plants, were surface sterilized for 15 s with 1% sodium hypochlorite solution, washed in sterile water and placed in Petri dishes containing different media: potato dextrose agar (PDA, Oxoid, UK) regular or acidified to pH 4.5, V8 juice agar (Mitchell and Kannwischer-Mitchell 1992) and Nash and Snyder's PCNB (Sigma-Aldrich, USA) (Nash and Snyder 1962). These media are routinely used in our laboratory for isolation of unknown agents.

The plates were kept at 25°C in the dark; emerging colonies were transferred to fresh PDA plates. After conidia formation monoconidial cultures were established. For morphological studies the fungus was cultured on modified Mathur's medium (MS) (Freeman and Katan 1997) supplemented with 2.5 g (a.i.) ml^{-1} of iprodione (Rovral 50WP, BASF, Germany) and acidified with 0.1% lactic acid to suppress growth of fungal contaminants and bacteria. Cultures grown on MS were incubated at 25°C in the dark. Size and shape of 150 conidia per isolate were evaluated and Simmonds' (1965) description was used for characterization. Conidia sizes were submitted to ANOVA and means were separated using least significant difference ($P \leq 0.05$).

Microscopic observations were made using light and phase contrast microscopy with an Olympus BH 2. Four, already characterized, *C. gloeosporioides* strains (data not shown) present in our mycological collection isolated from apple (*Malus domestica* 'Annurca') and the four *C. acutatum* oleander isolates were grown on benomyl (Benlate 50 WP Du Pont, France) -amended agar at four concentrations (0, 0.1, 0.5 and 1 µl l^{-1}) to evaluate sensitivity to benomyl in order to try to differentiate *C. gloeosporioides* from *C. acutatum*.

Pathogenicity test and host range

4 months-old healthy oleander plants, 20 for each of four *C. acutatum* oleander isolates chosen for this work and 20 as uninoculated control, were transplanted into 30 cm-diameter pots containing a sterilized mixture (1: 1: 1, v/v) of sand, peat and soil. Inoculation was made by spraying leaves with a conidial suspension (10^5 conidia ml^{-1}) or applying on the surface of stems agar plugs (5 mm), sealed with parafilm (Parafilm M, USA) to avoid drying of plugs, on 10 oleander stems on five plants, for each of the four oleander isolates. Stems were previously wounded with a sterile scalpel producing wounds of 5 mm long and about 3 mm depth. The conidial suspension was prepared as follows: fungal cultures were shaken with sterile distilled water, filtered through four layers of cheesecloth and the conidial concentration then adjusted to 2×10^5 conidia ml^{-1} using a haemocytometer (Burker-Turk Superior, Germany). Mycelial plugs, to inoculate stems, were taken from the growing margins of the fungal culture at day 10. Symptoms were assessed after 15 days and 1 month for leaves and stems, respectively.

Host range was evaluated by inoculating detached leaves of peach (*Prunus persica* 'Fayette'), apple (*Malus domestica* 'Stark delicious'), sweet orange (*Citrus sinensis* 'Washington navel') and pear (*Pyrus communis* 'Spadona'); plants of pepper (*Capsicum annuum* 'Almuden'), tomato (*Lycopersicon esculentum* 'Sorrentino'), strawberry (*Fragaria ananassa* 'Camarosa') and lupine (*Lupinus albus*); and ripen fruits of the same cultivars of peach, apple, sweet orange pear, pepper, tomato, strawberry using the same method described for oleander leaves inoculation.

Symptoms were observed on fruits and leaves of the above-mentioned species 4 days post inoculation (dpi). In preliminary experiments, inoculated pepper fruits showed no symptoms. In the present experiment two pepper ecotypes ("Cazzone") with red and yellow fruits and one hybrid (Eppo) with yellow fruits were inoculated after wounding, in order to confirm results. Severity was evaluated on the basis of spots dimension after 4 days as follows: high >10 mm, medium 5-9 mm and low < 5 mm.

DNA extraction

At least 100 mg of mycelium was crushed in liquid nitrogen in a pre-chilled mortar, and the resulting powder was treated as described by Raeder and Broda (1985), with the following modifications: DNA extraction was made with no sonication of tissues; after the addition of 1.5 ml extraction buffer the samples were kept at 60°C for 40 min and then centrifuged at 13,000 rpm for 15 min. The supernatant was transferred to a fresh tube and one volume of phenol: chloroform: isoamyl alcohol was added, and after mixing,

Table 1 Isolates of *Colletotrichum acutatum* used in this study and group assignment.

Isolate	EMBL Accession	Origin	Host (as reported by authors)	Group*	Author
IMI348494	AF272785	France	*Fragaria ananassa*	I	Freeman *et al.* 2001
U.S.-ALM-4	AF207793	USA	*Prunus dulcis*	I	Freeman *et al.* 2001
APL2	AF272787	USA	*Malus domestica*	I	Freeman *et al.* 2001
PCH8	AF272788	USA	*Prunus persica*	I	Freeman *et al.* 2001
PCN5	AF272786	USA	*Carya illinoensis*	I	Freeman *et al.* 2001
NL12A	AF272781	The Netherlands	*Anemone coronaria*	I	Freeman *et al.* 2001
Hv835	AF272782	Israel	*Anemone*	II	Freeman *et al.* 2001
TUT 5954	AF207794	Israel	*Fragaria ananassa*	II	Freeman *et al.* 2001
STR3	AF272784	USA	*Fragaria ananassa*	II	Freeman *et al.* 2001
IMI 223120	AF272783	Australia	*Anemone*	II	Freeman *et al.* 2001
GL-118-94111	AF081292	USA	*Olea europea*	II	Freeman *et al.* 2001
CECT20120	AF090853	USA	*Fragaria ananassa*	II	Freeman *et al.* 2001
ALM-KSH-10	AF207791	Israel	*Prunus dulcis*	III	Freeman *et al.* 2001
GRAY	AY826765.1	USA	*Vitis* spp. *Marquis*	III	Catal *et al.* 2004
213	FJ478084.1	Canada	*Vaccinium macrocarpon*	III	Polashock *et al.* 2008
XoI	-	Italia	*Nerium oleander*	III	-
IMI345026	AF272789	Spagna	*Strawberry*	III	Freeman *et al.* 2001
PT715	AM991135.1	Portugal	*Olea europea*	III	Talhinhas *et al.* 2008
06-133	AM991136	Portugal	*Olea europea*	III	Talhinhas *et al.* 2008
BBA 71320	AJ301969.1	-	*Hordeum vulgare*	III	Hagedorn *et al.* 2000
BBA 71427	AJ301987	-	*Camelia* sp.	IV	Hagedorn *et al.* 2000
BBA 71383	AJ301983	-	*Juglans regia*	IV	Hagedorn *et al.* 2000
BBA 71331	AJ301971	-	*Prunus cerasus*	IV	Hagedorn *et al.* 2000
BBA 71286	AJ301963	-	*Lycopersicon* sp.	IV	Hagedorn *et al.* 2000
BBA 70820	AJ301956	Germany	*Hepatica acutiloba*	IV	Hagedorn *et al.* 2000
BBA 70486	AJ301936	-	*Bergenia* sp.	IV	Hagedorn *et al.* 2000
BBA 70345	AJ301917	The Netherlands	*Prunus cerasus*	IV	Hagedorn *et al.* 2000
BBA 70342	AJ301914	-	*Fragaria*	IV	Hagedorn *et al.* 2000
BBA 69645	AJ301906	-	*Primula florindae*	IV	Hagedorn *et al.* 2000

the samples were centrifuged at 6500 rpm for 15 min. The DNA of each sample was amplified using primers (ITS1-ITS4) specific for nuclear ribosomal DNA internal transcribed spacer region (Glass and Donaldson 1995) acquired from Eurofins-MWG-Operon (Germany).

Polymerase chain reaction analysis

The PCR was modified from White *et al.* (1990) and contained the following components for reaction: dNTPs (0.2 mM), 10X reaction buffer (Tris-HCl pH 9.0 100 mM, KCl 500 mM, Triton X-100), $MgCl_2$ (2 mM), 0.2 mM of each primer, 0.5 U *Taq* DNA polymerase (GE Healthcare), and 100 ng of DNA. PCR conditions were 95°C for 4 min, then 35 cycles of 95°C for 60 s, 60°C for 60 s and 72°C for 60 s. PCR was performed in a Master thermocycler (Eppendorf). Afterward, 10 µl of reaction mixture was fractionated in a 1% agarose gel (Sambrook *et al.* 1989). Amplified DNA-fragments were sequenced by Eurofins-MWG-Operon and the sequences obtained were compared with those present in the data bank of the National Center for Biotechnology Information (NCBI) using BLASTn (Altschul *et al.* 1997).

Molecular characterization of oleander isolates

Table 1 shows the 29 *C. acutatum* isolates used for sequence analysis. Isolates used were those available in the EMBL nucleotide sequence database with a known host, and a ≥ 99% homology with isolates obtained from diseased oleander plants. The 29 isolates were then compared with one oleander isolate (XoI) and an outgroup (*C. gloeosporioides*). Only one oleander isolate was chosen because of its clonal origin.

Sequence comparisons of the ITS1-2 region was carried out using the program BLAST2 on NCBI GeneBank and cluster analysis was performed by applying distance matrix, and maximum-likelihood methods using DataDesk software (Data Description, Inc., Ithaca, NY).

RESULTS

Isolation, morphological characterization and sensitivity to benomyl

Up to 80% of isolations yielded white colonies on PDA that later became covered with orange conidial masses. All isolates from different parts of diseased oleander plants (stems and leaves) produced acute conidia with an average size of $14.8 \pm 1.2 \times 5.2 \pm 0.4$ µm. This morphology is almost typical of *C. acutatum* grown on supplemented MS or PDA as reported by Freeman and Katan (1997). Morphology of cultures and conidia sizes were not significant different among oleander isolates and are comparable with those reported in literature (Sutton 1992), but we were not sure to assign our isolates to *C. acutatum* using these characteristics, due to the high variability observed that showed non significant results of these parameters. The amended agar method showed variability in sensitivity of *C. gloeosporioides* isolates to benomyl; in fact one isolate grew in amended media, thus questioning the assignment of our isolates to *C. acutatum*.

Molecular identification of oleander isolates

The identification was confirmed by means of analysis of the internal transcribed spacers sequences. The amplicons from the total genomic DNA extracted from oleander isolates, using the primers ITS1 and ITS4, were 600-650 bp in length. The amplified fragments were sequenced and PCR products were analysed for homology using BLAST2 software. Significant homology (99%) was found with many isolates of *Colletotrichum acutatum* in Genebank. Oleander isolates showed 100% homology among them indicating a clonal origin, so 1 isolate was chosen for molecular characterization.

Pathogenicity test and host range

Pathogenicity tests confirmed the ability of oleander isolates to reproduce symptoms both on leaves and stems of oleander (**Fig. 2**). **Table 2** reports the results of inoculation tests on several host species chosen among those grown in the same area of symptomatic oleander plants. All species

Fig. 2 Reproduction of symptoms obtained by artificial inoculation of *Colletotrichum acutatum* on stem and leaves of oleander plants.

Table 2 Pathogenicity of isolates of *C. acutatum* on several crops grown close to diseased oleander plants.

Species	Leaves	Fruits
Peach (*Prunus persica*)*	+	+
Apple (*Malus domestica*)*	+++	+++
Sweet orange (*Citrus sinensis*)*	+	+
Pear (*Pyrus communis*)*	++	+++
Pepper (*Capsicum annuum*) **	-	-
Tomato (*Lycopersicon esculentum*)	++	+++
Strawberry (*Fragaria ananassa*)	+++	+++
Lupine (*Lupinus albus*)	+	NT

* inoculation on detached leaves; ** inoculation with and without wounding
NT = not tested;
+ = low severity; ++= medium severity; +++ = high severity

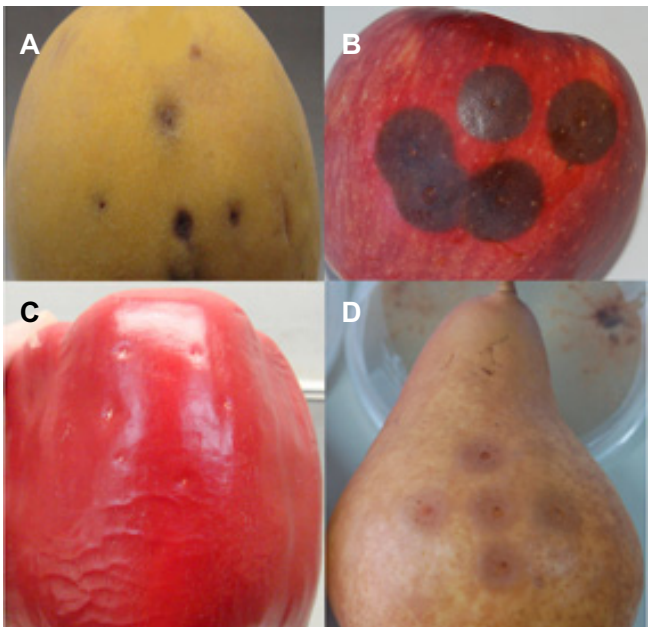

Fig. 3 Pepper fruits showing no symptoms (C) after inoculation with *Colletotrichum acutatum* isolated from oleander. Fruits of apple **(B)**, pear **(D)** and peach **(A)** with symptoms after inoculation with *C. acutatum*.

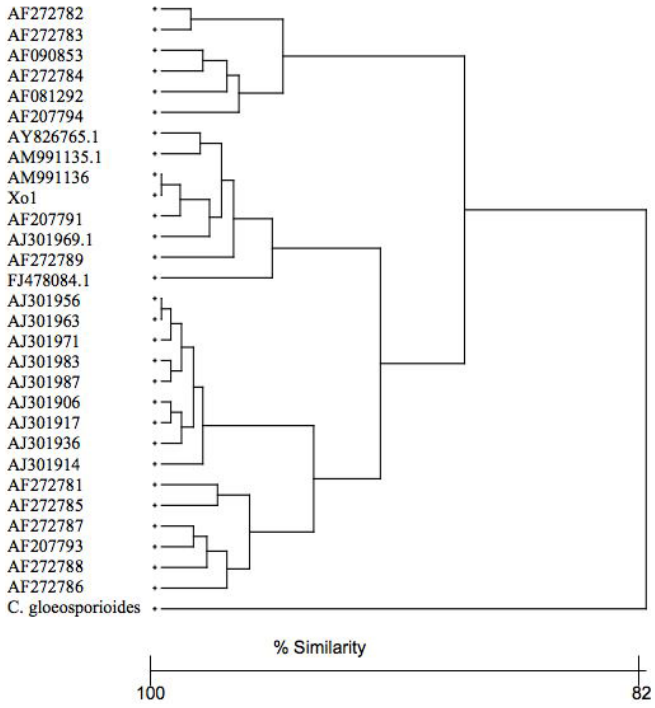

Fig. 4 Internal transcribed spacers 1-2 based phylogenetic dendrogram of *Colletotrichum acutatum* published sequences, showing homology of >99% with oleander isolate. Dendrogram was produced using the neighbor-joining algorithm.

tested showed symptoms both on leaves and fruits, except pepper fruits that remained healthy even when wounded before inoculation (**Fig. 3**).

Molecular characterization of oleander isolates

On the basis of the homology obtained among the isolates, a cluster analysis was performed on the similarity of sequences of the isolates (**Table 1**). A dendrogram was constructed in which the *C. acutatum* isolates clustered within four groups (**Fig. 4**), three of them similar to those reported by Freeman *et al.* (2001), except for an isolate from anemone, which clustered in group I and not in a separate group in our analysis. Group IV contains isolates deposited by Hagedorn in 2000 from different hosts with unknown origins. The isolate *XoI* from oleander clustered in the Subgroup III of Freeman *et al.* (2001) together with *C. acutatum* from almond (Israel), strawberry (Spain), vine (USA) and two isolates from olive (Portugal).

DISCUSSION

The main aim of the present work was to identify the specie responsible for anthracnose of oleander in the North of Italy. In previous work *Phoma exigua* var. *heteromorpha* has been reported as etiological agent of a similar disease (Alvarez *et al.* 2005). The present is the first report of *Colletotrichum acutatum* J.H. Simmonds as etiological agent of oleander anthracnose. *C. acutatum* infects plants of at least 24 families worldwide (Peres *et al.* 2005) and due to its high aggressiveness and yield losses caused in many crops *C. acutatum* is on the list of quarantine pathogens. In Italy, this pathogen is not only etiological agent of highly destructive diseases of many crops, but it has also been reported to cause leaf spots of Azalea (Garibaldi *et al.* 2004). Morphological characteristics (Sutton 1980) or optimal growth temperature and growth rate, (Gunnell and Gubler 1992; Sutton 1992) are not always reliable in identification of isolates, due to the existence of isolates from different species, such as *C. acutatum* and *C. gloeosporioides*, with intermediate forms and traits (Maclean *et al.* 1993). Nonetheless, morphological results indicated that the causal agent could be *C. acutatum*, but were not definitive enough to separate the two species.

In contrast with the data reported in other works (Adaskaveg and Hartin 1997; Peres *et al.* 2004) benomyl resistance could not be used in this study to assign the pathogen to *C. acutatum*; in fact one isolate of *C. gloeosporioides* from apple was resistant to benomyl. This is not a new finding because resistance to benomyl in *C. gloeosporioides* has previously been reported in the literature (Maymon *et al.* 2004, 2006).

In this study sequencing of the amplicons obtained by PCR using specific primers (ITS1-ITS4) confirmed that *C. acutatum* was the agent isolated from diseased oleander plants. The DNA nucleotide sequences of the isolate had a homology of 99% with *C. acutatum* isolates in the NCBI gene bank, while it was ≤ 96% for *C. gloeosporioides*. Several studies to describe sub-specific groups and the relationships among isolates related to different hosts were made (Freeman *et al.* 2001; Peres *et al.* 2005; MacKenzie *et al.* 2009), but no conclusive results have been obtained yet.

Further characterization of our isolates was made using host range and the analysis of ITS1-2 according to Freeman *et al.* (2001). In the present study we obtained isolates from one location, so characterization is only related to oleander in that specific environment and is far from representing the oleander situation in general. The oleander isolates from the environment in this study are clonal and polyphagous, but they failed to infect pepper leaves and fruits even when wounded, indicating the unique behaviour of such isolates. In addition, a previous study reported that all bell pepper cultivars tested for response to *C. acutatum* were susceptible in field trials and, although they varied in degree of susceptibility, no resistant cultivars were found (Lewis Ivey *et al.* 2004). The analysis of ITS 1-2 put our isolates in group III reported by Freeman *et al.* (2001) together with five other isolates from different hosts. In Bulgaria for example the population obtained from symptomatic strawberry, tomato, pepper and weed hosts was genetically uniform and apparently clonal in origin (Jelev *et al.* 2008), while genetic diversity was observed in strawberry when collected worldwide (Denoves-Rothan *et al.* 2003). Our findings, even though based on oleander isolates from one location, demonstrated once more that some sub-groups may exist, but are not related to host or geographic origin. Studies based both on safe exchange of isolates and collaborative experiments using the same set of host species, morphological and molecular tools should be conducted in the future.

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