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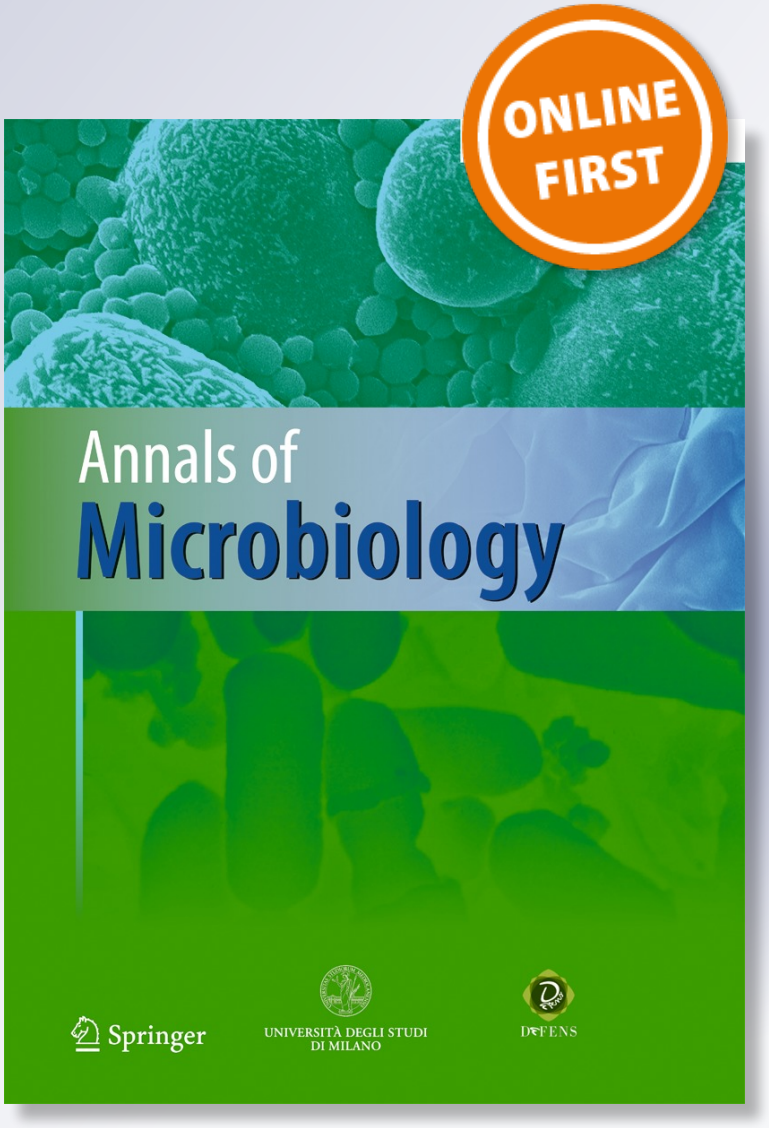
Annals of Microbiology

ISSN 1590-4261

Ann Microbiol
DOI 10.1007/s13213-015-1082-5



Springer

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DOI 10.1007/s13213-015-1082-5

ORIGINAL ARTICLE

Identification of *Acremonium* isolates from grapevines and evaluation of their antagonism towards *Plasmopara viticola*

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Received: 8 July 2014 / Accepted: 1 April 2015

Abstract Some endophytic fungal genera in *Vitis vinifera*, including *Acremonium*, have been reported as antagonists of *Plasmopara viticola*. Endophytic *Acremonium* isolates from an asymptomatic grapevine cultivar Inzolia from Italy were identified by morphological features and multigene phylogenies of ITS, 18S and 28S genes, and their intra-specific genomic diversity was analyzed by RAPD analysis. Culture filtrates (CFs) obtained from *Acremonium* isolates were tested in vitro for their inhibitory activity against the *P. viticola* sporangia germination. Among 94 isolates, 68 belonged to the *Acremonium persicinum* and 26 to the *Acremonium sclerotigenum*. RAPD analysis grouped the *A. persicinum* isolates into 15 clusters and defined 31 different strains. The *A. sclerotigenum* isolates, instead, were clustered into 22 groups and represented 25 strains. All *A. persicinum* CFs inhibited sporangia germination of *P. viticola*, while not all those of *A. sclerotigenum* had inhibitory effect. A different degree of inhibition was observed between strains of the same species, while some strains of different species showed identical inhibitory effect. No correlation was found between RAPD groups and inhibitory activity in both *Acremonium* species.

Keywords Fungal endophytes · Phylogeny · RAPD · Inhibition · Sporangia germination · *Vitis vinifera*

Introduction

Fungal endophytes, both clavicipitaceous and non-clavicipitaceous groups, colonize plant tissues without causing visible disease symptoms and have a profound impact on plant communities (Petrini 1991; Arnold et al. 2003; Schulz and Boyle 2006; Sanchez Marquez et al. 2007; Hyde and Soytong 2008; Rodriguez et al. 2009). These microorganisms display a remarkable diversity, in fact, more than 100 endophytic *taxa* have been detected in some plant species (Stone et al. 2004). Endophytic fungi can also show host, organ and tissue specificity (Chapela et al. 1991; Bettucci et al. 1997; Mostert et al. 2000; Ragazzi et al. 2004; Schulz and Boyle 2006; Peršoh et al. 2010; Yuan et al. 2011).

Several studies have shown the ability of fungal endophytes to promote growth of their host plants (Rahman and Saiga 2005). Moreover, they can also protect their hosts from herbivores, insects and pathogens (Leuchtmann et al. 2000; Arnold and Lewis 2005; Rubini et al. 2005; Schulz and Boyle 2005). Endophytic microorganisms may play a key role in host-pathogen interactions even prior to the triggering of the disease. In particular, some endophytes can induce systemic resistance mechanisms in their hosts, as well as the expression of defence genes against some pathogens (Gwinn and Gavin 1992; Arnold et al. 2003). In addition, endophytic fungi may produce secondary metabolites active against pathogens, constituting an important potential source of biocontrol (Li et al. 2000; Tan and Zou 2001; Schulz et al. 2002; Kongue Tatong et al. 2014).

Several authors have investigated the diversity and ecological role of endophytic fungal communities in *Vitis vinifera* L.

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Published online: 30 April 2015

(von Tiedemann et al. 1988; Mostert et al. 2000; Casieri et al. 2009; González and Tello 2010). Among these communities, the genera *Acremonium*, *Alternaria*, *Epicoccum* and *Fusarium* have been shown to be effective as biocontrol agents of *Plasmopara viticola* (Berk. & Curtis) Berl. & De Toni (Falk et al. 1996; Kortekamp 1997; Bakshi et al. 2001; Assante et al. 2005; Musetti et al. 2006; Burruano et al. 2008). In this regard, *Acremonium* sp. strain A20, isolated from a leaf of asymptomatic grapevine cultivar Inzolia and identified wrongly as *Acremonium byssoides* W. Gams & G. Lim, has been reported as hyperparasite of *P. viticola* gamic and agamic structures (Burruano et al. 2008) and producer of secondary metabolites, named acremines (A-N), that inhibit sporangia germination (Assante et al. 2005; Arnone et al. 2008, 2009).

The aim of the present study was to identify a collection of endophytic *Acremonium* isolates from grapevine cv. Inzolia, to analyze the genetic variability among these isolates, and to evaluate their antagonistic activity towards *P. viticola* sporangia.

Materials and methods

Fungal isolates

A total of 94 endophytic fungal isolates belonging to the *Acremonium* genus, collected in 2008 from asymptomatic grapevine cv. Inzolia located in southern Italy (province of Palermo, Sicily), were used in this study (Burruano et al. 2008). Particularly, 58 isolates were from leaves, 20 from buds, 11 from shoots, four from petioles and one from seed (Table 1). All fungi were maintained on 2 % Malt Extract Agar (MEA; Oxoid, Milan, Italy) at 28 °C±1 °C.

Fungal identification and genetic variability

Morphological characterization

Single-conidial cultures of each isolate were obtained in 2 % MEA plates incubated at 28 °C±1 °C, alternating light and darkness (12 h of each), for 7–14 days up to 1 month. Identification at the species level of isolates was performed on the basis of their macroscopic (morphology, colour and growth rate of colony) and microscopic (size, colour, shape of conidia and phialides) features in culture, by comparison of above criteria with those given by Gams (1971) and Domsch et al. (1980, 2007). Microscopic characteristics were examined using a light microscope (Axioskop, Zeiss, Germany), coupled to an AxioCam MRc5 (Zeiss) digital camera. Images were captured using the software AxioVision 4.6 (Zeiss).

DNA extraction and PCR amplification

Single-conidial cultures were also used for DNA extraction. Genomic DNA was extracted from these pure cultures using a standard CTAB-based protocol (O'Donnell et al. 1998).

The internal transcribed spacer (ITS) regions, ITS1 and ITS2, and the 5.8S gene of the ribosomal DNA (rDNA) operon, were amplified using primers ITS1F (fungal specific: 5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns 1993) and ITS4 (universal: 5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). The amplification reaction was performed in a total reaction volume of 40 μl containing 50–100 ng of DNA template, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.3 μM of each primer, 0.5 U of Dream Taq (Fermentas, Milan, Italy) and 1× Dream Taq buffer (Fermentas). The amplification reaction was carried out in T1 Thermocycler (Biometra, Göttingen, Germany) under the following conditions: initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 20 s, extension at 72 °C for 1 min and final extension at 72 °C for 5 min.

To amplify the 18S rRNA gene (18S) and D1/D2 domains of the 28S of the nuclear rRNA gene (28S), the primer pairs NS1 (5'-GTAGTCATATGCTTGTCTC-3')/NS2 (5'-GGCTGCTGGCACCAGACTTGC-3') and NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3')/NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (White et al. 1990; O'Donnell 1993) were used, respectively. The reaction volume (50 μl) contained 50 ng of DNA template, 2 mM of $MgCl_2$, 0.25 mM of dNTPs, 0.2 μM of each primer, 1.5 U of Dream Taq (Fermentas) and 1× Dream Taq buffer (Fermentas). The amplification programs consisted of 94 °C for 8 min; 35 cycles of 94 °C for 40 s, 48 °C for 40 s, 72 °C for 45 s; 72 °C for 5 min for 18 rRNA gene, and of 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 50 °C for 30 s, 72 °C for 1 min; 72 °C for 10 min for the 28S rRNA gene. PCR products were analyzed by electrophoresis on 1.5 % (w/v) agarose gel in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) buffer. Gels were stained with SYBR Safe DNA gel stain (Invitrogen, Milan, Italy), visualized by UV transilluminator and acquired by a Gel Doc 1000 Video Gel Documentation System (BioRad, Richmond, V USA). Standard molecular markers were 1 kb Plus DNA Ladder (Invitrogen) and GeneRuler 100 bp Plus DNA Ladder (Fermentas).

Sequencing and phylogenetic analysis

PCR products were sequenced in both directions with the same primers used for amplification reported in the previous paragraph. Nucleotide sequences were compared to GenBank sequences through BLASTn searches (Altschul et al. 1997). Sequences were read and edited by using the Sequencher software (Version 4.7, Gene Codes Corporation, Ann Arbor, MI),

Table 1 Isolation tissue and nucleotide sequences deposited in GenBank of endophytic *Acremonium* isolates from grapevine cv. Inzolia and their inhibitory activity against the *Plasmopara viticola* sporangia germination

Strain code	Species	Tissue	GenBank Accession no.			Inhibition sporangia germination	
			ITS	18S	28S	50 % CFs	25 % CFs
A1	*A. persicinum*	leaf	KP720661	KP720755	KP720849	92.30	87.63
A2	*A. persicinum*	leaf	KP720662	KP720756	KP720850	95.44	86.21
A3	*A. persicinum*	leaf	KP720663	KP720757	KP720851	96.48	84.73
A4	*A. persicinum*	leaf	KP720664	KP720758	KP720852	94.64	82.98
A7	*A. persicinum*	leaf	KP720665	KP720759	KP720853	92.78	79.77
A8	*A. persicinum*	leaf	KP720666	KP720760	KP720854	87.94	78.03
A9	*A. persicinum*	leaf	KP720667	KP720761	KP720855	86.77	77.72
A10	*A. persicinum*	leaf	KP720668	KP720762	KP720856	86.47	71.18
A11	*A. persicinum*	bud	KP720669	KP720763	KP720857	85.10	71.18
A12	*A. persicinum*	leaf	KP720670	KP720764	KP720858	84.00	68.62
A16	*A. persicinum*	leaf	KP720671	KP720765	KP720859	83.31	68.19
A18	*A. persicinum*	leaf	KP720672	KP720766	KP720860	80.88	68.81
A20	*A. persicinum*	leaf	KP720673	KP720767	KP720861	92.97	70.48
A21	*A. persicinum*	bud	KP720674	KP720768	KP720862	78.83	66.08
A22	*A. persicinum*	leaf	KP720675	KP720769	KP720863	79.08	66.51
A23	*A. persicinum*	leaf	KP720676	KP720770	KP720864	75.95	64.44
A24	*A. persicinum*	leaf	KP720677	KP720771	KP720865	77.08	68.06
A25	*A. persicinum*	leaf	KP720678	KP720772	KP720866	74.17	56.40
A26	*A. persicinum*	leaf	KP720679	KP720773	KP720867	67.59	67.68
A27	*A. persicinum*	leaf	KP720680	KP720774	KP720868	56.17	49.48
A29	*A. persicinum*	leaf	KP720681	KP720775	KP720869	95.20	59.49
A30	*A. persicinum*	leaf	KP720682	KP720776	KP720870	95.95	50.40
A32	*A. persicinum*	leaf	KP720683	KP720777	KP720871	79.01	64.56
A33	*A. persicinum*	bud	KP720684	KP720778	KP720872	31.09	14.83
A34	*A. persicinum*	leaf	KP720685	KP720779	KP720873	83.68	74.80
A35	*A. persicinum*	leaf	KP720686	KP720780	KP720874	86.68	79.27
A36	*A. persicinum*	leaf	KP720687	KP720781	KP720875	91.58	87.13
A38	*A. persicinum*	leaf	KP720688	KP720782	KP720876	96.14	85.01
A42	*A. persicinum*	leaf	KP720689	KP720783	KP720877	95.55	83.49
A43	*A. persicinum*	leaf	KP720690	KP720784	KP720878	87.17	66.77
A44	*A. persicinum*	leaf	KP720691	KP720785	KP720879	87.94	61.55
A45	*A. persicinum*	leaf	KP720692	KP720786	KP720880	82.04	71.71
A47	*A. persicinum*	bud	KP720693	KP720787	KP720881	94.16	81.57
A48	*A. persicinum*	leaf	KP720694	KP720788	KP720882	73.59	60.76
A49	*A. persicinum*	leaf	KP720695	KP720789	KP720883	30.88	0.12
A50	*A. persicinum*	leaf	KP720696	KP720790	KP720884	48.86	47.11
A52	*A. persicinum*	leaf	KP720697	KP720791	KP720885	95.95	81.90
A53	*A. persicinum*	leaf	KP720698	KP720792	KP720886	0.00	79.36
A54	*A. persicinum*	leaf	KP720699	KP720793	KP720887	85.37	68.16
A55	*A. persicinum*	leaf	KP720700	KP720794	KP720888	86.79	87.00
A56	*A. persicinum*	leaf	KP720701	KP720795	KP720889	91.78	82.56
A60	*A. persicinum*	bud	KP720702	KP720796	KP720890	89.61	78.60
A62	*A. persicinum*	leaf	KP720703	KP720797	KP720891	87.90	76.22
A64	*A. persicinum*	leaf	KP720704	KP720798	KP720892	88.21	73.27
A66	*A. persicinum*	leaf	KP720705	KP720799	KP720893	86.69	69.73
A67	*A. persicinum*	bud	KP720706	KP720800	KP720894	82.32	69.10
A68	*A. persicinum*	leaf	KP720707	KP720801	KP720895	87.04	68.63

Table 1 (continued)

Strain code	Species	Tissue	GenBank Accession no.			Inhibition sporangia germination	
			ITS	18S	28S	50 % CFs	25 % CFs
A70	*A. persicinum*	leaf	KP720708	KP720802	KP720896	82.59	67.27
A72	*A. persicinum*	leaf	KP720709	KP720803	KP720897	78.43	67.90
A73	*A. persicinum*	leaf	KP720710	KP720804	KP720898	77.40	66.74
A74	*A. persicinum*	leaf	KP720711	KP720805	KP720899	72.46	57.93
A75	*A. persicinum*	leaf	KP720712	KP720806	KP720900	51.24	56.65
A76	*A. persicinum*	bud	KP720713	KP720807	KP720901	51.19	51.19
A77	*A. persicinum*	leaf	KP720714	KP720808	KP720902	30.43	14.38
A78	*A. persicinum*	leaf	KP720715	KP720809	KP720903	91.62	80.23
A79	*A. persicinum*	leaf	KP720716	KP720810	KP720904	88.64	78.27
A80	*A. persicinum*	bud	KP720717	KP720811	KP720905	87.36	77.26
A81	*A. persicinum*	leaf	KP720718	KP720812	KP720906	87.68	72.30
A82	*A. persicinum*	leaf	KP720719	KP720813	KP720907	86.12	69.49
A83	*A. persicinum*	leaf	KP720720	KP720814	KP720908	82.76	69.20
A84	*A. persicinum*	leaf	KP720721	KP720815	KP720909	82.08	69.47
A85	*A. persicinum*	leaf	KP720722	KP720816	KP720910	82.08	67.84
A86	*A. persicinum*	bud	KP720723	KP720817	KP720911	77.91	67.63
A87	*A. persicinum*	leaf	KP720724	KP720818	KP720912	78.08	69.81
A88	*A. persicinum*	bud	KP720725	KP720819	KP720913	76.72	66.40
A89	*A. persicinum*	leaf	KP720726	KP720820	KP720914	73.74	56.65
A90	*A. persicinum*	leaf	KP720727	KP720821	KP720915	57.34	50.01
A91	*A. persicinum*	leaf	KP720728	KP720822	KP720916	30.65	14.74
A5	*A. sclerotigenum*	shoot	KP720729	KP720823	KP720917	0.00	0.00
A6	*A. sclerotigenum*	petiole	KP720730	KP720824	KP720918	0.00	0.00
A13	*A. sclerotigenum*	bud	KP720731	KP720825	KP720919	83.85	75.53
A14	*A. sclerotigenum*	bud	KP720732	KP720826	KP720920	18.78	0.00
A15	*A. sclerotigenum*	bud	KP720733	KP720827	KP720921	40.21	15.24
A17	*A. sclerotigenum*	shoot	KP720734	KP720828	KP720922	0.00	2.25
A19	*A. sclerotigenum*	bud	KP720735	KP720829	KP720923	36.78	0.00
A28	*A. sclerotigenum*	shoot	KP720736	KP720830	KP720924	90.71	77.26
A30/05	*A. sclerotigenum*	bud	KP720737	KP720831	KP720925	7.27	0.00
A31	*A. sclerotigenum*	shoot	KP720738	KP720832	KP720926	30.08	25.49
A37	*A. sclerotigenum*	shoot	KP720739	KP720833	KP720927	79.89	44.23
A39	*A. sclerotigenum*	shoot	KP720740	KP720834	KP720928	91.78	88.04
A40	*A. sclerotigenum*	shoot	KP720741	KP720835	KP720929	50.22	48.65
A41	*A. sclerotigenum*	bud	KP720742	KP720836	KP720930	90.67	62.18
A46	*A. sclerotigenum*	petiole	KP720743	KP720837	KP720931	98.33	94.86
A48/05	*A. sclerotigenum*	bud	KP720744	KP720838	KP720932	35.80	20.08
A50/05	*A. sclerotigenum*	shoot	KP720745	KP720839	KP720933	45.16	40.94
A51	*A. sclerotigenum*	bud	KP720746	KP720840	KP720934	86.95	51.96
A57	*A. sclerotigenum*	petiole	KP720747	KP720841	KP720935	16.38	0.00
A58	*A. sclerotigenum*	bud	KP720748	KP720842	KP720936	92.77	86.27
A59	*A. sclerotigenum*	seed	KP720749	KP720843	KP720937	98.39	94.32
A61	*A. sclerotigenum*	shoot	KP720750	KP720844	KP720938	48.43	33.76
A63	*A. sclerotigenum*	bud	KP720751	KP720845	KP720939	73.52	69.18
A65	*A. sclerotigenum*	petiole	KP720752	KP720846	KP720940	2.34	0.00
A69	*A. sclerotigenum*	shoot	KP720753	KP720847	KP720941	0.00	0.00
A71	*A. sclerotigenum*	shoot	KP720754	KP720848	KP720942	72.42	25.36

aligned by Clustal W (Geneious v. 6.1.6; Biomatters Ltd.) and manual adjustments of alignments were made where necessary. The final phylogenetic tree was based on a combined alignment of the ITS, 18S and 28S genes. Phylogenetic analyses were conducted by MEGA5 (v.5.2.1; The Biodesign Institute) and the neighbor-joining method was used. Evolutionary distances were calculated via the Kimura 2-parameter method (Kimura 1980). Bootstrap values were inferred from 1,000 replicates (Felsenstein 1985). For comparison, additional sequences (ITS, partial 18S and 28S genes, respectively) were selected from GenBank to be included in the alignment, as follows: *Acremonium persicinum* (Nicot) W. Gams CBS 310.59 (FN706554, HQ232201, HQ232077), *Acremonium sclerotigenum* (Moreau & R. Moreau ex Valenta) W. Gams CBS 124.42 (FN706552, HQ232209, HQ232126), *Acremonium spinosum* (Negroni) W. Gams CBS 136.33 (HE608637, HQ232210, HQ232137), *Sarocladium kiliense* (Grütz) Summerbell CBS 122.29 (AJ621775, HQ232198, HQ232052) and *Sarocladium strictum* (W. Gams) Summerbell CBS 346.70 (GQ376096, HQ232211, HQ232141). Tree was rooted with *Acremonium curvulum* W. Gams CBS 430.66 (HE608638, HQ232188, HQ232026) as outgroup strain.

RAPD analysis

Genomic DNA was analyzed by random amplified polymorphic DNA (RAPD) analysis. The decamer primers (Invitrogen) OPA03 (5'-AGTCAGCCAC-3'), OPA05 (5'-AGGGGTCTTG-3') and OPA07 (5'-GAAACGGGTG-3') were individually added to a reaction mixture (final volume 25 μl) containing 10 ng of template DNA, 100 μM of each dNTP, 0.4 μM of primer, 1 U of Dream Taq (Fermentas) and 1× Dream Taq buffer (Fermentas). Amplifications were performed by means of T1 Thermocycler (Biometra, Göttingen, Germany) programmed as follows: an initial step at 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 5 min. To ensure reproducibility, all RAPD reactions were performed twice based on two different DNA extractions. RAPD profiles were separated by electrophoresis on 1.5 % (w/v) agarose gel in 1× TBE buffer and visualized as above. Gel images were analyzed using GelCompar II software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). For comparison of RAPD-PCR patterns, cluster analysis was performed using the Dice similarity coefficient with a 1.5 % band matching tolerance, and the dendrograms were generated by means of the Unweight Pair Group Method with Arithmetic averages (UPGMA) method.

Analysis of antagonistic activity

Culture filtrates

All *Acremonium* isolates were grown at 28 °C in the dark for 3 weeks in Erlenmeyer flasks each containing 100 ml of Malt Extract Broth 2 % (MEB, Oxoid). CFs were obtained by sterile-filtering the cultures in a vacuum on a 500 ml Stericup (0.22 μm HV Durapore membrane, Millipore Co., Bedford, MA, USA) and stored at 4 °C.

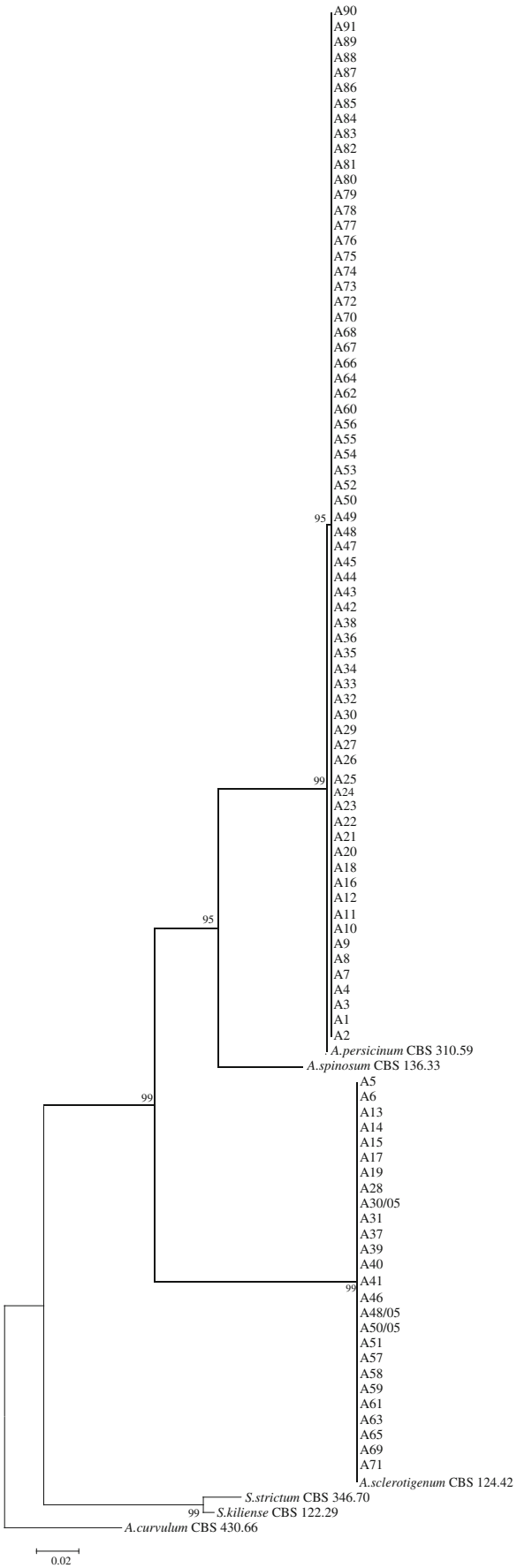
In vitro growth of P. viticola

A *P. viticola* isolate from a naturally infected leaf of cv. Inzolia was grown in the laboratory by transferring it from leaf to leaf of grapevine. In particular, healthy leaves were surface-sterilized in 5 % (v/v) NaClO for 5 min, rinsed in sterile distilled water, dried and placed into Petri dishes (9 cm diam) containing 5 ml of sterile distilled water, with the lower leaf surface facing up. The inoculum, consisting in a suspension of *P. viticola* sporangia prepared by shaking sporulating lesions of infected leaves in distilled sterile water, was maintained at 20±2 °C until zoospore liberation. Ten drops of the suspension were placed separately in the spaces between leaf veins and removed the following day. Inoculated leaves were exposed to daylight and kept at 20±2 °C until the evasion of pathogen (Conigliaro et al. 1996).

In vitro inhibition of P. viticola sporangia germination with Acremonium culture filtrates

The inhibition of *P. viticola* sporangia germination was conducted with the CFs diluted to half (50 %) and a quarter (25 %) of the initial concentration in a *P. viticola* sporangia suspension freshly prepared in distilled sterile water (10^4 ml^{-1}). Controls were prepared with sporangia suspensions in MEB half and one quarter diluted with water. The bioassay was performed in multi-well plates (100 μl per well), using three wells per treatment. Multi-well plates were then maintained at 20±2 °C and, after 2 h (Conigliaro and Burruano 2001), sporangia germination was halted by the addition of Amman's lactophenol (10 g phenol crystal, 10 ml lactic acid, 20 ml glycerol and 10 ml water).

Germination of *P. viticola* sporangia was observed by light microscopy, distinguishing germinated (empty) and non-germinated (degenerated) sporangia. Germination percentage was determined by comparing the number of germinated sporangia to the 100 propagules analyzed in each replicate. Two independent bioassays were performed, scoring a total of 600 sporangia per treatment.



◀ **Fig. 1** Phylogenetic analysis of *Acremonium* isolates based on ITS, 18S and 28S sequence data, using the neighbor-joining statistical method. Bootstrap support values are reported at the nodes. Sequences obtained from GenBank are indicated by their CBS number, while isolates obtained in this study by their strain code

In order to evaluate the inhibitory effect of CFs on the germination of *P. viticola* sporangia, the following formula (Rocha et al. 2011) was used:

$$\%\ inhibition = \left[1-\left(T\middle/C\right)\right] \times 100$$

where T is the mean percentage of sporangia germination in test wells and C is the mean percentage of germination in control wells for each *Acremonium* isolate. In particular, each 50 % culture filtrate (CF) was compared with 50 % MEB, while 25 % CF with 25 % MEB.

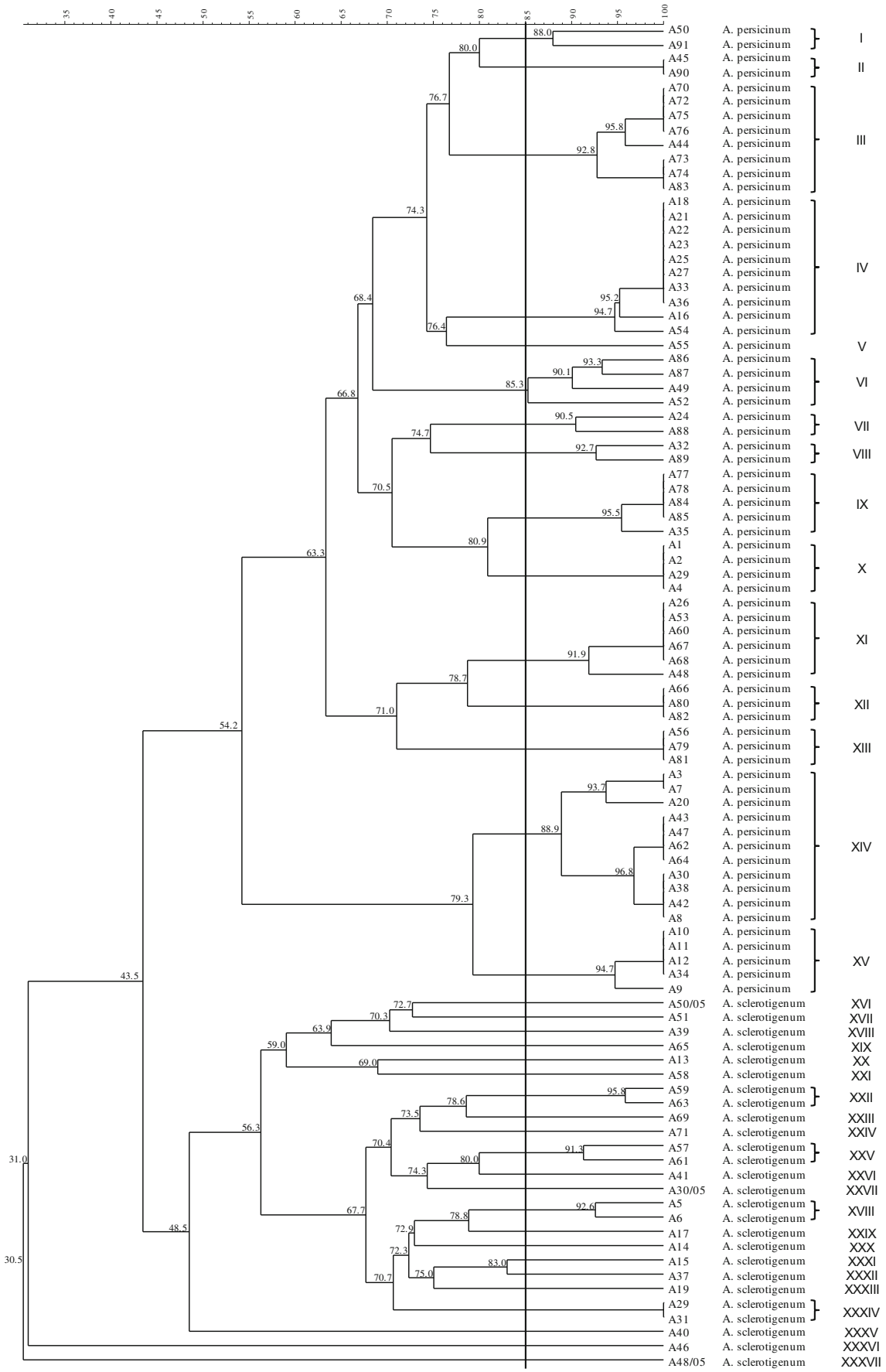
The inhibitory activity of the CFs on *P. viticola* germination was rated into five classes: no activity (0 to 4 % inhibition); weak activity (5 to 20 %); low activity (21 to 50 %); medium activity (51 to 80 %); high activity (81 to 100 %).

Data relating to antagonistic activity of *Acremonium* isolates were subjected to a multivariate analysis to simultaneously consider the variables of the inhibition value of 50 % and 25 % CFs. The approach for grouping *Acremonium* isolates into homogeneous groups according to their overall activities was achieved by hierarchical clustering analysis. The analysis was performed with the average quantitative activity data of the 94 *Acremonium* isolates assayed in this work. Statistical data elaboration was carried out by Statistica (data analysis software system), version 10 StatSoft, Inc. (2011, Tulsa, OK, USA).

Results

Morphological characterization

All 94 *Acremonium* isolates were initially clustered into two morphological groups according to their appearance in culture and microscopic features. A group of 68 isolates produced colonies with fluffy mycelium, initially white becoming light brown with age. Microscopic observations showed erect phialides, smooth-walled, 4.7–26.7 μm long, gradually tapering from 0.7–1.0 μm at the base to 0.2–0.5 μm. Conidia were one-celled, hyaline, globose, with dimensions of 1.2–2.3 × 1.2–2.3 μm. Based on these features, isolates were identified as *A. persicinum*. The other group of 26 isolates showed colonies with whitish and floccose mycelium. Phialides were simple, erect from the substratum, with a chromophilic septum at the base, 9.8–47.5 μm long,



◀ **Fig. 2** Dendrogram obtained by combination of RAPD profiles generated with the primers OPA03, OPA05 and OPA07 from *Acremonium* isolates. The scale indicates the Dice similarity coefficient

tapering from 1.2–1.7 μm to 0.5–1.0 μm. Conidia were one-celled, hyaline, cylindrical, straight or curved, arranged in slimy heads, with dimensions of 3.0–5.0 × 0.7–1.8 μm. Yellowish sclerotia, formed within the agar, were globose with diameter of 30–60 μm. These isolates were identified as *A. sclerotigenum*.

Phylogenetic analysis

Nucleotide sequences of ITS, 18S and 28S genes of all *Acremonium* isolates assayed were deposited in GenBank (Table 1). The alignment of the sequences generated in this study revealed a high similarity with reference sequences from GenBank. In particular, 68 sequences showed 100 % identity with the following sequences of *A. persicinum*: ITS (KP131528), 18S (HQ232201), 28S (HQ232076). The remaining 26 sequences resulted identical to *A. sclerotigenum*: ITS (KJ194115 with 100 % identity), 18S (KJ194117 with 100 % identity), 28S (HQ232134 with 99 % identity). The combined tree of ITS, 18S and 28S genes showed two main clades (Fig. 1). The first included the 68 *A. persicinum* isolates and the isotype strain of *A. persicinum*, the second was formed by remaining *A. sclerotigenum* isolates, including the type strain of *A. sclerotigenum*. Moreover, for both species, no genetic variation among our isolates was observed. The topology of the combined tree was no similar to those observed in the trees of individual genes analyzed (data not shown). In fact, the sequences of 68 isolates of *A. persicinum* were identical to that of the isotype strain of *A. persicinum* only for the ITS region and 18S gene, while differed for four nucleotides in the 28S gene. By contrast, the our *A. sclerotigenum* sequences were identical to that of the *A. sclerotigenum* type strain in each of the three loci studied. Bootstrap values showed strong support for all branches of the combined tree.

RAPD analysis

All three primers successfully amplified genomic DNA from all *Acremonium* analyzed. Each primer generated different profiles characterized by 1 to 15 reproducible bands, with a molecular size in the range of 200–3000 bp, approximately. The resulting dendrogram (Fig. 2) showed two main clusters for the two *Acremonium* species. The first group included the isolates belonging to *A. persicinum* species. The second main cluster contained all *A. sclerotigenum* isolates, except isolates A46 and A48/05. At a Dice coefficient of 85 %, the 68 *A. persicinum* isolates were found to represent 31 different strains and were clustered into 15 groups (I-XV). At the same similarity level, the 26 *A. sclerotigenum* cultures, represented by 25 strains, were grouped into 22 clusters (XVI-XXXVII).

In vitro activity against P. viticola

The inhibitory activity of all CFs of *Acremonium* isolates against sporangia germination of *P. viticola* is reported in Table 1. Forty-four of the 68 *A. persicinum* 50 % CFs inhibited germination of *P. viticola* sporangia above 80 %, seventeen exhibited an inhibitory effect between 51 and 80 %, and seven between 21 % and 50 %. Regarding the 26 *A. sclerotigenum* 50 % CFs, eight had antagonistic effect over 80 %, only four between 51 % and 80 %, six between 21 and 50 %, three between 5 and 20 %, and five had no activity (Table 2). The bioassays carried out with 25 % CFs showed a slight decrease of inhibitory activity of isolates belonging to both species of *Acremonium*. Considering the *A. persicinum* CFs, twelve maintained a high inhibitory effect, fifty-two and four showed medium and weak activity, respectively. About the *A. sclerotigenum* CFs, five high inhibition, six medium, six low, four weak, and five exhibited no inhibitory activity (Table 2).

Based on the cluster analysis results, two isolates, A53 and A71, belonging to *A. persicinum* and *A. sclerotigenum*, respectively, resulted different from the others, which, instead, clustered in two main groups (Fig. 3). In particular, only A53 25 % CFs inhibited germination sporangia, while no antagonistic activity was observed for the A53 50 % CF. By contrast, the A71 50 % CF showed an inhibition three times greater than the A71 25 %-CF. The first cluster grouped isolates (A31, A91, A77, A33, A48/05, A15, A49, A19, A57, A14, A30/05, A65, A17, A69, A6, A5) with a 26 % similarity to the relative linkage distance. All the remaining isolates were clustered together according to a relative linkage distance below 31 %.

Discussion

In this paper we report the identification, based on morphological features and multigene phylogenies of ITS, 18S and 28S genes, of endophytic *A. persicinum* and *A. sclerotigenum* isolates from asymptomatic grapevine cv. Inzolia from Sicily. In particular, the strain A20, previously identified as *A. byssoides* (Burruano et al. 2008), was here identified as *A. persicinum* on the basis of DNA sequence data analysis. For *A. sclerotigenum*, genetic variation among our isolates and the type strain was not observed, while the sequences of our *A. persicinum* isolates were not identical to that of isotype strain. These differences suggest a possible intraspecific variation, as already reported in the *Acremonium* genus (Giraldo et al. 2014).

Cluster analysis of RAPD patterns obtained by using three primers allotted all *Acremonium* isolates to two main groups,

Table 2 *Acremonium persicinum* and *Acremonium sclerotigenum* inhibitory activity on *Plasmopara viticola* sporangia germination: number of culture filtrates for each inhibition class

Inhibition class	*Acremonium persicinum*		*Acremonium sclerotigenum*	
	50 %-CFs	25 %-CFs	50 %-CFs	25 %-CFs
No activity	0	0	5	5
Weak activity	0	4	3	4
Low activity	7	0	6	6
Medium activity	17	52	4	6
High activity	44	12	8	5

that allowed to clearly distinguish *A. persicinum* from *A. sclerotigenum*. RAPD analysis also showed a high genetic variability within both *Acremonium* species, especially *A. sclerotigenum*. In fact, two strains of *A. sclerotigenum* were not grouped in either of the two main clusters, because they showed a similarity level considerably lower than that noted for all other *A. sclerotigenum* strains. This fact is not surprising, since, as reported by other authors (Martínez-Culebras et al. 2004; Al-Wadai et al. 2013), strains belonging to the same species could show a high level of genetic diversity. Furthermore, Santos et al. (2010) clearly showed that isolates of the ascomycete fungus *Diaporthe* Nitschke, obtained from single ascospores belonging to the same perithecium, presented high numbers of nucleotide substitution in ITS region. Thus, it's possible to assume that results obtained by genotypic strain typing of anamorphic fungi reflect a high variability.

The species *A. persicinum* has been reported as forest soil fungus in Iran (Sarookhani and Moazzami 2007), but never as endophytic fungus. The species *A. sclerotigenum* is known to be a common soil fungus, but its presence as endophyte has only been described in *Quercus ilex* (Collado et al. 1999). Among other *Acremonium* species, *Acremonium charticola* (Lindau) W. Gams and *Acremonium ochraceum* (Onions & G.L. Barron) W. Gams have been indicated as grapevine endophytes in South Africa (Halleen et al. 2007). To the best of our knowledge, this is the first report of *A. persicinum* and *A. sclerotigenum* as endophytes in grapevine.

Moreover, this work reports the antagonism of *A. persicinum* and *A. sclerotigenum* towards *P. viticola*. Our results indicate that all the *A. persicinum* strains showed inhibitory activity on *P. viticola* sporangia germination, unlike *A. sclerotigenum*. For both species, the antibiosis decreased with CF dilution. The hierarchical clustering analysis proved to be useful tool in the management of the large amount of data generated in this study in relation to the different inhibitory effect of *Acremonium* CFs. Moreover, this analysis

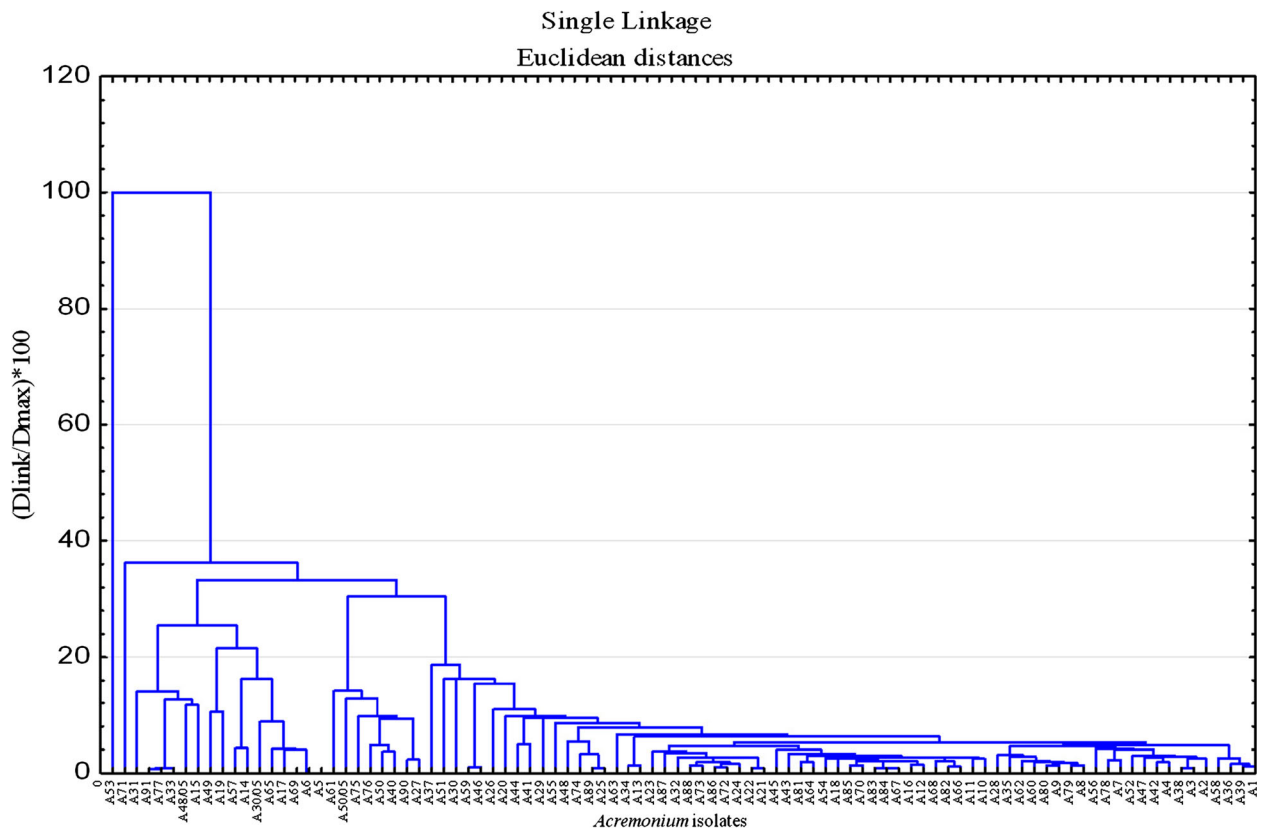


Fig. 3 Hierarchical clustering analysis grouping the *Acremonium* isolates according to their inhibitory activity toward germination of *Plasmopara viticola* sporangia

indicated that strains belonging to different species showed identical inhibitory activity, as A13 and A34, belonging to *A. sclerotigenum* and *A. persicinum*, respectively. The species *A. persicinum* has been described as inhibitor of uredospores germination in *Puccinia arachidis* Speg. (Ghewande 1990), while *A. sclerotigenum* has never been reported as an antagonist. However, antifungal action has been described for other *Acremonium* species such as *Acremonium implicatum* (J.C. Gilman & E.V. Abbott) W. Gams against *Ascochyta rabiei* (Pass.) Labr. (Rajakumar et al. 2005) and *Acremonium strictum* W. Gams towards *Magnaporthe grisea* (T.T. Hebert) M.E. Barr, *Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker, *Botrytis cinerea* Pers. (Kim et al. 2002) and *Colletotrichum musae* (Berk. & M.A. Curtis) Arx (Ragazzi and Turco 1997). To our knowledge, we reported for the first time *A. persicinum* and *A. sclerotigenum* as antagonists of *P. viticola*.

The different degree of inhibition on *P. viticola* sporangia germination, observed between strains of the same species, was not correlated to RAPD groups. Moreover, the identical inhibitory activity detected between strains belonging to different species suggests that the antagonistic activity of both species could be strain specific rather than species specific. This variability observed within both species emphasizes the importance of future analyses to determine the genes involved in biosynthesis of substances active in the antagonistic process.

In conclusion, our results suggest a potential role of *A. persicinum* and *A. sclerotigenum*, endophytes in Sicilian asymptomatic grapevines, as biocontrol agents of *P. viticola*. Further studies are needed to identify and characterize the secondary metabolites active in the antagonism produced by the strains of *A. persicinum* and *A. sclerotigenum* tested in this study. In this respect, only from *A. persicinum* strain A20, formerly named *A. byssoides* A20, twelve novel secondary metabolites (acremines A-N), involved in the inhibition of *P. viticola* sporangia germination, have been identified and characterized (Assante et al. 2005; Arnone et al. 2008, 2009). Moreover, in vivo tests are required to confirm the suitability of these organisms or their metabolites in the biocontrol of *P. viticola* in field.

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