

Identification and characterization of *Alternaria* species causing leaf spot on cabbage, cauliflower, wild and cultivated rocket by using molecular and morphological features and mycotoxin production

Ilenia Siciliano · Giovanna Gilardi · Giuseppe Ortu ·
Ulrich Gisi · Maria Lodovica Gullino ·
Angelo Garibaldi

Accepted: 22 February 2017
© Koninklijke Nederlandse Planteziektenkundige Vereniging 2017

Abstract *Alternaria* species are common pathogens of fruit and vegetables able to produce secondary metabolites potentially affecting human health. Twenty-nine isolates obtained from cabbage, cauliflower, wild and cultivated rocket were characterized and identified based on sporulation pattern and virulence; the phylogenetic analysis was based on the β -tubulin gene. Isolates were identified as *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. brassicicola* and *A. japonica*. Pathogenicity was evaluated on plants under greenhouse conditions. Two isolates showed low level of virulence on cultivated rocket while the other isolates showed medium or high level of virulence. Isolates were also characterized for their mycotoxin production on a modified Czapek-Dox medium. Production of the five *Alternaria* toxins, tenuazonic acid, alternariol, alternariol monomethyl ether, altenuene and tentoxin were evaluated. Under these conditions, about 80% of the isolates showed the ability to produce at least one mycotoxin.

Keywords Crucifers · Toxins · Leaf spot · Tenuazonic acid

Introduction

Most *Alternaria* species are saprophytes and ubiquitous in the environment, however some are plant pathogenic, inducing diseases on a large variety of economically important crops like cereals, oil-crops, vegetables and fruits (Pitt and Hocking, 1997). Most *Alternaria* spp. produce chains of conidia with transverse and longitudinal septa with a tapering apical cell. Conidial size, presence and size of a beak, the pattern of catenation and longitudinal and transverse septation are key taxonomic features for this genus (Joly 1964; Ellis 1971 and 1976, Simmons 1992). Simmons (1992) proposed different species groups each with a typified representative, e.g. the *Alternata* group with small, catenate spores, while the *porri* species-group has large, long-beaked, non-catenate spores. Other species-groups include the *brassicicola*, the *cheiranthi*, the *infectoria*, and the *tenuissima* group (Simmons 1995, Woudenberg et al. 2015). In addition, some *Alternaria* spp. have a clinical significance producing toxic secondary metabolites that are involved in cancer development in mammals (Ostry 2008). *Alternaria* toxins can be divided into five different chemical classes: dibenzo- α -pyrones; tetramic acid derivatives; perylenes; AAL-toxins (abbreviation for *A. alternata* f. sp. *lycopersici* toxins) and a class containing miscellaneous structures. *A. alternata* in

I. Siciliano (✉) · G. Gilardi · G. Ortu · U. Gisi ·
M. L. Gullino · A. Garibaldi
Agroinnova – Centre of Competence for the Innovation in the
Agro-Environmental Sector, University of Turin, Largo Paolo
Braccini 2, 10095 Grugliasco, Turin, Italy
e-mail: ilenia.siciliano@unito.it

M. L. Gullino
DISAFA - Dipartimento di Scienze Agrarie, Forestali e
Alimentari, University of Turin, Largo Paolo Braccini 2, 10095
Grugliasco, Turin, Italy

particular is also known as a human pathogen in immunocompromised patients (Rossman et al. 1996). Furthermore, *Alternaria* spores are well known as one of the most important airborne allergens (Thomma 2003). *Alternaria* diseases of crucifers are mainly caused by two species, *A. brassicicola*, and *A. brassicae*, and occasionally also by *A. alternata* (Kohl et al. 2010). Depending on the species involved, the symptoms on crucifers are referred to as black, grey or dark leaf spot. Brassica hosts can be affected in all stages of growth and typical symptoms include black necrotic lesions surrounded by chlorotic areas on seedlings, leaves, stems and siliquae (Neergaard 1945; Humpherson-Jones and Maude 1982; Humpherson-Jones 1989; Mac Kinon et al. 1999). Spots on leaf caused by *A. brassicicola* are generally similar to those caused by *A. brassicae* except that the lesions are gray-black in colour (Kolte and Awasthi 1987; Verma and Saharan, 1994). Spots can vary in size from 1.1 to 3.0 mm dark circular spots, when young, to black, brown or tan spots from 5 to 7.5 cm when older (Mac Kinon et al. 1999; Singh et al. 2012). Yellow halos may or may not surround leaf lesions. Larger spots may have a dark green-black coloration of fuzzy growth in the spots, usually concentrated in the center (Neergaard 1945). *A. japonica* has been reported on cultivated and wild rocket (Garibaldi et al. 2011), mainly as seed infection, causing reduced germination and seedling vigour, in addition to pre- and post-emergence damping-off (Gilardi et al. 2015). Symptoms caused by *A. japonica* on wild and cultivated rocket are usually black-brown lesions, 1 to 30 mm in diameter, which progressively turned black. Lesions usually start on the upper side of older leaves at the leaf margins and tips and developed a yellow halo. Eventually, lesions also affect leaf veins and stems (Garibaldi et al. 2011). However, *A. japonica* is not as widespread as *A. brassicicola* (CABI 2007; Humpherson-Jones 2007).

The present work was aimed at studying the variability within *Alternaria* isolates originating from different *Brassica* host plants and contaminated seeds, by using molecular and morphological characteristics, pathogenicity, virulence and mycotoxin production.

Materials and methods

Isolates collection

Alternaria spp. were isolated from diseased wild and cultivated rocket and *Brassica* (cabbage, cauliflower)

plants (15 isolates) and infected seeds (14 isolates) listed in Tables 1 and 2. *Alternaria* isolates were obtained by plating infected tissue (from wild and cultivated rocket, cabbage and cauliflower) onto Potato Dextrose Agar (PDA) medium, incubated at 25 °C for 7 days. Isolates from seeds were obtained by testing different seed samples; 400 seeds per seed sample were placed on Petri plates (10 seeds/plate) as described by Maude and Humpherson-Jones (1980). Isolations were made from seeds either non disinfected or surface disinfected for 1 min in 1% sodium hypochlorite, washed in sterile water for 5 min and dried under a sterile hood. The Petri dishes were incubated at 22 °C in 12 h light and 12 h darkness at 75% R.H. for 7–10 days. The fungal colonies developing from seeds, morphologically identified as *Alternaria* sp. were transferred from Potato Dextrose Agar to Potato Carrot Agar (PCA).

After incubation, the isolates were transferred onto Potato Carrot Agar (PCA) medium amended with 0.5 mg/ml streptomycin sulphate, and incubated for 7 days at 25 °C. Single-spore cultures were prepared for each isolate by dilution of conidial suspension; about 50 conidia were plated on PDA medium. Germinated conidia were picked under the stereomicroscope and transferred again to PDA plates. The different isolates were maintained on PDA at 8 °C. Isolates were re-grown on PCA at 25 °C for 10 days immediately before being tested for their pathogenicity/virulence.

Pathogenicity assay in host plants

The virulence of isolates, obtained from leaves and seeds, was tested on the host plants of origin. The plants, grown in pots, were inoculated, when 30–40 day-old, by spraying leaves with a conidial suspension at 1.5×10^5 conidia/ml. Inoculated plants were covered with plastic bags for 5–7 days and kept in greenhouse and/or in growth chambers at 25 ± 1 °C. Ten to fifteen days after artificial inoculation, the percentage of infected leaf area of ten leaves per plant was estimated by using a disease index scale ranging from 0 to 100. Isolates tested were classified as non-pathogenic (NP); low virulent: 10–30% leaf necrosis (L); moderately virulent 31–60% leaf necrosis (M); and highly virulent: 61–100% (H) (Van der Waals et al. 2004).

DNA extraction

DNA extraction was carried out using the NucleoSpin Plant kit (Macherey-Nagel GmbH and Co., Duren, DE,

Table 1 Isolates of *Alternaria* spp. obtained from leaves of different hosts in northern Italy with accession numbers of β -tubulin gene deposited on Genbank

Isolate code	Host	Farm of origin, province	Accession number
Cav 2/10	Cauliflower cv. White excell	CReSO-Experimental center, Boves (CN)	KT920427
Cav 3/10	Cabbage cv. Morama	Pagliero- Moncalieri(TO)	KT920426
Cav 5/10	Cabbage cv.Estoryl	Porcellana (AT)	KT920423
Cav. 7/10	Cabbage cv. Morama	Sacchetto G.-Savigliano (CN)	KT920425
Cav. 12/10	Cauliflower cv. White excell	Dutto-Giordanengo-Boves (CN)	KT920424
Cav. 15/10	Cabbage cv. Dama	CReSO-Experimental center, Boves (CN)	KT920428
Ruc 1/10	Cultivated rocket	Berruto-tunnel 1Moncalieri (TO)	KJ909926
Ruc 2/10	Cultivated rocket	Bagnolo (TO)	KT920415
Ruc 4/10	Wild rocket cv. Frastagliata	Albenga (SV)	KT920413
Ruc 5/10	Cultivated rocket	Berruto-tunnel 1, Moncalieri (TO)	KT920412
Ruc 6/10	Cultivated rocket	Berruto-tunnel 3, Moncalieri (TO)	KT920414
Ruc 9/10	Cultivated rocket	Berruto-tunnel 8, Moncalieri (TO)	KT920411
Ruc 12/10	Cultivated rocket	Berruto-tunnel 8, Moncalieri (TO)	KT920417
Ruc 13/10	Cultivated rocket	Berruto-tunnel 8, Moncalieri (TO)	KT920416
Ruc 1/11	Wild rocket	Berruto, Moncalieri (TO)	KJ909927

CN Cuneo province, TO Torino province, AT Asti province

USA), according to the manufacturer's instructions. Fresh mycelium was scraped from the surface of the colony and transferred into a 2 mL tube containing 400 μ L of lysis buffer and two tungsten beads (Qiagen Stainless Steel Beads, 5 mm). Mycelium was homogenized using Qiagen TissueLyser for 3 min at 28 repetitions per minute; the lysate obtained was used for DNA extraction. DNA concentration was measured using a NanoDrop spectrophotometer and stored at -20°C until further use.

PCR amplification

Amplification of β -tubulin was performed with the primers T1 5'-AACATGCGTGAGATTGTAAGT-3' (O'Donnell and Cigelnik, 1997) and Beta-tub-2 5'-ATCATGTTCTTGGGGTCGAA-3' (Peever et al., 2004) able to produce a 1100 bp amplicon. PCR reactions were performed using a T100 Thermal Cycler (Biorad) in a 20 μ L reaction mixture containing: 30 ng/ μ L of genomic DNA, 1 μ L (10 μ M stock, Invitrogen) of each primer, 1 unit of *Taq* DNA Polymerase (Qiagen), 2 μ L of PCR buffer (10X, Qiagen), 1 μ L of dNTPs (10 μ M, VWR), and 0.8 μ L of MgCl_2 (25 mM, Qiagen). The cycling conditions included an initial denaturing step at 94°C for 5 min, followed by 35 cycles of

denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 7 min. A negative control (no template DNA) was included in all experiments. Amplifications were verified by electrophoresis on 0.8% agarose gel (Agarose D-1 LOW EEO, Eppendorf). After purification with QIAquick PCR purification kit (Qiagen), PCR products were measured using a NanoDrop spectrophotometer and sent to BMR genomics sequencing service (<http://www.bmr-genomics.it/>). Sequence contigs were assembled using DNAbaser software, and sequences were deposited at GenBank with accession numbers reported in Table 3.

Alignment and phylogenetic analysis

Similarity searches (blastn, default parameters) were performed for all sequences. The sequences obtained were used for CLUSTALW multiple sequence alignments through MEGA6 software set to default parameters. Manual corrections were performed for each alignment in order to delete trimmer regions and incomplete sequences were discarded. Phylogenetic tree was constructed with MEGA6 (Tamura et al., 2007) using Maximum Likelihood method with 1000 bootstrap repeats and pairwise

Table 2 Isolates of *Alternaria* spp. obtained from seeds of different hosts with accession numbers of β -tubulin gene deposited on Genbank

	Isolate code	Host	Origin (seed company)	Accession number
FC Forlì-Cesena province, VE Venezia province, BG Bergamo province	Ruc PMP 4	Cultivated rocket	Cesena (FC), Italy	KT920420
	Ruc PMP 8	Cultivated rocket	Cesena (FC), Italy	KT920419
	Ruc PMP 9	Cultivated rocket	Cesena (FC), Italy	KT920418
	Ruc PMP 12	Cultivated rocket	Cesena (FC), Italy	KT920422
	Ruc PMP 19	Cultivated rocket	Cesena (FC), Italy	KT920421
	Ruc PMP 19j	Cultivated rocket	Cesena (FC), Italy	KJ909928
	36Q-4NL	Wild rocket	Vegetable seed (VE), Italy	KJ909929
	37Q-13NL	Wild rocket	Vegetable seed (VE), Italy	KJ883443
	37Q-16NL	Wild rocket	Vegetable seed (VE), Italy	KJ909930
	37Q-22NL	Wild rocket	Vegetable seed (VE), Italy	KJ883441
	38Q-1NL	Wild rocket	Mazzocchi, Lodi, Italy	KJ883442
	38Q-9NL	Wild rocket	Mazzocchi, Lodi, Italy	KJ909931
	38Q-19NL	Wild rocket	Mazzocchi, Lodi, Italy	KJ909932
	43Q-1NL	Wild rocket	Orosem, Azzano (BG), Italy	KJ883440
	43Q-2NL	Wild rocket	Orosem, Azzano (BG), Italy	KJ909933

deletion. The evolutionary distances were computed using the Kimura 2-parameter model method and were given as units of the number of base substitutions per site. In each analysis sequences derived from reference *Alternaria* isolates obtained from the CBS-KNAW Fungal Biodiversity Centre (<http://www.cbs.knaw.nl/>) were included together with the sequence of *Pleospora herbarum* (CBS 191.86) used as outgroup (Woudenberg et al. 2013).

Morphological evaluation

The *Alternaria* monoconidial isolates were grown in Petri dishes with PCA media amended with streptomycin sulphate (25 mg/L) as reported by Simmons (2007). Isolates were maintained in growth chambers

with cool white fluorescent illumination at 23 °C and 10/14 h light/dark cycle for 7 days. The observations of conidia morphology were carried out with a NIKON (Eclipse55t) microscope at 40× magnification. Conidial characteristics, body and beak length, shape, and number of longitudinal and transverse septa of twenty conidia per isolate were measured. The type of sporulation was evaluated under Stereo microscope (Leica M165C) by considering the length of conidial chains and type, if present, of branching.

Secondary metabolites production

Production of secondary metabolites was tested by growing isolates on a modified Czapek-Dox liquid medium according to Brzonkalik et al. (2011). Cultures were inoculated with three mycelial plugs in 20 mL. All cultures were performed in triplicate and statically incubated in the dark at 28 °C. After 8 days, cultures were filtered and the clear medium was analyzed.

Chemicals and standard preparation

Standards of tenuazonic acid (TeA) copper salt from *A. alternata* (purity ≥98%), alternariol (AOH) from *Alternaria* spp. (purity ≥94%), alternariol monomethylether (AME) from *Alternaria alternata* (purity ≥98%), altenuene (ALT) from *Alternaria*

Table 3 Accession numbers of β -tubulin gene deposited on Genbank of CBS strains used in this work

Isolates	Specie	Accession numbers
CBS 239.73	<i>Alternaria brassicae</i>	Y17074.1
ICPM 1120–77	<i>Alternaria brassicicola</i>	Y17084.1
CBS 124278	<i>Alternaria tenuissima</i>	KT920410
CBS 124274	<i>Alternaria arborescens</i>	KT920429
CBS 115152	<i>Alternaria alternata</i>	KT920430
CBS 118390	<i>Alternaria japonica</i>	KJ883438
CBS 191.86	<i>Pleospora herbarum</i>	AY749032.1

spp. (purity $\geq 98\%$) and tentoxin (TEN) from *Alternaria tenuis* (purity $\geq 99\%$) were purchased from Sigma-Aldrich (St Louis, MO, USA) in crystallized form. For each mycotoxin, a stock solution of 1000 $\mu\text{g/mL}$ and a working solution of 10 $\mu\text{g/mL}$ were prepared in methanol and kept at $-20\text{ }^{\circ}\text{C}$. Standard solutions for HPLC calibration and for addition experiment were prepared by diluting the working solution in mobile phase ($\text{H}_2\text{O}:\text{CH}_3\text{OH}$ 6:4 both with NH_4HCO_3 2 mM).

Extraction of secondary metabolites from fungal cultures

Alternaria mycotoxins were extracted by liquid-liquid extraction. Each sample was adjusted to pH 2 with HCl, and an aliquot (5 mL) was transferred in a separating funnel. Ten mL of dichloromethane was added three times, and the mixture was shaken for 1 min, then the lower dichloromethane extracts were collected in a flask. The final extract was evaporated to dryness in a rotary

Table 4 Identity, sporulation group, virulence and mycotoxin production of *Alternaria* isolates obtained from different hosts

Isolate	<i>Alternaria</i> species based on β -tubulin	<i>Alternaria</i> species based on morphology	Sporulation group	Host	Virulence ^a	Mycotoxins
Ruc 1/10	<i>japonica</i>	<i>japonica</i>	-	Cultivated rocket	H	TeA, AOH, AME, ALT
Ruc 2/10	<i>alternata</i> complex	<i>alternata</i>	4	Cultivated rocket	H	TeA, AOH, AME, ALT
Ruc 4/10	<i>alternata</i> complex	Not tested	Not tested	Cultivated rocket	M	TeA, AOH, AME
Ruc 5/10	<i>alternata</i> complex	<i>arborescens</i>	3	Cultivated rocket	M	TeA, AOH, AME
Ruc 6/10	<i>alternata</i> complex	Not tested	Not tested	Cultivated rocket	M	TeA
Ruc 9/10	<i>alternata</i> complex	<i>arborescens</i>	3	Cultivated rocket	M	TeA, AOH, AME, ALT, TEN
Ruc 12/10	<i>alternata</i> complex	<i>arborescens</i>	3	Cultivated rocket	M	TeA, AOH, AME, ALT, TEN
Ruc 13/10	<i>alternata</i> complex	<i>tenuissima</i>	5	Not tested	Not tested	TeA, AOH, AME, ALT
Ruc 1/11	<i>japonica</i>	<i>japonica</i>	-	Not tested	Not tested	-
Ruc PMP 4	<i>brassicicola</i>	<i>brassicicola</i>	5	Cultivated rocket	M	TeA
Ruc PMP 8	<i>alternata</i> complex	<i>alternata</i>	4	Cultivated rocket	L	TeA
Ruc PMP 9	<i>alternata</i> complex	<i>alternata</i>	4	Cultivated rocket	L	TeA, AOH, AME, ALT
Ruc PMP 12	<i>alternata</i> complex	<i>alternata</i>	4	Cultivated rocket	M	TeA, AOH, AME, ALT
Ruc PMP 19	<i>Alternata</i> complex	<i>brassicinae</i>	5	Cultivated rocket	M	TeA, AOH, AME, ALT, TEN
Ruc PMP 19 J	<i>japonica</i>	Not tested	Not tested			TeA
Cav 2/10	<i>alternata</i> complex	<i>alternata</i>	4	Cauliflower	M	TeA, AOH, AME, ALT
Cav 3/10	<i>alternata</i> complex	<i>tenuissima</i>	5	Cabbage	H	TeA
Cav 5/10	<i>alternata</i> complex	<i>arborescens</i>	3	Cabbage	H	TeA, AOH, AME, ALT
Cav 7/10	<i>alternata</i> complex	<i>brassicinae</i>	5	Cabbage	M	TeA, AOH, AME, ALT
Cav 12/10	<i>alternata</i> complex	<i>tenuissima</i>	5	Cauliflower	H	TeA, AOH, AME, ALT, TEN
Cav 15/10	<i>alternata</i> complex	<i>tenuissima</i>	5	Cabbage	M	TeA, AOH, AME, ALT
36Q-4 NL	<i>japonica</i>	Not tested	Not tested	Wild rocket	H	-
37Q-13 NL	<i>japonica</i>	Not tested	Not tested	Wild rocket	M	-
37Q-16 NL	<i>japonica</i>	<i>japonica</i>	-	Wild rocket	M	-
37Q-22NL	<i>japonica</i>	Not tested	Not tested	Wild rocket	M	-
38Q-1 NL	<i>japonica</i>	<i>japonica</i>	-	Wild rocket	H	TeA, AOH, AME, ALT
38Q-9 NL	<i>japonica</i>	<i>japonica</i>	-	Wild rocket	M	-
38Q-19 NL	<i>japonica</i>	<i>japonica</i>	-	Wild rocket	M	-
43Q-1NL	<i>japonica</i>	<i>japonica</i>	-	Wild rocket	H	-
43Q-2NL	<i>japonica</i>	<i>japonica</i>	-	Wild rocket	M	-

^a L, low virulence (10–30% of infected leaves); M, moderate virulence (31–60% of infected leaves); H, high virulence (61–100% of infected leaves)

evaporator at 35 °C. The residue was dissolved in 1 mL of H₂O:CH₃OH 1:1 for the HPLC-MS/MS analysis.

Analysis of secondary metabolites

Chromatographic separation was carried out using a 1260 Agilent Technologies system consisting of a binary pump and a vacuum degasser, connected to a Varian autosampler Model 410 Prostar (Hansen Way, CA, USA) equipped with a 20 µL loop. The separation was performed using a Kinetex PFP (100 × 2.10 mm 2.6 µ, Phenomenex) under a flow of 200 µL/min and with a temperature set at 35 °C. Solvent A was H₂O with 2 mM NH₄HCO₃, solvent B was CH₃OH with 2 mM NH₄HCO₃. A linear gradient from 40% to 100% of solvent B in 12 min was used. Detection was done by using Varian 310-MS TQ Mass Spectrometer equipped with an electrospray (ESI) ion source operating in negative ion mode. For the Multiple Reaction Monitoring (MRM) experiments two transitions were selected for each compound. MRM transition used for quantification were m/z 196 > 139 CE 20 V for TeA, m/z 257 > 213 CE 22 V for AME, m/z 271 > 256 CE 22 V for AOH, m/z 291 > 229 CE 12 V for ALT, and m/z 413 > 271 CE 16 V for TEN.

Results

Pathogenicity assay

First *Alternaria* symptoms were observed on old leaves. All isolates were pathogenic, but expressed different degrees of virulence on their host of origin (Table 4). Isolates Ruc PMP8 and Ruc PMP9 showed a low virulence, all other isolates were moderately virulent (18 isolates) or highly virulent (eight isolates). There was no correlation between degree of virulence and host plants and/or *Alternaria* species.

Phylogenetic analysis

Sequence analysis of β -tubulin gene as molecular marker showed a Maximum Likelihood tree with 4 different *Alternaria* clades (Fig. 1). The first clade included isolates identified by sporulation pattern as *A. alternata*, *A. arborescens* and *A. tenuissima*. This first clade also included the reference strains. The second clade included only the Ruc PMP 4 isolate with a reference strain of

Fig. 1 Phylogenetic relatedness (tree) of *Alternaria* spp. based on β -tubulin gene and analyzed by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (−3253.8466) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. All positions with less than 95% site coverage were eliminated

A. brassicicola while the third clade represent the *A. brassicae* reference strain. In the last group, we found all isolates with sporulation pattern close to *A. japonica*.

Morphological evaluation

On PCA medium all isolates tested showed moderate to high sporulation with the exceptions that did not produce any conidia (Table 5). Isolates from seeds of wild rocket, 37Q-16NL, 38Q-1NL, 38Q-9 NL, 38Q-19NL, 43Q-1 L showed dark colonies. The presence of numerous, intercalary chains of hyphal chlamydospores of 8.9–17.2 (mean 12.58) µm in length were easily recognized. Ruc PMP4 showed the same conidial length to those identified as *A. japonica*, but had fewer longitudinal septa and did not show any chlamydospores.

Isolates Ruc 5/10, Ruc 9/10, Ruc 12/10 showed a conidia chain length ranging from 12 to 37.6 (mean 29.3) µm, with 3–8 transversal septa, while occasionally longitudinal septa were present. Young conidia were verrucose. Conidia were produced in branched chains with long primary conidiophores. Those characters correspond to *A. arborescens* morphotype.

For isolates Cav 3/10, Cav 12/10, Cav 15/10, Ruc 5/10 and Ruc 13/10, conidia were in chains of 7 to 20 elements. Mature conidia were golden brown and showed frequently a median septum, with a total body length ranging from 19.0 to 50.8 (mean 34.4 µm). Those characters correspond to *A. tenuissima* morphotype. Conidia of Cav 7/10 and Ruc PMP 19 were clearly smaller than the previously described isolates and produced mature conidia with a body length of 16.6 to 34.1 (mean 27.0 µm) in chains of up of 20 units, which correspond to *A. brassicinae* morphotype. *Alternaria* isolates coded Ruc 2/10, Ruc PMP 12, and Ruc PMP 9 showed conidia chains with lateral branches with a conidia size ranging from 16.7 to 40.1 (mean 27.7 µm).

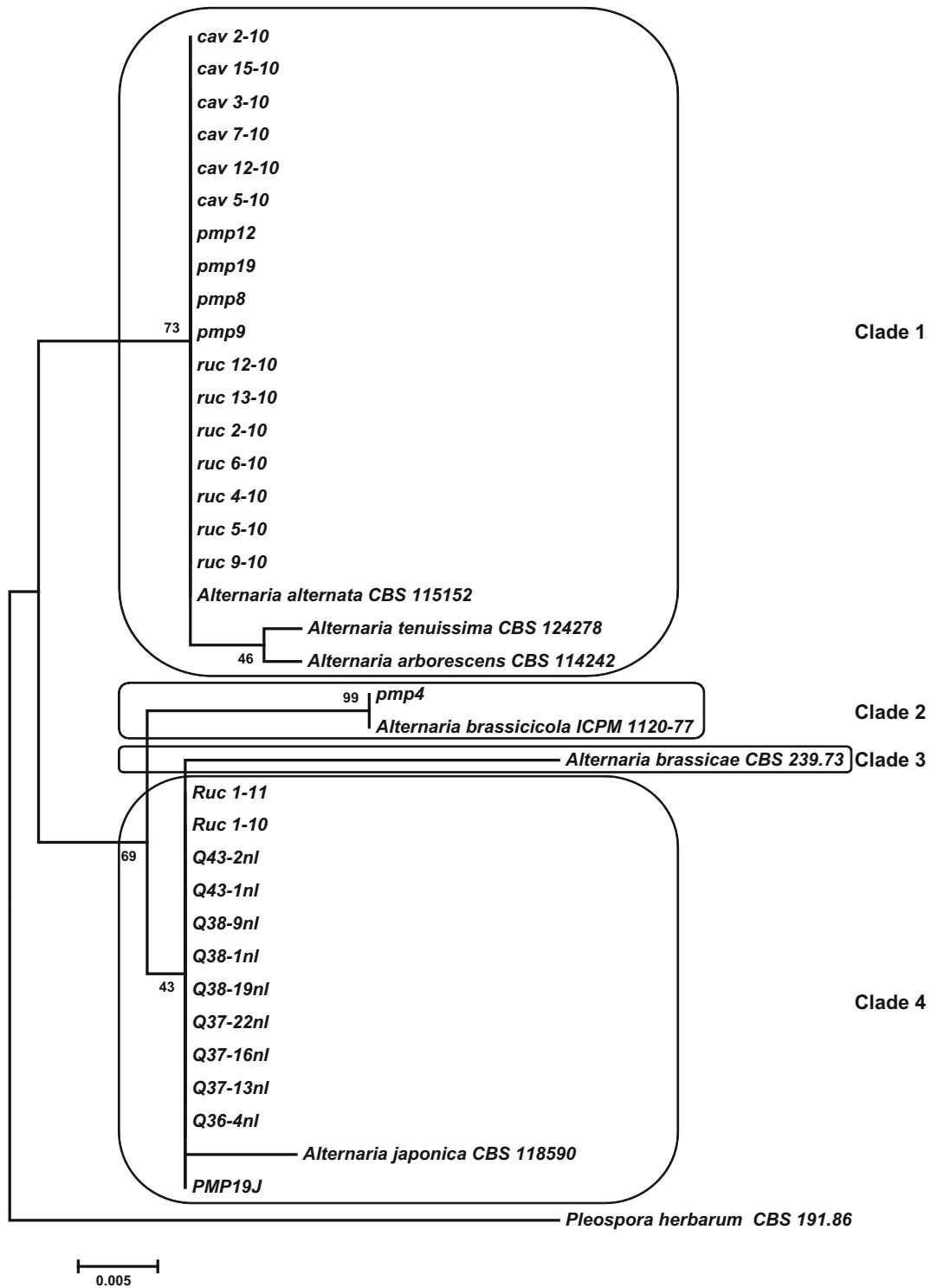


Table 5 Size of conidia (μm , mean in parenthesis) of *Alternaria* spp. on PCA media

Strain	<i>Alternaria</i> species based on β -tubulin	<i>Alternaria</i> species based on morphology	Size mm		Beak	Total body
			Length			
Cav 2/10	<i>Alternata complex</i>	<i>alternata</i>	11.9–30.9 (19.1 $\pm$ 4.7)		2.2–6.5 (3.7 $\pm$ 1.4) c-e	15.4–36.8 (24.6 $\pm$ 5.2) ab
Cav 3/10	<i>alternata complex</i>	<i>tenuissima</i>	21–37.8 (28.9 $\pm$ 5.3)		2.3–13.0 (4.6 $\pm$ 2.8) e	24.0–50.8 (33.5 $\pm$ 6.6) d-g
Cav 5/10	<i>alternata complex</i>	<i>arborescens</i>	22.3–36.1 (29.8 $\pm$ 4.2)		1.7–7.8 (3.6 $\pm$ 1.7) b-e	25.2–35.3 (30.6 $\pm$ 3.5) b-g
Cav 7/10	<i>alternata complex</i>	<i>brassicinae</i>	14–31.8 (23.8 $\pm$ 4.7)		2.1–5.0 (3.3 $\pm$ 0.8) c-e	16.6–34.1 (27.0 $\pm$ 4.9) a-e
Cav 12/10	<i>alternata complex</i>	<i>tenuissima</i>	22.4–43.9 (31.1 $\pm$ 7.2)		2.6–5.2 (3.9 $\pm$ 0.8) c-e	21.2–46.5 (34.9 $\pm$ 7.1) f-g
Cav 15/10	<i>alternata complex</i>	<i>tenuissima</i>	18.3–43.9 (31.9 $\pm$ 7.7)		2.1–7.2 (3.9 $\pm$ 1.3) c-e	21.2–46.5 (35.9 $\pm$ 8.0) g
Ruc 1/10	<i>japonica</i>	<i>japonica</i>	13.9–39.0 (26.6 $\pm$ 6.5)		0.0–4.9 (2.6 $\pm$ 1.5) a-d	19.8–34.7 (26.4 $\pm$ 4.6) a-d
Ruc 1/11	<i>japonica</i>	<i>japonica</i>	17.7–37.5 (28.8 $\pm$ 5.8)		0.0–6.3 (2.6 $\pm$ 1.3) a-d	19.4–40.7 (30.0 $\pm$ 5.7) a-g
Ruc 2/10	<i>alternata complex</i>	<i>alternata</i>	14.0–28.2 (22.0 $\pm$ 3.9)		2.0–4.0 (3.0 $\pm$ 0.5) a-e	16.8–31.0 (25.0 $\pm$ 3.8) a-c
Ruc 5/10	<i>alternata complex</i>	<i>arborescens</i>	18.9–37.6 (26.5 $\pm$ 4.9)		2.4–6.7 (3.7 $\pm$ 1.2) c-e	23.4–42.1 (30.2 $\pm$ 4.8) a-g
Ruc 9/10	<i>alternata complex</i>	<i>arborescens</i>	12.0–28.1 (23.2 $\pm$ 4.9)		3.0–6.0 (4.3 $\pm$ 0.9) c-e	26.5–34.5 (30.3 $\pm$ 2.2) a-g
Ruc 12/10	<i>alternata complex</i>	<i>arborescens</i>	16.9–30.6 (24.5 $\pm$ 4.1)		2.9–7.0 (4.2 $\pm$ 1.1) c-e	19.9–34.5 (28.7 $\pm$ 4.0) a-g
Ruc 13/10	<i>alternata complex</i>	<i>tenuissima</i>	15.8–33.5 (28.0 $\pm$ 6.7)		2.1–6.5 (4.3 $\pm$ 1.0) c-e	19.0–50.5 (32.2 $\pm$ 7.0) c-g
Ruc PMP 4	<i>brassicicola</i>	<i>brassicicola</i>	15–38.7 (26.3 $\pm$ 6.9)		0.0–7.8 (3.0 $\pm$ 2.4) a-e	18.5–40.8 (30.1 $\pm$ 5.9) a-g
Ruc PMP 8	<i>alternata complex</i>	<i>Alternata</i>	18.0–36.5 (27.1 $\pm$ 5.9)		2.7–5.0 (3.9 $\pm$ 0.9) c-e	21.9–36.9 (28.3 $\pm$ 4.5) a-f
Ruc PMP 9	<i>alternata complex</i>	<i>Alternata</i>	17.0–35.6 (28.9 $\pm$ 6.0)		1.1–4.6 (2.4 $\pm$ 0.9) a-c	19.7–36.0 (26.9 $\pm$ 5.5) a-e
Ruc PMP 12	<i>alternata complex</i>	<i>Alternata</i>	14.5–31.0 (20.3 $\pm$ 4.7)		2.7–7.0 (4.3 $\pm$ 1.1) c-e	16.7–34.6 (24.5 $\pm$ 4.5) ab
37Q-16 NL	<i>japonica</i>	<i>japonica</i>	16.8–26.5 (23.8 $\pm$ 4.3)		0.0–4.2 (2.5 $\pm$ 1.1) a-c	19.6–36.2 (26.3 $\pm$ 4.5) a-d
38Q-1 NL	<i>japonica</i>	<i>japonica</i>	17.4–33.2 (25.7 $\pm$ 5.7)		0.0–3.1 (1.3 $\pm$ 0.9) a	18.3–34.8 (27.0 $\pm$ 5.7) a-e
38Q-9 NL	<i>japonica</i>	<i>japonica</i>	18.9–32.5 (26.3 $\pm$ 5.5)		1.0–2.1 (1.6 $\pm$ 0.4) ab	19.1–34.5 (27.9 $\pm$ 5.4) a-f
38Q-19 NL	<i>japonica</i>	<i>japonica</i>	22.8–33.2 (29.4 $\pm$ 3.4)		0.0–3.3 (1.1 $\pm$ 1.3) a	25.0–36.1 (30.5 $\pm$ 3.4) b-g
43Q-1NL	<i>japonica</i>	<i>japonica</i>	17.0–28.9 (21.8 $\pm$ 3.1)		0.0–3.1 (1.2 $\pm$ 1.3) a	17.0–28.9 (23.0 $\pm$ 3.6) a
43Q-2NL	<i>japonica</i>	<i>japonica</i>	19.9–33.2 (25.7 $\pm$ 5.6)		0.0–3.1 (1.3 $\pm$ 0.9) a	18.3–34.3 (27.0 $\pm$ 5.7) a-e
Pmp 19	<i>Alternata complex</i>	<i>brassicinae</i>	15.7–31.7 (24.7 $\pm$ 4.2)		1.6–6.2 (3.4 $\pm$ 1.2) b-e	17.4–36.4 (28.1 $\pm$ 4.5) a-f
CBS34-015	<i>tenuissima</i>	<i>tenuissima</i>	21.4–39.0 (29.5 $\pm$ 5.8)		2.2–10 (4.5 $\pm$ 2.2) d-e	25.2–44.4 (34.0 $\pm$ 6.6) a-g
CBS118390	<i>japonica</i>	<i>japonica</i>	16.0–33.4 (25.2 $\pm$ 5.7)		0–2.1 (1.0 $\pm$ 0.6) a	16.0–33.9 (26.2 $\pm$ 5.6) a-d
CBS124274	<i>arborescens</i>	<i>arborescens</i>	12–49.3 (24.9 $\pm$ 6.7)		2.2–9.2 (4.4 $\pm$ 1.7) d-e	14.6–46.4 (29.3 $\pm$ 7.8) a-g
CBS34-016	<i>alternata</i>	<i>alternata</i>	17.9–28.5 (22.3 $\pm$ 3.7)		2.2–6.2 (3.6 $\pm$ 1.4) c-e	20.2–29.5 (25.6 $\pm$ 2.8) a-c

Strain	Size mm	Number of septa		Chlamydospore	Sporulation group*
		Transversal	Longitudinal		
Cav 2/10	5.7–11.8 (7.5 $\pm$ 1.6)	3–6 (3.6 $\pm$ 0.8)	0–2 (0.5 $\pm$ 0.6)		4
Cav 3/10	6.5–11.5 (8.4 $\pm$ 1.4)	3–6 (4.1 $\pm$ 0.9)	0–3 (1.2 $\pm$ 1.2)		5
Cav 5/10	5.7–10.8 (8.2 $\pm$ 1.5)	3–7 (5.2 $\pm$ 1.4)	0–2 (0.5 $\pm$ 0.8)		3
Cav 7/10	6.5–9 (7.6 $\pm$ 0.9)	3–7 (4.3 $\pm$ 1.2)	0–1 (0.2 $\pm$ 0.4)		5

Table 5 (continued)

Strain	Size mm		Number of septa		Chlamydospore	Sporulation group*
	Wide		Transversal	Longitudinal		
Cav 12/10	5.1–12.4 (8.5 ± 2.0)		3–8 (4.6 ± 1.6)	0–1 (0.5 ± 0.6)		5
Cav 15/10	5.1–12.6 (8.6 ± 1.9)		2–7 (4.0 ± 1.5)	0–2 (0.5 ± 0.6)		5
Ruc 1/10	4.7–11 (7.8 ± 1.8)		3–6 (4.9 ± 1.3)	0–3 (0.6 ± 1.0)		-
Ruc 1/11	6.6–10 (10.0 ± 1.9)		1–4 (3.4 ± 0.8)	0–0 (0.0 ± 0)	10.0–18.0 (13.6 ± 2.3)	-
Ruc 2/10	5.6–13.0 (8.5 ± 1.9)		2–5 (3.3 ± 0.9)	0–1 (0.2 ± 0.4)		4
Ruc 5/10	2.9–8.4 (7.0 ± 1.0)		3–7 (4.6 ± 1.5)	0–2 (0.2 ± 0.4)		3
Ruc 9/10	6.0–8.3 (7.1 ± 0.6)		3–6 (4.1 ± 0.8)	0–1 (0.1 ± 0.3)		3
Ruc 12/10	5.5–10.1 (7.5 ± 1.0)		4–8 (5.0 ± 1.2)	0–1 (0.2 ± 0.4)		3
Ruc 13/10	6.6–11.0 (7.7 ± 1.1)		3–8 (5.0 ± 1.5)	0–2 (0.8 ± 0.7)		5
Ruc PMP 4	6.0–11.1 (8.6 ± 1.5)		1–5 (3.1 ± 1.3)	0–1 (0.1 ± 0.3)		5
Ruc PMP 8	5.9–0.1 (7.8 ± 1.5)		4–7 (4.9 ± 1.0)	0–3 (0.7 ± 0.8)		4
Ruc PMP 9	5.5–11 (7.7 ± 1.2)		4–7 (4.6 ± 1.1)	0–3 (0.6 ± 0.8)		4
Ruc PMP 12	6.2–8 (6.9 ± 0.5)		3–6 (3.7 ± 1.0)	0–1 (0.1 ± 0.4)		4
37Q-16 NL	6.9–8.5 (7.8 ± 0.5)		2–5 (3.3 ± 0.9)	0–1 (1.0 ± 0.7)	11.9–15.2 (13.6 ± 1.1)	**
38Q-1 NL	5.9–7.8 (6.6 ± 0.8)		5–7 (4.7 ± 1.2)	0–1 (0.5 ± 0.5)	8.9–13.7 (11.1 ± 1.4)	-
38Q-9 NL	5.9–9.4 (7.4 ± 1.3)		3–7 (4.7 ± 1.3)	0–3 (0.9 ± 0.9)	9.1–14.1 (12.7 ± 0.9)	-
38Q-19 NL	6.1–7.9 (7.1 ± 0.6)		4–7 (5.3 ± 1.2)	0–1 (0.5 ± 0.5)	10.4–16.6 (14.1 ± 1.9)	-
43Q-1NL	6.9–11 (8.4 ± 1.2)		2–6 (4.1 ± 1.2)	0–2 (0.9 ± 0.8)	10–17.2 (12.5 ± 2.0)	-
43Q-2NL	5.8–8.7 (6.9 ± 0.7)		2–6 (4.4 ± 1.2)	0–1 (0.5 ± 0.5)	11.7–15.4 (13.8 ± 2.2)	-
Pmp 19	4.2–10 (8.1 ± 1.5)		3–7 (4.0 ± 1.0)	0–1 (0.4 ± 0.5)		5
CBS34.015	4.7–11.0 (7.8 ± 1.8)		4–8 (5.1 ± 1.3)	1–3 (0.8 ± 1.0)		5
CBS118390	5.7–7.8 (6.4 ± 0.7)		3–7 (4.9 ± 1.6)	0–1 (0.3 ± 0.5)		-
CBS124274	6.6–10 (7.6 ± 0.9)		3–6 (3.9 ± 0.7)	0–1 (0.2 ± 0.4)		3
CBS34-016	5.5–8.9 (7.0 ± 1.2)		3–6 (3.9 ± 0.7)	0–2 (0.3 ± 0.6)		

No conidia were developed for Ruc 6/10. No microscopic observations for 37Q-22NL; 37Q-13 NL; 36Q-4 NL; Ruc PMP 19. * Sporulation groups according to Simmons and Roberts (1993). ** Conidia mainly solitary o with chains of 2–3 spores

Average size of conidia of *Alternaria* isolates varied within a broad range (Table 5). The length of conidia body and beak (Gannibal et al. 2014), used to draw an approximate division among the *Alternaria* species identified in the present study, provided evidence of a good correlations among the morphological parameters and the *Alternaria* species (Fig. 2). Moreover, the identification performed by sporulation pattern yielded a good correspondence to the clustering in the phylogenetic tree.

Production of secondary metabolites

The analytes were investigated using the external calibration method, and a calibration curve was built for each analyte. Good linearity was obtained for all analytes ($R^2 > 0.999$). Recovery experiments were done spiking the matrix before extractions with a standard solution at 100 µg/L for each mycotoxin. The calculated recovery ranged between 80 and 100%. TeA was the main mycotoxin produced and was detected in more than 80% of analyzed *Alternaria* isolates. Benzopyrone derivatives, AOH, AME, and ALT, were identified in more than 50% of samples. TEN was produced only by four isolates (Cav 3/10, Cav 12/10, Ruc 9/10 and Ruc 12/10) (Table 4). As showed in Fig. 3, samples in clade 1 and 4 have a different production. Only two samples (Ruc PMP 19 J and 38Q1NL) in clade 4 produced mycotoxins, while all the strains in clade 1 produce at least one mycotoxin. In fact, all strains were able to produce TeA, the benzopyrone derivatives (AOH, AME and ALT) were synthesized by almost the same strains. AOH and AME were produced by more than 70% of the strains, with some differences in the concentrations. Ruc PMP 4 belonging to clade 2 produced only TeA.

Discussion

Within the genus *Alternaria*, taxonomy is rather complex and species differentiation is usually based on a combination of morphological observations and molecular characterization (Bessadat et al. 2014; Simmons 2007). However, especially within the small-spored *Alternaria* species, a large variation in morphological aspects was observed depending mainly on growth conditions and even with molecular methods species identification is difficult (Andersen et al. 2009). In some cases, separation has been achieved at species-group level (Andersen et al.

2002; Polizzotto et al. 2012). Lawrence et al. (2013) identified *A. arborescens* and the *A. tenuissima* species-groups together with *A. alternata* into one section, with more than 50 additional species that were not always correlated with the species-group based on morphological characters (Woudenberg et al. 2013). More recently, Woudenberg et al. (2015) have identified *A. arborescens* species complex, whereas *A. alternata* and *A. tenuissima* have been joined together in the section *Alternaria*. In our experimental setup based on β -tubulin sequences, we found two big groups of isolates, the first including *A. alternata*, *A. arborescens* and *A. tenuissima*, and the second closely related to *A. japonica*. Interestingly, no isolate close to the *A. brassicae* was found, while only one isolate was related to *A. brassicicola* (isolate Ruc PMP 4 obtained from rocket). In most cases, three *Alternaria* species (*A. brassicicola*, *A. brassicae* and *A. japonica*) have been reported as the causal agents of black spot disease of crucifers (Peruch et al. 2006; Reis and Boiteux 2010; Gilardi et al. 2015). Also, Aneja et al. (2014) described *A. brassicae*, *A. brassicicola* and *A. alternata* to be present on oilseed Brassicas with *A. brassicae* as a dominant species. However, *A. tenuissima* was found as a principal species on cabbage in Iran (Rahimloo and Ghosha 2015).

In general, morphological observations and in particular the sporulation pattern confirmed the results obtained by phylogenetic analysis (clustering) except for isolate Cav 7/10 that featured an atypical morphological aspect. However, there were no statistical differences between different clusters when mean conidia size was considered.

Our results show that *Alternaria* isolates from rocket and cabbage plants are able to infect their original host with different levels of virulence. *A. japonica* was found to be the most prevalent specie within the isolates of *Alternaria* from wild and cultivated rocket plants and seeds, however *A. tenuissima*, *A. brassicicola* and *A. arborescens* have been here also identified. The results of the present study suggest that these species are a potential threat of rocket. However, it is well known the broad range of hosts from which these species may be isolated (Neergaard 1945; Simmons 2007; Farr and Rossman 2016).

At the same time, almost all isolates were able to produce at least three different mycotoxins under in vitro conditions. However, there was no clear correlation between virulence and mycotoxin production. In

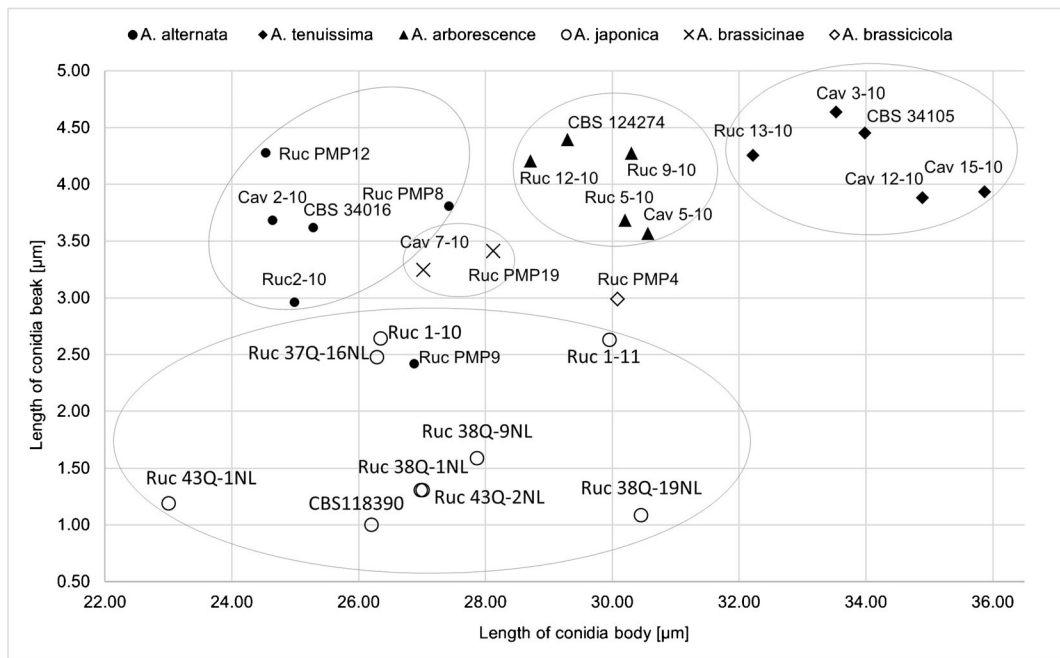


Fig. 2 Length of conidia body and beak of *Alternaria* sp. isolates obtained on PCA media

a wide range of plant pathogens, virulence level has been described to be independent of mycotoxin production, whereas virulence have been associated to mycotoxin production for other plant pathogens: Desjardins et al. (1989) have demonstrated the essential role of trichothecene 2 during pathogenesis of *Fusarium sporotrichioides*. In their experiments, the authors showed that a mutation blocking the T2 toxin biosynthesis resulted in a non-pathogenic phenotype, whereas pathogenicity was restored when the same mutant was complemented by the wild type. In *Alternaria* spp., the possible role of mycotoxins on virulence and/or pathogenicity was investigated in a study on the impact of osmotic stress on tomato that revealed that AOH

biosynthesis is an important factor that supporting the tissue colonization of tomatoes (Graf et al. 2012; Geisen et al. 2015). Most isolates of our study produced TeA in vitro confirming results obtained in our previous work (Siciliano et al. 2015). In fact, TeA contamination at rather high levels of tomatoes grown in southern Italy can be a major issue, while alternariol and alternariol methyl ether were present at lower levels (Bottalico and Logrieco 1998). Stinson et al. (1980, 1981) reported a high level of tenuazonic acid in infected tomatoes.

Mycotoxin production depends on several factors (Sanchis and Magan 2004). Water activity (a_w) played an important role for the TeA, AME and AOH production in *A. alternata* with an optimum value greater than

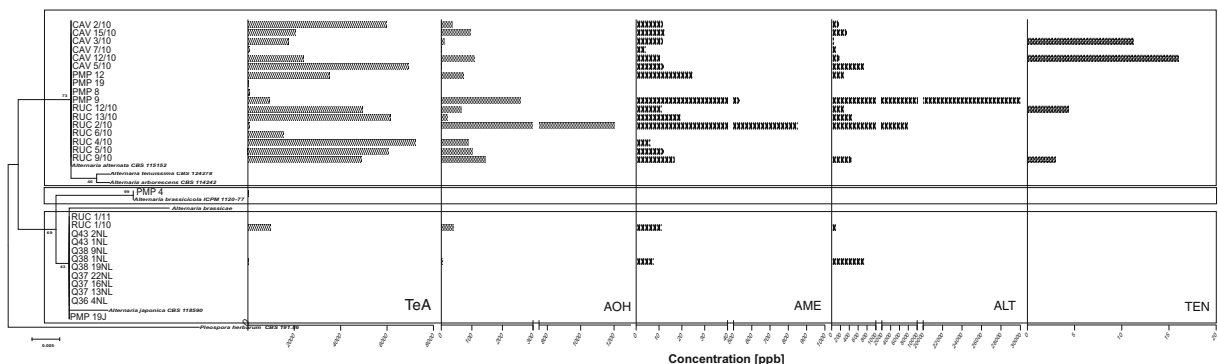


Fig. 3 Mycotoxin production of *Alternaria* sp. isolates obtained on inductive media incubated at 28 °C for 8 days in the dark

0.97. Furthermore, temperature affected mycotoxin production by *A. alternata* in synthetic medium with an optimum around 28 °C for AOH and AME and 21 °C for TEA (Hasan 1996). Moreover, AOH production has been shown to be influenced by light exposure with a reduced mycotoxin production compared to cultures grown in the dark (Häggbloom and Unestam 1979; Häggbloom and Niehaus 1986). Finally, cultivation conditions, in particular carbon and nitrogen sources can influence mycotoxin production by *A. alternata* (Brzonkalik et al. 2011). Thus, mycotoxin production under natural conditions is strongly influenced by the species and cultivar of host plants, by growth, harvesting and environmental conditions (Lauren and Fadwa 2008). For all these reasons, it is not surprising that there was no obvious correlation between our in vitro mycotoxin and virulence data set.

Acknowledgements Work supported by the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n. 261752, PLANTFOODSEC "Plant and Food Biosecurity, Network of Excellence".

References

- Andersen, B., Krøger, E., & Roberts, R. G. (2002). Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. *Mycological Research*, 106, 170–182.
- Andersen, B., Sørensen, J. L., Nielsen, K. F., van den Ende, B. G., & de Hoog, S. (2009). A polyphasic approach to the taxonomy of the *Alternaria* Infectoria species-group. *Fungal Genetics and Biology*, 46, 642–656.
- Aneja, J. K., Agarwal, A. A., & Agnihorti, A. A. (2014). Inter and intra-specific diversity in *Alternaria* species infecting oilseed brassicas in India. *Journal of Oilseed Brassica*, 5, 102–117.
- Bessadat, N., Simoneau, P., Benichou, S., Setti, B., Kihal, M., & Henmi, D. E. (2014). Morphological, physiological and pathogenic variability of small-spore *Alternaria* sp. causing leaf blight of Solanaceous plants in Algeria. *African Journal of Microbiology Research*, 8, 3422–3434.
- Bottalico, A., & Logrieco, A. (1998). Toxigenic *Alternaria* species of economic importance. In K. K. Sinha & D. Bhatnagar (Eds.), *Mycotoxins in agriculture and food safety* (pp. 65–108). New York: Marcel Dekker.
- Brzonkalik, K., Herrling, T., Syltatk, C., & Neumann, A. (2011). The influence of different nitrogen and carbon sources on mycotoxin production in *Alternaria alternata*. *International Journal of Food Microbiology*, 147, 120–126.
- CABI. (2007). *Crop protection compendium* (2007th ed.). Wallingford: CABI International.
- Desjardins, A. E., Spencer, G. F., Plattner, R. D., & Beremand, M. N. (1989). Furanocoumarin phytoalexins, trichothecene toxins, and infection of *Pastinaca sativa* by *Fusarium sporotrichioides*. *Phytopathology*, 79, 170–175.
- Ellis, M. B. (1971). *Dematiaceous hyphomycetes*. Kew, UK: Commonwealth Mycological Institute.
- Ellis, M. B. (1976). *More Dematiaceous Hyphomycetes*. Kew, UK: Commonwealth Mycological Institute.
- Farr, D. F., Rossman, A. Y. (2016). Fungal databases - fungal databases, systematic mycology and microbiology laboratory. ARS, USDA.
- Gannibal, P. B., Orina, A. S., Mironenko, N. V., & Levitin, M. M. (2014). Differentiation of closely related species, *Alternaria solani* and *A. tomatophila*, by molecular and morphological features and aggressiveness. *European Journal of Plant Pathology*, 139, 609–623.
- Garibaldi, A., Gilardi, G., Bertoldo, C., & Gullino, M. L. (2011). First report of leaf spot of wild (*Diplotaxis tenuifolia*) and cultivated (*Eruca vesicaria*) rocket caused by *Alternaria japonica* in Italy. *Plant Disease*, 95, 1316.
- Geisen, R., Graf, E., & Schmidt-Heydt, M. (2015). HogA and PacC regulated alternariol biosynthesis by *Alternaria alternata* is important for successful substrate colonization. *Acta Horticulturae*, 1144, 141–148.
- Gilardi, G., Demarchi, S., Ortu, G., Gullino, M. L., & Garibaldi, A. (2015). Occurrence of *Alternaria japonica* on seeds of wild and cultivated rocket. *Journal of Phytopathology*, 163, 419–422.
- Graf, E., Schmidt-Heydt, M., & Geisen, R. (2012). HOG MAP kinase regulation of alternariol biosynthesis in *Alternaria alternata* is important for substrate colonization. *International Journal of Food Microbiology*, 157, 353–359.
- Häggbloom, P., & Niehaus, W. G. (1986). Light effects on polyketide metabolism in *Alternaria alternata*. *Experimental Mycology*, 10, 252–255.
- Häggbloom, P., & Unestam, T. (1979). Blue light inhibits mycotoxin production and increases total lipids and pigmentation in *Alternaria alternata*. *Applied and Environmental Microbiology*, 38, 1074–1077.
- Hasan, H. A. (1996). *Alternaria* mycotoxins in black rot lesion of tomato fruit: Conditions and regulation of their production. *Acta Microbiologica et Immunologica Hungarica Journal*, 43, 125–133.
- Humpherson-Jones, F. M. (1989). Survival of *Alternaria brassicae* and *Alternaria brassicicola* on seed debris of oilseed rape and cabbage. *Annals of Applied Biology*, 115, 45–50.
- Humpherson-Jones, F. M. (2007). The incidence of *Alternaria* spp. and *Leptosphaeria maculans* in commercial brassica seed in the United Kingdom. *Plant Pathology*, 34, 385–390.
- Humpherson-Jones, F. M., & Maude, R. B. (1982). Studies on the epidemiology of *Alternaria brassicicola* in *Brassica oleracea* seed production crops. *Annals of Applied Biology*, 100, 61–71.
- Joly, P. (1964). Le genre *Alternaria*: recherches physiologiques, biologiques et systématiques. *Encyclopédie Mycologique*, 33, 1–250.
- Kohl, J., van Tongeren, C. A. M., Groenenboom-de Haas, B. H., van Hoof, R. A., Driessen, R., & van der Heijden, L. (2010). Epidemiology of dark leaf spot caused by *Alternaria brassicicola* and *A. brassicae* in organic seed production of cauliflower. *Plant Pathology*, 59, 358–367.
- Kolte, S. J., Awasthi, R. P., Vishwanath (1987). Assessments of yield losses due to *Alternaria* blight in rapeseed and mustard. *Indian Phytopathology*, 40, 209–211.

- Lauren, S. J., & Fadwa, A. T. (2008). Factors affecting mycotoxin production in fruits. In R. Barkai-Golan & N. Paster (Eds.), *Mycotoxins in fruit and vegetables* (pp. 75–104). London: Academic Press Is an Imprint of Elsevier.
- Lawrence, D. P., Gannibal, P. B., Peever, T. L., & Pryor, B. M. (2013). The sections of *Alternaria*: Formalizing species-groups concepts. *Mycologia*, 105, 530–546.
- Mac Kinon, S. L., Keifer, P., & Ayer, W. A. (1999). Components from the phytotoxic extracts of *Alternaria brassicicola*, a black spot pathogen of canola. *Phytochemistry*, 51, 215–221.
- Maude, R. B., & Humpherson-Jones, F. M. (1980). Studies on the seed-borne phases of dark leaf spot (*Alternaria brassicicola*) and grey leaf spot (*Alternaria brassicae*) of brassicas. *Annals of Applied Biology*, 95, 311–327.
- Neergaard, P. (1945). Danish Species of *Alternaria* and *Stemphylium*. Copenhagen: E. Munksgaard.
- O'Donnell, K., & Cigelnik, E. (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution*, 7(1), 103–116.
- Ostry, V. (2008). *Alternaria* mycotoxins: An overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxins Journal*, 1, 175–188.
- Peever, T. L., Salimath, S. S., Su, G., Kaiser, W. J., & Muehlbauer, F. J. (2004). Historical and contemporary multilocus population structure of *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) in the Pacific Northwest of the United States. *Molecular Ecology*, 13(2), 291–309.
- Peruch, L. A. M., Michereff, S. J., & Araújo, I. B. (2006). Survey of the intensity of *Alternaria* black spot and black rot on brassica species under organic farming systems in Pernambuco and Santa Catarina states, Brazil. *Horticultura Brasileira*, 24(4), 464–469.
- Pitt, J. I., & Hocking, A. D. (1997). *Fungi and Food Spoilage*. Sydney: Academic Press. doi:10.1007/978-1-4615-6391-4.
- Polizzotto, R., Andersen, B., Martini, M., Grisan, S., Assante, G., & Musetti, R. (2012). A polyphasic approach for the characterization of endophytic *Alternaria* strains isolated from grapevines. *Journal of Microbiological Methods*, 88, 162–171.
- Rahimloo, T., & Ghosta, Y. (2015). The occurrence of *Alternaria* species on cabbage in Iran. *Žemdirbystė (Agriculture)*, 102(3), 343–350.
- Reis, A., & Boiteux, L. S. (2010). *Alternaria* species infecting *Brassicaceae* in the Brazilian neotropics: Geographical distribution, host range and specificity. *Journal of Plant Pathology*, 92(3), 661–668.
- Rossmann, S. N., Cernoch, P. L., & Davis, J. R. (1996). Dematiaceous fungi are an increasing cause of human disease. *Clinical Infectious Diseases*, 22, 73–80.
- Sanchis, V., & Magan, N. (2004). Environmental conditions affecting mycotoxins. In N. Magan & M. Olsen (Eds.), *Mycotoxins in food: Detection and control* (pp. 174–189). Cambridge: Woodhead Publishing.
- Siciliano, I., Ortu, G., Gilardi, G., Gullino, M. L., & Garibaldi, A. (2015). Mycotoxin production in liquid culture and on plants infected with *Alternaria* spp. isolated from rocket and cabbage. *Toxins*, 7, 743–754.
- Simmons, E. G. (1992). *Alternaria* taxonomy: current status, viewpoint, challenge. In J. Chelkowski & A. Viskonti (Eds.), *Alternaria: Biology, Plant Diseases and Metabolites* (pp. 1–35). Amsterdam: Elsevier.
- Simmons, E. G. (1995). *Alternaria* themes and variations. *Mycotaxon*, 55, 55–163.
- Simmons, E. G. (2007). *Alternaria: An Identification Manual*. St Paul: APS PRESS.
- Simmons, E. G., & Roberts, R. G. (1993). *Alternaria* themes and variations. *Mycotaxon*, 48, 109–140.
- Singh, R. C., Mohan, C., & Sharma, V. K. (2012). Morphological and pathological variations in isolates of *Aletnaria brassicae* causing leaf blight of rapeseed and mustard. *Plant Disease Resistance*, 27, 22–27.
- Stinson, E. E., Bills, D. D., Osman, S. F., Siciliano, J., Ceponis, M. J., & Heisler, E. G. (1980). Mycotoxin production by *Alternaria* species grown on apples, tomatoes and blueberries. *Journal of Agricultural and Food Chemistry*, 28, 960–963.
- Stinson, E. E., Osman, S. F., Heisler, E. J., Siciliano, J., & Bills, D. D. (1981). Mycotoxin production in whole tomatoes, apples, oranges and lemons. *Journal of Agricultural and Food Chemistry*, 29, 790–792.
- Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24(8), 1596–1599.
- Thomma, B. P. H. J. (2003). *Alternaria* spp.: from general saprophyte to specific parasite. *Molecular Plant Pathology*, 4, 225–236.
- Van der Waals, J. E., Korsten, L., & Slippers, B. (2004). Genetic diversity among *Alternaria solani* isolates from potatoes in South Africa. *Plant Disease*, 88, 959–964.
- Verma, P. R., & Saharan, G. S. (1994). *Monograph on Alternaria diseases of crucifers*. Ottawa, Canada: Research Branch, Agriculture and Agri-Food Canada.
- Woudenberg, J. H. C., Groenewald, J. Z., Binder, M., & Crous, P. W. (2013). *Alternaria* redefined. *Studies in Mycology*, 75, 171–212.
- Woudenberg, J. H., Seidl, M. F., Groenewald, J. Z., de Vries, M., Stielow, J. B., Thomma, B. P., & Crous, P. W. (2015). *Alternaria* section *Alternaria*: Species, formae speciales or pathotypes? *Studies in Mycology*, 82, 1–21.