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

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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

Siciliano Ilenia[1], Giovanna Gilardi[1], Giuseppe Ortu[1], Ulrich Gisi[1], Maria Lodovica Gullino[1,2], Angelo Garibaldi[1]

[1] *Agroinnova – Centre of Competence for the Innovation in the Agro-Environmental Sector, University of Turin, Largo Paolo Braccini 2, 10095 Grugliasco, Turin, Italy*.

[2] *DISAFA - Dipartimento di Scienze Agrarie, Forestali e Alimentari, University of Turin, Largo Paolo Braccini 2, 10095 Grugliasco, Turin, Italy*

Corresponding Author: e-mail: ilenia.siciliano@unito.it**, Tel.**: +390116708546

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.

Key words: Crucifers, toxins, leaf spot, tenuazonic acid.

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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 of *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 particular is also known as an human pathogen in immunocompromised patients (Rossman 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*. 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 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 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., 2014). 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 spaying leaves with a conidial suspension at 1-5x10^5 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, 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 µl 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 µl reaction mixture containing: 30 ng/µL of genomic DNA, 1 µL (10 µM stock, Invitrogen) of each primer, 1 unit of *Taq* DNA Polymerase (Qiagen), 2 µL of PCR buffer (10X, Qiagen), 1 µL of dNTPs (10 µM, VWR), and 0.8 µL 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 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 et al. (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 40x 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* spp. (purity ≥ 98%) and tentoxin (TEN) from *Alternaria tenuis* (purity ≥ 99%)

were purchased from Sigma-Aldrich (St Louis, MO, USA) in crystallized form. For each mycotoxin, a stock solution of 1000 µg/mL and a working solution of 10 µg/mL were prepared in methanol and kept at -20 °C. Standard solutions for HPLC calibration and for addition experiment were prepared by diluting the working solution in mobile phase (H_2O:CH_3OH 6:4 both with NH_4HCO_3 2mM).

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 evaporator at 35 °C. The residue was dissolved in 1 mL of H_2O:CH_3OH 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 x 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 H_2O with 2mM NH_4HCO_3, solvent B was CH_3OH with 2mM NH_4HCO_3. 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 20V for TeA, m/z 257>213 CE 22V for AME, m/z 271>256 CE 22V for AOH, m/z 291>229 CE 12V for ALT, and m/z 413>271 CE 16V 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 (8 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 *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-1L 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).

Average size of conidia of *Alternaria* isolates varied within a broad range (Table 5). The length of conidia body and beak, used to draw an approximate boundary line among the *Alternaria* species identified in the present

study, provided evidence of a good correlations among the morphological parameters and the *Alternaria* species (Figure 2). Moreover, the identification performed by sporulation pattern yielded a good correspondence to the clustering in the phylogenic 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 19J 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 (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 identify *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 Ghosta, 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 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 thrichotecene 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 (Botalico 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 (aw) played an important role for the TeA, AME and AOH production in *A. alternata* with an optimum value greater than 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 (Häggblom, 1979; 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 Thaer, 2008). For all these reasons, it is not surprising that there was no obvious correlation between our *in vitro* mycotoxin and virulence data set.

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Table captions

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. (CN=Cuneo province; TO=Torino province; AT=Asti province)

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. Momma	Pagliero- Moncalieri(TO)	*KT920426*
Cav 5/10	Cabbage cv.Estoryl	Porcellana (AT)	*KT920423*
Cav. 7/10	Cabbage cv. Momma	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

Table 2 Isolates of *Alternaria* spp. obtained from seeds of different hosts with accession numbers of *β-tubulin* gene deposited on Genbank. (FC=Forlì-Cesena province; VE= Venezia province; BG=Bergamo province)

Isolate code	Host	Origin (seed company)	Accession number
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*

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	*Alternaria brassicae*	*Y17074.1*
ICPM 1120–77	*Alternaria brassicicola*	*Y17084.1*
CBS 124278	*Alternaria tenuissima*	*KT920410*
CBS 124274	*Alternaria arborescens*	*KT920429*
CBS 115152	*Alternaria alternata*	*KT920430*
CBS 118390	*Alternaria japonica*	*KJ883438*
CBS 191.86	*Pleospora herbarum*	*AY749032.1*

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

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

[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)

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

Strain	Size µm	Number of septa		Chlamydospore	Sporulation group*
	Wide	Transversal	Longitudinal		
Cav 12.3.0	*5.1–12.4 (8.5 ± 2.0)*	*3–8 (4.6 ± 1.6)*	*0–1 (0.5 ± 0.6)*		5
Cav 15.1.0	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 12.1.0	*5.5–10.1 (7.5 ± 1.0)*	*4–8 (5.0 ± 1.2)*	*0–1 (0.2 ± 0.4)*		3
Ruc 13.1.0	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	5.9–9.1 (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
CBS34.0.15	*4.7–11.0 (7.3 ± 1.3)*	*4–8 (5.1 ± 1.3)*	*1–3 (0.8 ± 1.0)*		5
CBS118.39.0	5.7–7.8 (6.4 ± 0.7)	3–7 (4.9 ± 1.6)	0–1 (0.3 ± 0.5)		-
CBS12.42.2.4	6.6–10 (7.6 ± 0.9)	3–6 (3.9 ± 0.7)	0–1 (0.2 ± 0.4)		3
CBS34.0.16	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

Figure caption

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

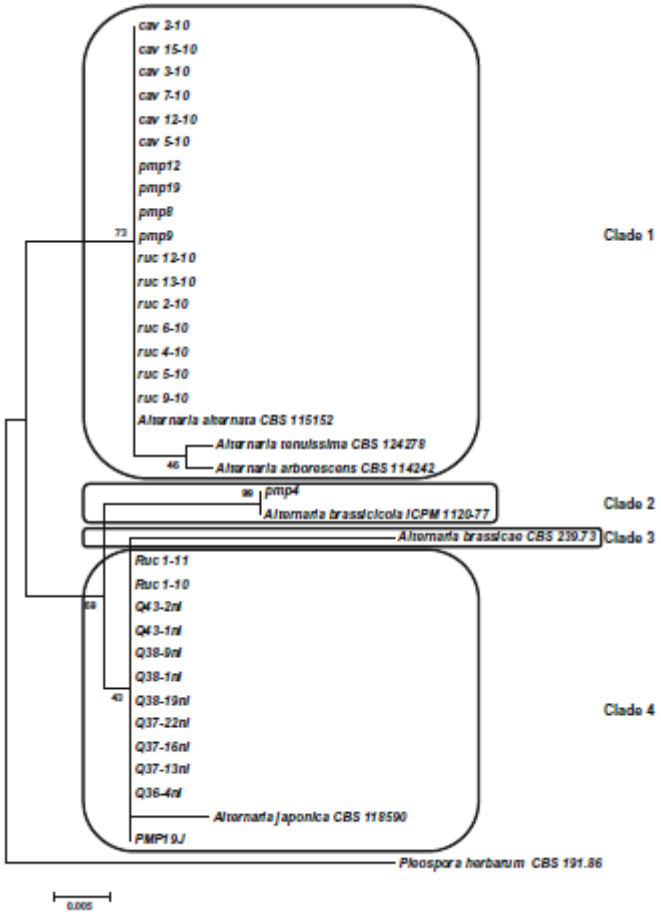

Fig. 2 Length of conidia body and beak of *Alternaria* sp. isolates obtained on PCA media.

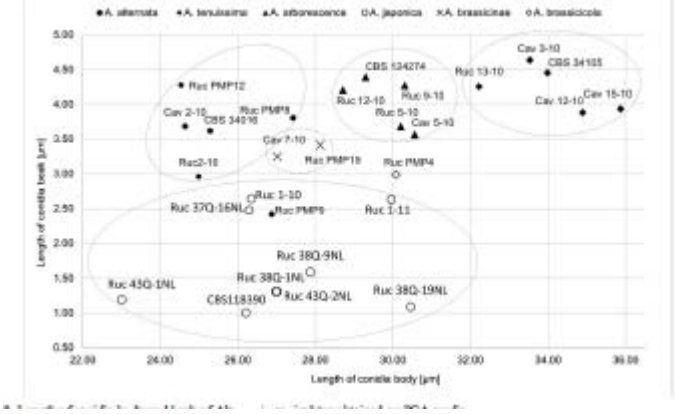

Fig. 3 Mycotoxin production of *Alternaria* sp. isolates obtained on inductive media incubated at 28 °C for 8 days in the dark.