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Molecular phylogeny and characterization of secondary metabolite profile of plant pathogenic *Alternaria* species isolated from basil

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Running head: Characterization of basil *Alternaria* isolates

Abstract

Alternaria leaf-spot is a new disease recently reported on basil in Italy. The correct identification of *Alternaria* species has suffered from many reclassifications in function of morphological features and molecular data. In our study, we performed an overall approach to obtain a better characterization of basil *Alternaria* isolates. Morphological characteristics, seven-genome region phylogenic analysis, and secondary metabolite profile differentiated the majority of the isolates as *A. alternata.* OPA 1-3 and OPA 10-2 were the best molecular regions to discriminate among the isolates. Morphological characteristics and sporulation groups helped to discriminate *A. tenuissima* from *A. alternata* isolates. All isolates in the *A.* sect. *Alternaria* were mycotoxigenic and pathogenic on basil, the production of mycotoxins was enhanced on basil compared to *in vitro* conditions used in this work.

Keywords: Multilocus analysis, *Ocimum basilicum,* HPLC-MS/MS, morphology, Alternaria leaf-spot

1. Introduction

Basil (*Ocimum basilicum* L.) is a popular aromatic herb crop, belonging to the *Lamiaceae* family, it is economically important for Mediterranean countries. Basil leaves can be used fresh or dried as a food ingredient. In Italy, as well as in many other countries, basil is appreciated for fresh consumption, in particular, it is used for industrial production of pesto sauce. In traditional medicine basil leaves and flowering tops are used as carminative and antispasmodic products (Javanmardi et al. 2002). Basil is also a source of essential oils that contain a wide variety of aromatic compounds; the European type is considered to have the highest quality aroma, containing linalool and methylchavicol as the major constituents (Simon et al. 1999). Essential oils from basil were investigated for biological properties and antimicrobial activity attributed to the presence of phenolic compounds (Barbosa et al. 2009). Basil is susceptible to several diseases like Fusarium wilt and Pythium damping-off (Garibaldi et al. 1997); moreover, new diseases, like downy mildew caused by

Peronospora belbahrii, have been reported in several countries (Garibaldi et al. 2004, McLeod et al. 2006). *Alternaria alternata* on basil causes brown-black lesions surrounded by yellow halo on older leaves, leading to progressive plant defoliation, rarely followed by plant death. *Alternaria* leaf spot of basil was first reported by Taba et al. (2009) in Japan. In Italy, a similar black spot caused by *A. alternata* was reported for the first time in 2011 by Garibaldi et al. (2011).

Alternaria Nees is a distributed worldwide and a pathogen with a broad host range. Many *Alternaria* species are saprophytes (Rotem 1994) while the majority are animal and plant pathogens (Hong and Pryor 2004, de Hoog and Horré 2002) causing extensive yield losses in agriculture, especially in pre- and post-harvest conditions (Peever et al. 2005, Wilson and Wisniewski 1994).

The identification of the genera is of great concern in the plant pathology field. Different approach to investigate the identity of *Alternaria* isolates were used. Different works revealed that various culturing conditions could greatly influence conidial morphology (Leach and Aragaki 1970; Zitter and Hsu 1990). For this reason, Simmons and Roberts (1993) highlighted the importance of standardization of growing conditions to produce consistent and reproducible sporulation patterns for morphological analysis. Due to the different re-classification caused by the plasticity of some species, molecular data used for identification of *Alternaria* species were useful but not resolutive to obtain a correct identification. In addition to morphology and molecular analysis, secondary metabolite profiling has been widely used to differentiate similar species within the genus (Smedsgaard and Frisved 1996) and chemotaxonomy was utilized also to distinguish closely-related species of *Alternaria* (Andersen and Thrane 1996). Several studies have demonstrated that metabolite profiling was a reliable tool to characterize and differentiate plant pathogenic fungi. *Alternaria species* are characterized by their ability to produce a wide range of secondary metabolites, some of them are mycotoxins (Scott 2001, Patriarca et al. 2007).

Alternaria mycotoxins were found in many crops such as tomato, orange, and lemon (Logrieco et al. 2003), olive (Visconti et al. 1986), grape and dried vine fruit (Swart et al. 1995). Only five out of 30 known toxins are common natural contaminants of food: the benzopyrene derivatives such as

alternariol (AOH), alternariol monomethylether (AME), and altenuene (ALT), the tetramic acid tenuazonic acid (TeA) and the perylene derivative altertoxin I (ATX-I) (Barkai-Golan 2008). However, it has been reported that the cooking process can affect the chemical structure of the mycotoxins reducing or removing their harmful effects. For these reasons, the non-cooked product may be a potential risk for people health. When fresh products are contaminated with *Alternaria* spp. there is a high risk that critical concentrations of mycotoxins are produced in an important Italian crop such as basil. The aim of this study was the precise and correct identification of *Alternaria* spp. on basil using morphological, molecular and metabolite profiling techniques allowing to formulate effective disease management strategies against this pathogen.

2. Experimental procedures

2.1 Inoculum production and pathogenicity test.

Monoconidial isolates of *Alternaria* spp. (Table 1) were grown on Potato Carrot Broth (PCB) in a growth chamber in darkness at 22-24 °C for two weeks. For the pathogenicity test, basil plants cv. Genovese gigante Italiko (Furia sementi, Parma, Italy) were produced by putting seeds into a steamed potting soil mixture (peat: composted broadleaf bark: clay, 60:20:20 *v*/*v*/*v*) in plastic pots (2 L capacity) and maintained at 22 °C, with 12 hours/day of fluorescent light. Forty-day-old plants were artificially inoculated by using a conidial and mycelial suspension at 1-5 $\times 10^5$ CFU/mL. Five plants were inoculated with each *Alternaria* isolate originally obtained from plants and seeds. Uninoculated plants were prepared similarly but sprayed with deionized water only. After artificial inoculation, the pots were placed in a plastic tray (100 ×100 × 50 cm) covered with a transparent polyethylene film (50 µm thick) in order to keep the relative humidity at 95-100 % and stored for 7 days in a greenhouse at 24±1 °C, and 8 hours/day fluorescent light. Plants were checked two weeks after inoculation for disease development by evaluating disease severity (DS, expressed as % of affected leaf area) on 20 leaves/plant by using a rating scale of 0 to 5 (0 = no symptom; 1 = up to 5 % of infected leaf area; 2

= 6 to 10 % of infected leaf area; 3 = 11 to 25 % of infected leaf area; 4 = 26 to 50 % of infected leaf area; 5= 51 to 100 % infected leaf area).

2.2 Morphological characterization

For morphological examination, the fungal plates were evaluated after 10 days at × 40 magnification with a NIKON (Eclipse55t) microscope. Conidial characteristics, body and beak length, shape, and number of longitudinal and transverse septa of twenty conidia per isolate were measured. The sporulation pattern was evaluated under the Stereo microscope (Leica M165C) considering the length of conidial chains and branching type (Simmons and Roberts 1993). The colony and sporulation characteristics of reference strains of *A. alternata*, *A. tenuissima* and *A. arborescens* were compared with those of the isolates from basil.

2.3 DNA Extraction and PCR amplification

Single-spore cultures of the isolates (Table 1) were grown in potato dextrose broth (PDB) (Sigma Aldrich, Germany) on a rotary shaker (120 rpm) for 10 days at 22 °C. The mycelial mats were collected by filtration through Whatman No.1 filter paper and stored at -20 °C. The total genomic DNA was obtained using the E.Z.N.A Fungal DNA mini kit (OMEGA Bio-Tek, Norcross, GA, USA), according to the manufacturer's instructions.

Twenty µl of reaction material using approximately 50 ng of genomic DNA were used for amplification of the different genes, using 2.5 mM deoxynucleotide triphosphates, 2.5 mM $MgCl_2$, 0.5 µM of each primer, QIAGEN reaction buffer diluted 1:10 and 1U of Taq DNA polymerase (QIAGEN, Chatsworth, CA, USA) in a T-100 thermal cycler (Bio-Rad Japan, Tokyo, Japan). The PCR products (Supplementary Table 1) were checked by gel electrophoresis at 1 % agarose, and amplicons purified through QIAqick Columns (QIAGEN, Valencia, California) following the manufacturer's instructions.

2.4 Phylogenetic analysis

DNA concentration was measured using Nanodrop 2000 (Thermo Fisher, USA), sequencing was performed in both directions using external service of Macrogen Europe (Amsterdam, The Netherlands). Contig of forward and reverse sequences were carried out using DNA Baser (Heracle BioSoft SRL, Romania). Sequences of the isolates and reference strains were deposited in GenBank, the accession numbers are listed in Table 1.

Sequences were aligned in CLUSTALW (multiple sequence alignment) using MEGA 6 program (Tamura et al. 2013) and further edited manually and trimmed obtaining a data set of 816, 331, 625, 454, 582, 563 and 762 bp for mtLSU, EF-1α, β-TUB, endoPG, melanin, OPA 10-2, OPA 1-3, respectively. 2888 bp concatenated data sets were obtained with the mtLSU, EF-1α, β-TUB, endoPG, melanin sequences, while OPA 10-2 and OPA 1-3 were included in the concatenated tree obtaining a 3869 bp data set to perform phylogenetic tree with higher resolution for isolates belonging to the *A.* sect *Alternaria*.

The best-fit nucleotide model for each dataset was determined using Findmodel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html). For mtLSU was used TrN (Tamura-Nei Plus Gamma); for EF-1α, β-TUB, endoPG, THN reductase and for the concatenated trees were used GTR (General Time Reversible and gamma-distributed rates (G)) while Kimura 2-Parameter and gamma-distributed rates (G) were used for OPA10-2 and OPA 1-3 trees. For Bayesian analysis (Huelsenbeck and Ronquist 2001), the best-fit model of each region was estimated using TOPALI v.2.5 (Milne *et al*. 2004): Kimura 80 (K80) model and gamma-distributed rates (G) was the model used for EF-1α, LSU, endoPG, OPA10-2, and OPA1-3; General Time Reversible and gamma-distributed rates (G) were used for β-TUB analysis; symmetrical model and gamma-distributed rates (G) (SYM+G) for THN reductase and concatenated trees.

Maximum likelihood analysis was carried out using Mega 6.0.6 (Gardiner et al. 2012) with 1000 bootstrap replicates to evaluate the stability of each individual locus. For the concatenated dataset analysis, an independent model of nucleotide evolution was used for each locus. CBS reference strains (Table 2) were included in the analyses, while other *Alternaria* spp. sequences were obtained

from GenBank. Bayesian analyses were performed using TOPALI v2.5 program (Milne et al. 2004) discarding the first 25 % of the iterations as burn-in and with 2,000,000 generations and sampling frequency of one tree every 1000 iteration. Tree topologies were adjusted using FigTree v1.4.3.

2.5 Mycotoxin extraction

According to Brzonkalik et al. (2011), mycotoxin production for each isolate was induced using a modified Czapek-Dox medium: 10 g/L glucose, 0.162 g/L NH_4NO_3, 1.7 g/L KH_2PO_4, 0.85 g/L $MgSO_4$, 0.425 g/L NaCl, 0.425 g/L KCl, 0.017 g/L $FeSO_4$, 0.017 g/L $ZnSO_4$ and 1.7 g/L yeast extract, final pH 5.5. Cultures were inoculated with three mycelia plugs (2 mm diam.) in 30 mL of medium, performed in triplicate and incubated in the dark at 28 °C. After 8 days, cultures were filtered, and a liquid-liquid extraction was performed. An aliquot of 5 mL was adjusted to pH 2 with HCl and transferred to a separating funnel. Ten mL of dichloromethane were used for *Alternaria* mycotoxin extraction for three times and the mixture was shaken for 1 min. each time. The dichloromethane extracts were mixed, collected in a flask and evaporated to dryness in a rotary evaporator at 35 °C. The residue was dissolved in 500 µL of H_2O:CH_3OH 1:1 for the HPLC-MS/MS analysis.

The production of *Alternaria* mycotoxins was evaluated also *in vivo*. Artificially inoculated leaves of basil were extracted by solid-liquid extraction. All samples were homogenized, and 3 g of each sample placed in a centrifuge tube with 500 µL of HCl and 20 mL of extraction solution (CH_3OH:CH_3CN:H_2O 10:45:45 v/v/v adjusted to pH 3 with *o*-phosphoric acid). The mixture was shaken for 30 min in an ultrasonic bath and then centrifuged at 6000 rpm for 5 min. Extracts were transferred to a new centrifuge tube with 20 mL of toluene, vortexed and centrifuged at 6000 rpm for 2 min. The organic phase was evaporated to dryness in a rotary evaporator at 60 °C and the residue dissolved in 500 µL of H_2O:CH_3OH 1:1 for the HPLC-MS/MS analysis.

2.6 Instrumental analysis

Analyses of mycotoxins were carried out by using a 1260 Agilent Technologies system consisting of a binary pump and a vacuum degasser, connected to a Varian auto-sampler Model 410 Prostar (Hansen Way, CA, USA) equipped with a 20 µL loop coupled with a Varian 310-MS TQ Mass Spectrometer. The separation of mycotoxins was performed using a Gemini-NX C18 (150 × 3.0 mm 3.0 µm, Phenomenex, Torrance, CA, USA) under a flow of 200 µL/min. Solvent A was H_2O and solvent B was CH_3OH, both with ammonium acetate 5 mM. HPLC analysis was performed using a linear gradient from 70 % to 100 % of solvent B in 7 min. Samples were ionized using 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 the analyses were: *m/z* 196 > 139 CE 20 eV and *m/z* 196 > 112 CE 24 eV for TeA; *m/z* 257 > 213 CE 22 eV and *m/z* 257 > 147 CE 34 eV for AOH; *m/z* 271 > 256 CE 22 eV an *m/z* 271 > 228 CE 28 eV for AME, *m/z* 413 > 271 CE 16 eV and *m/z* 413 > 141 CE 18 eV for TTX, *m/z* 351 > 297 CE 25 eV and *m/z* 351 > 263 CE 35 eV for ATX-I. The collision gas (Ar) pressure was set at 2 mbar for all experiments.

2.7 Method validation

The developed analytical method was evaluated for recovery, limit of detection (LOD), limit of quantification (LOQ) and matrix effect (ME) for TeA, AOH, AME, and TTX in accordance with Matuszewski et al. (2003); the standard of ATX-I was not available, the quantification of this analyte was done using the AME calibration curve. Three sets of samples were prepared: (i) standards of the analytes were dissolved in mobile phase; (ii) the extraction was performed from uninoculated basil leaves, afterward, the analytes were added; (iii) standards were added before extraction procedure at three concentration levels on uninoculated leaves. Signal-to-noise method was used to determine limits of detection (LOD) and quantification (LOQ) for each matrix. S/N ratio 3:1 was used for the determination of LOD, while 10:1 for LOQ.

3. Results

3.1 Phylogeny

Five-gene phylogeny, including β-TUB, endoPG, mtLSU, THN reductase and EF-1α was used for the basil isolates and CBS reference strains to their relatedness (Figure 1). Maximum likelihood and Bayesian analysis were carried out to compare the two different phylogenetic approaches; the same isolate clustering was observed for both cases (Supplementary files). However, some isolates produced a low sequence quality and were not included in the analysis, as Bas 19-BI and the CBS 477.83 for endoPG and Bas 1/13 for THN reductase gene. All phylogenetic trees were deposited on TreeBASE with the submission ID 21392.

Twelve out of 18 basil isolates grouped together with *A. alternata* reference CBS strains CBS 916.96, CBS 115152, CBS 102.47, CBS106.24, while 3 isolates (Bas G1, Bas 31371L7, and Bas 31369L36) grouped together with the references strains of *A. arborescens* species complex (AASC) CBS 109730, CBS 116329, CBS 124274, ATCC 34509 (Figure 1). On the other hand, the isolate Bas 1/13 grouped with 86 % bootstrap with CBS 477.83 identified as *A. cichorii* belonging to *A.* sect *Porri* (Woudenberg et al. 2014). Individual phylogeny of EF-1α and mtLSU regions were performed to identify Bas 19-BI (Figure 2). The mtLSU phylogeny identified Bas 19-BI as a species within the *A.* sect. *Dianthicola*, together with *A. elegans, A. simsimi* and *A. dianthicola* (data not shown), while the EF-1α clustered the same isolate within *A. cheiranthi* within the *A.* sect. *Cheiranthi* (Woudenberg et al. 2013).

The mtLSU phylogeny provided a reduced resolution compared to the other regions analyzed clustering 15 out of 18 isolates in the same clade with references strains of *A.* sect. *Alternaria*. Bas G1 clustered in a second subclade together with *A. arborescens* reference strains, while Bas 23-1BA represented an independent lineage (data not shown). The EF-1α, β-TUB and endoPG phylogeny clustered the majority of isolates within *A. alternata*, Bas G1, Bas 31371L7, Bas 31369L36 were part of the *A. arborescens* species complex, while THN reductase phylogeny clustered only Bas 31369L36 and Bas G1 in the *A. arborescens* species complex (data not shown).

Phylogeny data generated with OPA 10-2 and OPA 1-3 alignments were analyzed only for the isolates previously identified within *A.* sect *Alternaria*. The combined tree with β-TUB, endoPG, mtLSU, THN reductase, EF-1α, OPA 10-2 and OPA 1-3 produced a 3869 bp dataset, which was analyzed using the maximum likelihood with GTR model and gamma distribution producing five different clades (Figure 3). The first major clade with isolates identified as *A. alternata,* was divided into three different subclades, the first included the reference CBS 115152 and the seven isolates Bas 27-1BA, Bas 4-1BA, Bas 2/10, Bas 31369L10, Bas 3169L14, Bas 31371L20, and Bas 31371L10, with a sub-group formed by Bas 27-1BA, Bas 4-1BA and Bas 2/10 with 65 % bootstrap. The second subclade was composed of four isolates grouped with four reference strains including CBS 118488, while the third clade was formed by only two isolates, Bas 31371L36 and Bas 1/10. The isolate CBS 918.96 identified as *A. tenuissima* did not cluster with basil isolates. A second clade was composed of three basil isolates and four reference strains identified as *A. arborescens* (Figure 3).

3.2 Morphology

The average conidia size of the *Alternaria* isolates varied within a broad range from 15.0 to 25.0 µm (variation 6.9-58.9 µm) for conidial length and from 18.8 to 30.5 µm (variation 11.7-70.7 µm) for total body length (incl. beak) (Table 3). There was a good correlation between total conidia body length and beak length, i.e. the longer the total body size the longer the beak length (Figure 4). The *Alternaria* isolates showed moderate to high sporulation except for Bas 1/10, Bas 1BA, Bas G1 and Bas 1/13 that did not produce any conidia. *Alternaria alternata* complex, *Alternaria arborescens*, and the isolates of other *Alternaria* species were differentiated based on catenulation characteristics and morphology of conidia (Table 3). Most of the isolates (Bas 6/10, Bas 23-1BA, Bas 31369L10, Bas 31369L14, Bas 31371L10, Bas 31371L17, Bas 31371L20 and Bas 31371L36) belonged to *A. alternata* species-group type 4 showing conidial chains of 4 to 10 conidia in length with abundant secondary and ternary branches. The *Alternaria* isolates Bas 2/10, Bas 31371L10, Bas 27-1BA and Bas 4-1BA showed conidial chains with 5 to 20 conidia in length with occasional secondary branches

suggesting a strong similarity to *A. tenuissima* characteristics. The isolates Bas 31369L36 and Bas 31371L7 showed conidial chain branches with short secondary conidiophores in a tree-like formation, with a long primary conidiophore, suggesting that they belong to *A. arborescens* species-group 3 (Table 3). The identification performed by sporulation pattern yielded a good relationship to the clustering in the phylogenic trees.

3.3 Mycotoxin analysis in vitro

A total of 18 *Alternaria* isolates were analyzed for mycotoxin production. At least one mycotoxin type was produced by 89 % of the tested strains, only two strains, Bas 1/13 and Bas 19-BI, did not produce any mycotoxins (Table 4). TeA, AOH, AME, ATX-I, and TTX were simultaneously produced by 50 % of the isolates. Bas 2/10, Bas 27-1BA and Bas 4-1BA produced TeA, ATX-I, and TTX, whereas Bas 1/10, Bas 1BA and Bas 31369L10 produced only TeA and ATX-I.

All studied isolates, except Bas 1/13 and Bas 19-BI, were able to synthesize TeA, which was the main mycotoxin produced with a concentration ranging between 33.4 and 9510 μg/L (Table 4). AOH and AME were always produced simultaneously with an average production of 27.4 and 24.0 μg/L, respectively. ALT was not produced by any isolate, whereas ATX-I was synthesized by all strains, except Bas 1/13 and Bas 19-BI. Twelve out of 18 isolates produced TTX, in a range between 3.48 and 2030 μg/L. Bas 6/10 was the isolate with highest mycotoxin production, with a total amount of more than 10,000 μg/L. On the contrary, Bas G1 showed the lowest concentration of mycotoxins, with 2.66 μg/L of ATX-I (Table 4).

3.4 Relationship between phylogeny, morphology and mycotoxin production

The question can now be addressed whether phylogenetic relatedness, mycotoxin production, and sporulation group exhibit any relationship among the different isolates (Figure 5). As shown in the phylogenetic analysis, three strains were grouped in the *A. arborescens* species complex. Bas 31369L36 and Bas 31371L7 clustered together, they also belong to the same sporulation group (3)

and produce the same mycotoxins. Phylogenetically, Bas G1 was different to Bas 31369L36 and Bas 31371L7, and this result was consistent with a different mycotoxin production pattern and sporulation group (Figure 5). Furthermore, isolates Bas 27-1BA and Bas 4-1BA were in another phylogenic subclade and are identical, they belong to the *A. tenuissima* group and exhibited the same mycotoxin profile and sporulation type (2). The other 11 isolates belonged phylogenetically to *A. alternate*. In the Subclade 1 was possible to observe the presence of two groups. Subclade 1 was formed by Bas 31369L10, Bas 31369L14, Bas 31371L20 and Bas 31371L10. The sporulation type was identical for the 4 isolates (type 4), also the mycotoxin profile was the same, except for Bas 31369L10 which formed a mycotoxin group by itself. Bas 23-1BA was the only isolate belonging to sporulation group 6, it was also phylogenetically different from the other isolates of subclade 2. The isolates of subclade 3 (Bas 31371L36 and Bas 1/10) differed from each other for both mycotoxin production and sporulation group.

3.5 Pathogenicity

The inoculation method used in this study resulted in disease severities, with consistent results in both sets of trials (Figure 6). The first leaf spot symptoms were observed 9-12 days after artificial inoculation with the isolates. When comparing disease severity among isolates belonging to different *Alternaria* species-groups, no significant differences have been detected (Figure 6). The highest disease severity was provided by Bas 31371L20 and Bas 6/10 (about 55 % affected leaf surface) belonging to *A. alternata*, while Bas 27-1BA (*A. tenuissima*) and Bas G1, Bas 31369L36 and Bas 31371L7 (*A. arborescens*) resulted in lowest values (20-24 % affected leaf surface), significantly lower than that of isolates in the *alternatae*-species-group.

3.6 Mycotoxin analysis on basil

In both liquid media and basil plants, the matrix effect had the same impact on TeA, AOH, and AME with a suppression of the ionization of the analytes (Supplementary Table 2). TTX in liquid media was not affected by matrix effect, while on basil an ionization enhancement was induced.

Of the 18 analyzed isolates, 16 were mycotoxin producers, whereas two, Bas 1/13 and Bas 19-BI, did not produce any of the studied analytes (Figure 6). The major mycotoxin producers were Bas 27-1BA, Bas 6/10 and Bas 23-1BA, with a total concentration (all 4 analytes together) of 17.0, 15.7 and 12.4 mg/L, respectively, whereas the lowest producers of mycotoxins were Bas G1, Bas 31368L14 and Bas 31369L10 with a total concentration of 0.4, 1.9 and 2.6 mg/L, respectively (Figure 6). On average, TeA was detected in basil plants at highest concentrations among all mycotoxins (with 2.4 mg/L per sample), while AME concentrations were lowest (0.5 mg/L). Bas 27-1BA produced the highest concentration of TeA (12.9 mg/L) of all isolates, whereas Bas 31369L10 and Bas 31369L36 did not produce TeA at all, and Bas 31371L7 only very low concentrations (0.3 mg/L). AOH was produced mainly by Bas 6/10 (11.3 mg/L), whereas 4 out of 16 isolates did not show the capability to produce this molecule. Bas 31371L7, Bas 6/10 and Bas 31369L36 showed the highest production of AME (1.7, 1.6 and 1.6 mg/L, respectively), while three isolates did not produce it at all. With a production of 4.2 mg/L, Bas 4-1BA produced by far the highest concentration of TTX, however, this mycotoxin was produced by isolates although in different concentrations.

4. Discussion

The economically most important pathogens belonging to the *Alternaria* genus are within the small-spored *Alternaria* species, embracing especially the *Alternaria* sect. *Alternaria* complex with over 60 host-specific species (Woudenberg et al. 2013) of which *A. alternata* is the most widespread within the genus. It is an important saprophyte on dead organic material but also an opportunistic human pathogen and a plant pathogen causing leaf spot disease in over 100 different host plant species (Rotem 1994). The *Alternaria* sect. *Alternaria* includes also additional plant pathogenic species such as *A. longipes* which is the principal cause of brown spot of tobacco, *A. mali* causing Alternaria blotch on apple, *A. gaisen* which is the causal agent of Japanese pear and *A. arborescens* which causes stem canker on tomato.

The taxonomy of the *Alternaria* genus suffered from many controversies until the current classification with 27 sections based on molecular phylogeny (Lawrence et al. 2016). Molecular analyses have some challenges to overcome as evolutionary differences caused by lineages sorting and recombination (Andrew et al. 2009), have produced incongruent results for each locus studied, which complicates the selection of suitable molecular markers for phylogeny and systematic analysis. Different loci such as elongation factor, mtLSU, coding gene sequence for β-TUB, calmodulin, actin, chitin synthase and non-coding regions as OPA 1-3, OPA 1-2 and OPA 10-2 (Peever et al. 2005, Andrew et al. 2009) have been widely used for characterization of *Alternaria* population studies in citrus (Peever et al. 2004), apple (Rotondo et al. 2012), pistachio (Pryor and Michailides 2002), and in *Brassicaceae* (Siciliano et al. 2017). However, calmodulin, actin, and chitin synthase gene sequences were unable to determine the variation among small-spored isolates, especially the *A.* sect. *Alternaria* (Peever et al. 2004), so these molecular markers were excluded in this study due to the low polymorphic rate compared with other molecular markers such as OPA1-3 and OPA10-2 (Stewart et al. 2013). Some authors described EF-1α and β-TUB as not being useful markers to differentiate the *Alternaria* sections due to their low probability of molecular evolution (Lawrence et al. 2013). However, for our basil isolates, these loci had enough phylogenetic signals to discriminate the *Alternaria alternata* species complex from other species of the *A.* sect. *Alternaria*, while mtLSU was a locus with low resolution. Woudenberg et al. (2015) using a multi-gene phylogeny described the *A.* sect *alternata*, synonymised 35 morphospecies as *A. alternata*. Our combined phylogenetic tree confirmed the difficulties in the identification of the different morphospecies in this section. In particular, *A. alternata* and *A. tenuissima* grouped together as in Woudenberg et al. (2015) work (Figure 3). Morphological characteristics have been used frequently for the identification and differentiation of the *Alternaria* species-groups. However, it is a time-consuming process and needs much mycological knowledge. In general, morphological observations and, in particular, the sporulation pattern allowed us to differentiate among *A. alternata*, *A. arborescens* and *A. tenuissima* isolates (Simmons 1995). Simmons and Roberts (1993) subdivided the small-spored *Alternaria* into

six morphological groups. *A. alternata* produce short bushy clumped conidiophores with extensive secondary sporulation, *A. tenuissima* is characterized by a larger and more robust spore with moderate to short conidial catenation (5–10), *A. arborescens* taxa produced short (50–70 μm) to very long (100–150 μm) conidiophores that produced copious amounts of secondary sporulation by the elongation of subconidium conidiophores.

Therefore, additional properties including molecular traits and secondary metabolites may be considered for species classification resulting in a combined approach useful for the characterization of the *Alternaria* species-group.

It is well known that mycotoxin production depends on several factors such as temperature, soil, water activity, light and plant nutrition. Production of TeA, AME, and AOH in *A. alternata* reached a maximum when water activity was 0.97 (Sanchis and Magan 2004). A temperature of 28 °C was the best for the production of AOH and AME while 21 °C was optimal for TeA (Hasan 1996). Häggblom and Niehaus (1986) described an inhibitory effect of low light intensity on the production of AOH and AME, which was also confirmed by Pruβ et al. (2014) for AOH, AME and ATX-I production. In addition, also carbon and nitrogen sources influence mycotoxin production in *A. alternata* (Brzonkalik et al. 2011), in particular, the production of polyketide compounds (AOH and AME) was affected both by carbon and nitrogen while TeA production was influenced strongly by carbon sources. Mycotoxin production under field conditions is strongly dependent on the species and cultivar of host plants, by growing, harvesting and environmental conditions (Lauren and Fadwa 2008). In our study, mycotoxin production in basil plants was different from *in vitro* conditions, in particular, AOH, AME, and TTX concentration generally increased in planta. This increase may have been induced both by host plant nutrients and light exposure, highlighting a potential increase in risks for human health when basil plants are infected by *Alternaria* under field conditions. In Table 4, analyses conducted on modified Czapek-Dox medium have shown different concentration of AOH, AME, and TTX among our isolates. The isolates classified, using the morphological features, as *A. tenuissima* (Bas 2/10, Bas 27-1BA and Bas 4-1BA) and three of *A. alternata* isolates (Bas 1/10, Bas

1-BA and Bas 31369L10) did not produce AOH and AME. Nevertheless, the TTX production was a significant factor that differentiates the isolates belonging to the two species, in fact, *A. tenuissima* isolates always produced this mycotoxin. Modified Czapek-Dox medium induced a different mycotoxins production compared to DRYES medium (data not shown), that was widely used for this type of analysis (Andersen et al., 2005; Polizzotto et al., 2012). Most of the 18 isolates from basil plants and seeds belonged to the *A. alternata* species-group, they were more aggressive than isolates of other species such as *A. arborescens* and *A. tenuissima.* The latter two species have not been previously known to cause disease in basil but were pathogenic on basil in this study probably as a result of expansion in host range or an increase of virulence.

In conclusion, this study contributed to reaching a successful classification of *Alternaria* isolates from basil because we examined a combination of morphological, pathological, biochemical and molecular properties of the isolates and analyzed them with appropriate technologies. In fact, multilocus analysis was not resolutive for the complete classification of our isolates. The controversy between morphological analysis and molecular data has boots us to follow more-detailed analytic pathway such as the secondary metabolite production profile. This approach combined with the previous data has been revealed as a new and useful tool to discriminate some of the species within *A.* sect. *Alternaria.* The majority of the isolates studied belong to the *A. alternata* species-group, however, we were able to differentiate also some other species within this species group such as *A. arborescens* and *A. tenuissima*. Particularly useful was the analysis of secondary metabolite production. AOH, AME, and TTX production helped us to differentiate the isolates of *A. tenuissima* from *A. alternata* and *A. arborescens*. These mycotoxins analysis could be a potential discriminant within *A. alternata* species complex.

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Table 1. List of the 18 *Alternaria* isolates collected from basil seeds and leaves in northern Italy and deposited accession numbers for the 7 molecular loci.

			GenBank Accession number						
Isolate code	Origin	Host	β-TUB	endoPG	EF-1α	THN reductase	mtLSU	OPA 1-3	OPA 10-2
Bas 1/10	Leaf	Basil cv. Superbo	MF070269	MF070304	MF070340	MF070376	MF070411	MF070441	MF070472
Bas 2/10	Leaf	Basil cv. Gecom	MF070270	MF070305	MF070341	MF070377	MF070412	MF070442	MF070473
Bas 6/10	Leaf	Basil cv. Italico	MF070271	MF070306	MF070342	MF070378	MF070413	MF070443	MF070474
Bas 1/13	Leaf	Basil cv. Profumo	MF070273	MF070308			MF070415		
Bas 19-BI	Seed	Basil cv. Genovese italiano classico	MF070258		MF070327	MF070363	MF070398		
Bas 1-BA	Seed	Basil cv. Genovese	MF070260	MF070293	MF070329	MF070365	MF070400	MF070430	MF070461
Bas 23-1BA	Seed	Basil cv. Genovese	MF070261	MF070294	MF070330	MF070366	MF070401	MF070431	MF070462
Bas 27-1BA	Seed	Basil cv. Genovese	MF070259	MF070292	MF070328	MF070364	MF070399	MF070429	MF070460
Bas 4-1BA	Seed	Basil cv. Genovese		MF070295	MF070331	MF070367	MF070402	MF070432	MF070463
Bas 31369L10	Seed	Basil cv. Genovese	MF070262	MF070296	MF070332	MF070368	MF070403	MF070433	MF070464
Bas 31369L14	Seed	Basil cv. Genovese	MF070263	MF070297	MF070333	MF070369	MF070404	MF070434	MF070465
Bas 31369L36	Seed	Basil cv. Genovese	MF070264	MF070298	MF070334	MF070370	MF070405	MF070435	MF070466
Bas 31371L10	Seed	Basil cv. Genovese	MF070266	MF070300	MF070336	MF070372	MF070407	MF070437	MF070468
Bas 31371L17	Seed	Basil cv. Genovese	MF070267	MF070301	MF070337	MF070373	MF070408	MF070438	MF070469
Bas 31371L20	Seed	Basil cv. Genovese		MF070302	MF070338	MF070374	MF070409	MF070439	MF070470
Bas 31371L36	Seed	Basil cv. Genovese	MF070268	MF070303	MF070339	MF070375	MF070410	MF070440	MF070471
Bas 31371L7	Seed	Basil cv. Genovese	MF070265	MF070299	MF070335	MF070371	MF070406	MF070436	MF070467
Bas G1	Seed	Basil cv. Genovese Albahaca	MF070272	MF070307	MF070343	MF070379	MF070414	MF070444	MF070475

Table 2. List of reference strains of *Alternaria* spp. and accession numbers.

				GenBank Accession number						
Isolate code	Specie	Host	Origin	β-TUB	endoPG	EF-1α	THN reductase	mtLSU	OPA 1-3	OPA 10-2
CBS 115152	*A. alternata*	*Psychotria serpens*	China	MF070245	MF070280	MF070315	MF070350	MF070386	MF070420	MF070449
CBS 116329	*A. alternata*	*Malus domestica*	Germany	MF070242	MF070277	MF070312	MF070347	MF070383	MF070417	MF070446
CBS 916.96	*A. alternata*	*Arachis hypogea*	India	MF070244	MF070279	MF070314	MF070349	MF070385	MF070419	MF070448
ATCC 34509	*A. alternata*	*Malus domestica*	Japan	MF070243	MF070278	MF070313	MF070348	MF070384	MF070418	MF070447
CBS 109730	*A. arborescens*	*Solanum lycopersicum*	The USA	MF070255	MF070289	MF070324	MF070360	MF070395	MF070427	MF070458
CBS 124274	*A. arborescens*	*Prunus* sp.	Denmark	MF070253	MF070287	MF070322	MF070358	MF070393	MF070425	MF070456
CBS 918.96	*A. arborescens*	*Dianthus* sp.	UK	MF070252	MF070286	MF070321	MF070357	MF070392	MF070424	MF070455
CBS 122591	*A. alycipyricola*	*Pyrus communis*	The USA	MF070239	MF070274	MF070309	MF070344	MF070380		
CBS 477.83	*A. chichorii*	*Cichorium intybus* var. *foliosum*	Netherlands	MF070249		MF070476	MF070354			
CBS 102.47	*A. citri*	*Citrus sinensis*	The USA	MF070246	MF070281	MF070316	MF070351	MF070387	MF070421	MF070450
CBS 90051	*A. gaisen*	*Pyrus pyrifolia*	Japan	MF070241	MF070276	MF070311	MF070346	MF070382	MF070416	
CBS 118488	*A. gaisen*	*Pyrus pyrifolia*	Japan	MF070254	MF070288	MF070323	MF070359	MF070394	MF070426	MF070457
CBS 123925	*A. hungarica*	*Triticum aestivum lesions*	Hungary	MF070257	MF070291	MF070326	MF070362	MF070397		
CBS 113.44	*Alternaria* sp.	*Matthiola incana*	Unknown	MF070240	MF070275	MF070310	MF070345	MF070381		MF070445
CBS 106.24	*A. mali*	*Malus sylvestris*	The USA	MF070248	MF070283	MF070318	MF070353	MF070389	MF070422	MF070452
CBS 112003	*A. radicicina*	*Daucus carota* L.	Netherlands	MF070247	MF070282	MF070317	MF070352	MF070388		MF070451
CBS 124278	*A. tenuissima*	*Prunus* sp.	Denmark	MF070256	MF070290	MF070325	MF070361	MF070396	MF070428	MF070459
CBS 124283	*A. tenuissima*	*Oryza* sp.	Russia	MF070250	MF070284	MF070319	MF070355	MF070390	MF070423	MF070453
CBS 121546	*A. ventricosa*	*Pyrus bretschneideri*	China	MF070251	MF070285	MF070320	MF070356	MF070391		MF070454

Table 3. Conidia size (µm, mean in parenthesis) and number of septa of *Alternaria* isolate grown on PCA media.

Isolate	*Alternaria* species	Size [µm]				Number of septa			Sporulation group*
		Length	Wide	Beak	Total body	Transversal	Longitudinal	Oblique	
Bas 2/10	*A. tenuissima*	15.8-39.4 (25.0±6.4)	5.7-13.6 (9.3±2.1)	0.0-12.5 (5.6±3.4)	18.8-51.7 (30.5±8.4)	1-4 (3.0±0.9)	0-2 (0.3±0.6)	0-0 (0.0±0.0)	2
Bas 6/10	*A. alternata*	13.4-36.4 (21.7±6.7)	8.5-14.9 (10.5±1.8)	1.3-17.8 (6.1±3.9)	16.7-43.5 (27.8±8.5)	1-4 (2.8±1.1)	0-2 (0.2±0.5)	0-1 (0.1±0.2)	4
Bas 19-BI	*Alternaria* sp.	11.6-18.0 (15.0±1.7)	9.8-16.2 (13.0±1.7)	9.8-16.2 (13.0±1.7)	21.4-33.3 (28.0±3.0)	0-1 (0.8±0.4)	0-1 (0.4±0.5)	0-1 (0.4±0.5)	Group not assigned
Bas 23-1BA	*A. alternata*	14.7-27.7 (19.9±3.5)	6.6-14.6 (10.7±2.5)	0.0-14.6 (5.2±4.2)	16.5-33.6 (25.2±4.9)	1-4 (2.7±0.8)	0-2 (0.3±0.6)	0-0 (0.0±0.0)	4
Bas 27-1BA	*A. tenuissima*	6.9-47.7 (21.9±7.8)	3.2-13.8 (8.3±2.5)	0-23.1 (5.6±4.7)	21.9-70.7 (30.2±11.5)	0-7 (2.5±1.6)	0-1 (0.1±0.2)	0-0 (0.0±0.0)	2
Bas 4-1BA	*A. tenuissima*	11.9-34.0 (20.9±5.4)	5.9-14.9 (10.1±2.2)	0.0-11.2 (5.4±3.1)	20.7-40.2 (29.2±5.2)	1-4 (2.6±1.1)	0-1 (0.2±0.5)	0-1 (0.1±0.2)	2
Bas 31369 L10	*A. alternata*	9.4-30.8 (19.2±6.4)	6.9-12.9 (9.7±1.6)	0.0-9.5 (5.6±2.4)	13.0-38.3 (24.8±7.0)	1-5 (2.8±1.1)	0-1 (0.1±0.3)	0-0 (0.0±0.0)	4
Bas 31369 L14	*A. alternata*	11.3-42.2 (18.6±6.5)	5.7-11.7 (8.1±1.3)	0.0-10.4 (4.6±2.6)	15.0-46.5 (23.2±7.1)	0-5 (2.0±1.1)	0-0 (0.0±0.0)	0-0 (0.0±0.0)	4
Bas 31369 L36	*A. arborescens*	8.9-27.4 (15.3±4.2)	6.4-12.4 (8.9±1.5)	0.0-8.7 (3.5±2.6)	11.7-33.9 (18.8±5.5)	1-3 (1.7±0.7)	0-1 (0.1±0.2)	0-2 (0.1±0.4)	3
Bas 31371 L10	*A. alternata*	8.6-58.9 (21.6±10.4)	7.4-18.0 (10.1±2.4)	0.0-11.0 (5.4±2.8)	17.9-67.4 (27.0±11.2)	1-5 (2.7±0.9)	0-1 (0.1±0. 3)	0-1 (0.1±0.3)	4
Bas 31371 L17	*A. alternata*	10.6-31.8 (20.3±6.7)	7.3-13.3 (10.0±2.4)	0.0-10.2 (4.9±2.6)	12.5-42.0 (25.2±8.3)	1-4 (2.6±1.0)	0-1 (0.1±0.3)	0-0 (0.0±0.0)	4
Bas 31371 L20	*A. alternata*	10.1-45.7 (24.3±9.5)	6.8-14.2 (11.4±2.1)	0.0-10.9 (4.6±2.2)	15.2-38.7 (25.7±6.5)	1-6 (3.4±1.3)	0-1 (0.3±0.4)	0-1 (0.1±0.2)	4
Bas 31371 L36	*A. alternata*	11.2-38.6 (21.8±9.2)	7.0-14.9 (10.6±1.7)	0.0-14.2 (5.0±3.8)	13.4-48.2 (26.8±10.7)	1-5 (3.0±1.2)	0-2 (0.4±0.7)	0-1 (0.1±0.2)	4
Bas 31371 L7	*A. arborescens*	11.8-47.5 (21.4±8.4)	6.2-13.7 (10.5±2.2)	0.0-6.8 (3.2±2.1)	13.3-34.0 (21.6±6.0)	1-6 (2.7±1.4)	0-1 (0.2±0.4)	0-1 (0.2±0.4)	3

No conidia were developed for Bas 1/10, Bas 1BA, Bas G1, and Bas 1/13
*Sporulation group assigned in accordance with Simmons & Roberts 1993

Table 4. Mycotoxin production [ng/mL] *in vitro* by *Alternaria* isolate after 8 days of incubation at 28 °C in the dark.

Isolates	Specie	TeA	AOH	AME	ALT	ATX-I	TTX
Bas 1/10	*A. alternata*	657±101	n.d.	n.d.	n.d.	91.2±19.4	n.d.
Bas 6/10	*A. alternata*	9510±454	30.0±2.79	26.6±2.32	n.d.	289±24.8	152±26.2
Bas 1BA	*A. alternata*	3040±558	n.d.	n.d.	n.d.	27.3±11.5	n.d.
Bas 23-1BA	*A. alternata*	6130±874	14.4±2.65	11.1±3.61	n.d.	37.8±11.6	250±52.6
Bas 31369L10	*A. alternata*	33.4±3.71	n.d.	n.d.	n.d.	11.0±3.82	n.d.
Bas 31369L14	*A. alternata*	4190±588	7.09±2.28	16.1±5.18	n.d.	61.6±6.31	2030±570
Bas 31371L10	*A. alternata*	7680±970	27.5±6.79	47.4±11.6	n.d.	63.5±7.81	297±84.4
Bas 31371L17	*A. alternata*	1640±378	2.08±0.28	0.45±0.17	n.d.	51.7±6.29	6.77±1.92
Bas 31371L20	*A. alternata*	5110±470	123±14.0	75.1±8.79	n.d.	117±4.98	3.48±0.563
Bas 31371L36	*A. alternata*	6480±852	26.1±5.16	17.9±8.50	n.d.	409±61.1	1880±265
Bas G1	*A. arborescens*	62.7±20.5	2.94±0.10	10.1±0.45	n.d.	2.66±0.74	n.d.
Bas 31369L36	*A. arborescens*	1510±290	10.2±3.59	13.4±4.48	n.d.	186±45.8	51.6±20.7
Bas 31371L7	*A. arborescens*	1250±187	6.32±2.77	7.80±0.24	n.d.	12.8±5.34	7.05±0.75
Bas 2/10	*A. tenuissima*	3730±453	n.d.	n.d.	n.d.	42.5±8.15	16.7±2.48
Bas 27-1BA	*A. tenuissima*	4630±600	n.d.	n.d.	n.d.	236±28.9	455±121
Bas 4-1BA	*A. tenuissima*	1940±431	n.d.	n.d.	n.d.	126±29.5	122±23.7
Bas 1/13	*Alternaria* sp.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Bas 19-BI	*Alternaria* sp.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Mean values ± standard error of three independent biological experiments consisting of three technical replicates each.
n.d. = not detected.

Supplementary table 1. Loci, primer and sequences used for the phylogenetic analysis of *Alternaria* isolates from basil.

LOCUS	PRIMERS	SEQUENCE	REFERENCE
Mitochondrial ribosomal large subunit (mtLSU)	LSU1Fd	GRATCAGGTAGGRATACCCG	Crous *et al.*, 2009 Vilgalys and Hester, 1990
	LR5	TCCTGAGGGAAACTTCG	
Elongation factor 1-alpha (EF-1α)	EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn, 1999
	EF1-986R	TACTTGAAGGAACCCTTACC	
Endopolygalacturonase (endoPG)	PG3	TACCATGGTTCTTTCCGA	Isshiki *et al.*, 1997, 2001; amplifications as Peeves *et al.*, 2004; 2005
	PG2b	GAGAATTCRCARTCRTCYTGRTT	
OPA 10-2	OPA 10-2R	GATTCGCAGCAGGGAAACTA	Andrew *et al., 2009*
	OPA 10-2L	TCGCAGTAAGACACA TTCTACG	
OPA 1-3	OPA 1-3L	CAGGCCCTTCCAATCCAT	Peeves *et al.*, 2004
	OPA 1-3R	AGGCC CTTCAAGCTCTCTTC	
β-tubulin (β-TUB)	T1	AACATGCGTGAGATTGTAAGT	O' Donnell and Cigelnik 1997 Peeves *et al.*, 2004
	β-tub-2	ATCATGTTCTTGGGGTCGAA	
1,3,8-trihydroxynaphthalene reductase (THN reductase)	melanin-3	TCAATCGAGCAGACATGGAG	Peeves *et al.*, 2004
	melanin-4	CAACGCAGTTGACGGTGAT	

Supplementary table 2. Validation parameters for four mycotoxins investigated in liquid medium and on basil plants.

	Liquid medium				**Basil**			
	TeA	AOH	AME	TTX	TeA	AOH	AME	TTX
LOD	5.79	2.31	2.20	1.34	18.6	8.48	3.29	6.98
LOQ	19.3	7.69	7.35	4.45	62.0	28.3	11.0	23.3
ME (%)	27.2	47.7	34.8	182	28.9	35.5	17.1	101

Figure captions

Figure 1. Phylogenetic tree based on beta tubulin (β-TUB), endopolygalacturonase (endoPG), mitochondrial ribosomal large subunit (mtLSU), 1,3,8-trihydroxynaphthalene reductase gene (THN reductase) and elongation factor- 1alpha (EF-1α) sequences. The phylogenetic tree was constructed with Maximum Likelihood analysis using General Time Reversible plus Gamma model for differentiating *Alternaria* isolates from basil and references strains.

Figure 2. Phylogenetic tree representing the relationship between the *Alternaria* isolate Bas 19BI and references strain obtained from GenBank. A. Maximum Likelihood analysis with Tamura-Nei Plus Gamma model, based on mitochondrial ribosomal large subunit (mtLSU) sequence. B. Maximum Likelihood Analysis General Time Reversible plus Gamma model based on elongation factor-1 alpha sequences (EF-1α).

Figure 3. Phylogenetic tree based on beta tubulin (β-TUB), endopolygalacturonase (endoPG), mitochondrial ribosomal large subunit (mtLSU), 1,3,8-trihydroxynaphthalene reductase gene (THN reductase), elongation factor- 1alpha (EF-1α), OPA 10-2 and OPA 1-3 sequences. The phylogenetic tree was constructed with Maximum Likelihood analysis using General Time Reversible plus Gamma model for differentiating *Alternaria* isolates from basil and references strains.

Figure 4. Length of conidia body and beak of *Alternaria* sp. isolates obtained on PCA media after 10 days of incubation.

Figure 5. Isolates differentiation based on maximum likelihood phylogenetic analyses based on β-TUB, endoPG, mtLSU, THN reductase, EF-1α, OPA 10-2 and OPA 1-3 sequences. The blue and green bars represent the mycotoxin production and the external circle indicates the sporulation group for each isolate.

Figure 6. Phylogeny, disease severity [%] and mycotoxin production [ng/g] by 16 *Alternaria* isolates on basil after 14 days grown under greenhouse conditions.

Bas 13/10 and Bas 19-BI did not produce any of the investigated metabolites.

*LV, low virulence (10-30 % of infected leaves); MV, moderate virulence (31-60 % of infected leaves).