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A polyphasic approach for the characterization of endophytic *Alternaria* strains isolated from grapevines

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ABSTRACT

A polyphasic approach was set up and applied to characterize 20 fungal endophytes belonging to the genus *Alternaria*, recovered from grapevine in different Italian regions.
Morphological, microscopical, molecular and chemical investigations were performed and the obtained results were combined in a pooled cluster analysis. Following morphological analyses, all strains were grouped according to their three-dimensional sporulation pattern on PCA and to the colony characteristics on different substrates. After DNA extraction, all strains were analyzed by RAPD-PCR and the resulting profiles were subjected to cluster analysis. The metabolites extracted from the 20 *Alternaria* endophytes were analyzed by a HPLC and the resulting metabolite profiles were subjected to multivariate statistic analyses. In comparison with reference 'small-spored' *Alternaria* species, the 20 strains were segregated into two morphological groups: one belonging to the *A. arborescens* species-group and a second to the *A. tenuissima* species-group. RAPD analysis also showed that grapevine endophytes belonged to either the *A. arborescens* or the *A. tenuissima* species-group and that they were molecularly distinct from strains belonging to *A. alternata*. Chemotaxonomy gave the same grouping: the grapevine endophytic strains belong to *A. arborescens* or *A. tenuissima* species-groups producing known metabolites typical of these species-groups. Interestingly, the 20 grapevine endophytes were able to produce also a number of unknown metabolites, whose characterization could be useful for a more precise segregation of the two species-groups.
The results show how complementary morphological, molecular and chemical data can clarify relationships among endophyte species-groups of low morphological divergence.

1. Introduction

Genus *Alternaria* Ness is ubiquitous, including species found worldwide in association with a large variety of substrates. Many species are saprophytes, animal/plant pathogens or postharvest pathogens. As a genus, *Alternaria* encompasses considerable morphological diversity and there have been a number of attempts to organize taxa into subgeneric groupings based on shared morphological characters. Above all, small-spored *Alternaria* species are a taxonomically challenging group of fungi with few morphological or molecular characters that allow unambiguous discrimination among taxa (Andrew et al., 2009).

A precise and correct identification of these species is necessary, not only because of our desire to classify and control, but also because the species name embodies a set of characters (e.g., growth preference, host interaction and metabolite production) that enables us to predict its behavior.

In spite of the numerous studies on different *Alternaria* spp., few reports deal with the characterization of the endophytic strains (Guo et al., 2004).

Studies have shown that small-spored *Alternaria* species often dominate among endophytic fungi isolated from leaves and other tissue parts of various plant groups (Dugan and Lupien, 2002; Dugan et al., 2002; Su et al., 2010). Depending on the plant group, different species-groups or complexes of *Alternaria* have been isolated. In grasses *A. infectoria* dominated (Dugan and Lupien, 2002), while *A. alternata* complex was the most frequent group found in grapevine (Mostert et al., 2000; Grisan et al., 2011).

In the case of the grapevine, it has been reported that some categories of endophytes may potentially provide control of important diseases (Kortekamp, 1997; Musetti et al., 2006; Bulgari et al., 2011); others are successfully used as biocontrol agents against postharvest pathogens (e.g., *Botrytis cinerea* and *Penicillium expansum*) (Schena et al., 1999). In particular, fungi belonging to the genus *Alternaria*, have been demonstrated to inhibit *Plasmopara viticola* sporulation in grapevine leaves in moisture chambers as well as in plants maintained in the greenhouse, producing secondary metabolites

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belonging to the class of Diketopiperazines (Musetti et al., 2007; Polizzotto et al., 2009).

Therefore, the correct classification of endophytic *Alternaria* acquires importance also under a practical point of view.

Gene sequence analyses of ITS and other household genes can distinguish *A. infectoria* from other small-spored *Alternaria* species (Pryor and Bigelow, 2003), but are not able to distinguish between *A. alternata*, the *A. arborescens* and the *A. tenuissima* species-groups (Hong et al., 2005). Characterization of *Alternaria* species based on morphological (Andersen et al., 2005) and molecular analyses (e.g., RAPD-PCR and PCR-RFLP) (Roberts et al., 2000; Pryor and Michailides, 2002; Hong et al., 2005) are important for a correct identification, but may not be enough to differentiate pathogenic strains from endophytic ones. It is known that many endophytes are able to produce secondary metabolites with biological activities and that they can contribute to their classification (Azevedo et al., 2000; Shultz et al., 2002). A classification based on the profile of secondary metabolites is very important as it reflects the major signals of importance for interactions with the environment. Furthermore, in itself the secondary metabolism is a powerful feature for fungal characterization and offers the opportunity to classify and identify also endophyte microorganisms presenting often difficulties in the differentiation of reproducing structures in laboratory conditions (Frisvad et al., 2008).

Segregation, therefore, must be based on other types of molecular analyses (e.g., RAMS, RAPD-PCR and PCR-RFLP) (Roberts et al., 2000; Pryor and Michailides, 2002; Guo et al., 2004; Hong et al., 2006) together with chemical and morphological methods suited for *Alternaria* (Andersen et al., 2002).

A polyphasic approach including morphology and molecular analyses and metabolite profiling should give a good basis to characterizing endophytes belonging to the genus *Alternaria*.

The aim of this work was to set up an integrated approach for the characterization of *Alternaria* endophytic strains isolated from different origins, using morphological, molecular and chemical methods in order to facilitate the understanding of endophyte properties and the interactions with the host plants. Another aim was to determine if endophytic *Alternaria* strains isolated from grapevine belong to known taxa or constitute new undescribed species.

2. Materials and methods

2.1. Strains used in this study

Thirty-two *Alternaria* strains were used in this study: twenty endophytic fungal strains belonging to the genus *Alternaria* that had been isolated from grapevine shoots in a previous study (Musetti et al., 2006; Martini et al., 2009) and twelve representative strains belonging to four 'small-spored' *Alternaria* species-groups. Identity and origin of all strains are given in Table 1. A temporary working collection of the strains was made from agar blocks with conidia from potato carrot agar (PCA) (Simmons, 1992) placed in cryo tubes and kept at 5 °C. All strains were maintained and stored in sterile water at about 5 °C and in glycerol 20% at −20 °C and are available from the authors' private collections at Department of Agricultural and Environmental Sciences (DISA), University of Udine, Italy and from the IBT collection at the Department of Systems Biology, DTU, Denmark.

2.2. Growth media and growth conditions

To ensure purity and generate inoculum, all 32 strains were inoculated on PCA and grown under standardized conditions (Andersen et al., 2005). The unsealed 9-cm plates were incubated in one layer for 7 days at 23 °C under an alternating light/dark cycle consisting of 8 h of cool-white fluorescent daylight and 16 h darkness. Conidia from PCA cultures of each strain were transferred to the following five media: PCA, Dichloran Rose Bengal Yeast Extract Sucrose agar [DRYES (Frisvad, 1983)], Potato Dextrose agar (PDA, DIFCO 213400), Malt Extract agar [MEA according to Blakeslee (Samson et al., 2002)] and Dichloran 18% Glycerol agar [DG18 (Andersen et al., 2009)], as three-point inoculations. All plates, except those with PCA, were staked (six in each) and put in perforated plastic bags and incubated for 14 days in the dark at 25 °C. The PCA plates were incubated unsealed as mentioned above. If sporulation was unsatisfactory, the plates were scarified and incubated longer.

Table 1
Alternaria strains used in the study.

Isolate code[a]	Species/species group[b]	Type	Host/substratum	Origin
BA961[c]	*A. arborescens* sp-grp	Saprophyte	Tomato fruit	–
BA925[c]	*A. tenuissima* sp-grp	–	Air	–
BA924[c]	*A. alternata*	Saprophyte	Banana	–
BA923[c]	*A. alternata*	Saprophyte	Datura	–
BA922[c]	*A. alternata*	Saprophyte	Peanut	–
BA879[c]	*A. tenuissima* sp-grp	Saprophyte	Moldy grape berry	Denmark
BA853[c]	*A. tenuissima* sp-grp	Saprophyte	Moldy tomato fruit	Denmark
BA1422[c]	*A. arborescens* sp-grp	–	Saltmarch	Slovenia
BA1382[c]	*A. arborescens* sp-grp	–	Insect gall, Nerium	Spain
BA1343[c]	*A. arborescens* sp-grp	Saprophyte	Grain, barley	Denmark
BA1208[c]	*A. incomplexa*	–	Mud	–
BA1240[d]	*A. infectoria* sp-grp	–	–	–
A-MELO[d]	*A. arborescens* sp-grp	Endophyte	Shoot, grapevine	Italy
M44B[d]	*A. tenuissima* sp-grp	Endophyte	Shoot, grapevine	Italy
M44C[d]	*A. tenuissima* sp-grp	Endophyte	Shoot, grapevine	Italy
P85A[d]	*A. arborescens* sp-grp	Endophyte	Shoot, grapevine	Italy
P85B[d]	*A. arborescens* sp-grp	Endophyte	Shoot, grapevine	Italy
P85Cd	*A. arborescens* sp-grp	Endophyte	Shoot, grapevine	Italy
P85D[d]	*A. arborescens* sp-grp	Endophyte	Shoot, grapevine	Italy
R42A[d]	*A. arborescens* sp-grp	Endophyte	Shoot, grapevine	Italy
R68B[d]	*A. tenuissima* sp-grp	Endophyte	Shoot, grapevine	Italy
R89A[d]	*A. arborescens* sp-grp	Endophyte	Shoot, grapevine	Italy
R94A2[d]	*A. tenuissima* sp-grp	Endophyte	Shoot, grapevine	Italy
T50C1[d]	*A. arborescens* sp-grp	Endophyte	Shoot, grapevine	Italy
T65A[d]	*A. arborescens* sp-grp	Endophyte	Shoot, grapevine	Italy
T65B[d]	*A. tenuissima* sp-grp	Endophyte	Shoot, grapevine	Italy
T65D[d]	*A. tenuissima* sp-grp	Endophyte	Shoot, grapevine	Italy
VP12A5B[d]	*A. tenuissima* sp-grp	Endophyte	Leaf, grapevine	Italy
VP12B1A[2]	*A. tenuissima* sp-grp	Endophyte	Leaf, grapevine	Italy
VP12B2A[d]	*A. tenuissima* sp-grp	Endophyte	Leaf, grapevine	Italy
VP13B2B[d]	*A. tenuissima* sp-grp	Endophyte	Leaf, grapevine	Italy
VP13B2B2[d]	*A. tenuissima* sp-grp	Endophyte	Leaf, grapevine	Italy

[a] Identification code as used in Figures.
[b] Sporulation group according to Simmons and Roberts (1993).
[c] IBT collection at the Department of System Biology, DTU, Denmark.
[d] DISA collection, University of Udine, Italy.

2.3. Morphological and culture characterisation

For morphological examination, the PCA cultures were observed after 7 days at ×50 magnification in a stereo-microscope. The sporulation pattern of each culture was examined directly on the plates according to the method of Simmons and Roberts (1993). Further examination was done at ×400 magnification using slides made in lactophenol with tape using a compound microscope. Colony colors on DRYES, PDA, MEA and DG18 were determined according to Kornerup and Wanscher (1978) and pictures were taken of the 7-days-old. Twenty micro- and macro-morphological characters (e.g., rough conidial texture, secondary conidiophores originating from apex, green color on DRYES) were recorded as binary data [present (1) or absent (0)] for cluster analysis.

2.4. Genomic DNA extractions

Mycelia for DNA extraction was extracted from 7-days-old PDA cultures of *Alternaria* isolates (Table 1) following the procedure of Lecellier and Silar (1994) modified by Martini et al. (2009).

The DNA pellet was suspended in 100 µl TE buffer and stored at −20 °C until further use. DNA concentrations were determined by spectrophotometer (Nanodrop 1000, Spectrophotometer, TermoScientific, Wilmington, USA).

2.5. RAPD-PCR amplification

Genomic DNA was amplified using four primers: OPR-02 (Roberts et al., 2000), OPR-12 (Roberts et al., 2000), OPA9 (Cooke et al., 1998) and OPA13 (Cooke et al., 1998). RAPD reactions were carried out in a 25 µl PCR reaction mixture containing 5 ng of DNA, 20 pmol of primer, 150 µM of DNTP's, 2 µM of $MgCl_2$ and 0.75 U of Go *Taq* Flexi DNA Polymerase (PROMEGA, USA). Amplification was performed in a thermal cycler (MJ Mini Personal Thermal Cycler, BIORAD) and consisted in an initial denaturation at 94 °C for 2 min followed by 36 cycles of denaturation at 94° for 1 min, annealing at 35 °C for 30 s, extension at 72 °C for 2 min, and with a final extension at 72 °C for 8 min. The PCR products (5 µl) were resolved by electrophoresis by using 1.5% agarose gel in 0.5× TBE buffer (45 mM Tris–borate, 1 mM EDTA pH 8.0), at 50 V for 5 h and visualized by UV illumination after staining in ethidium bromide. One kb DNA Ladder (Fermentas, Lituania) was used as a DNA size marker.

RAPD bands were scored as binary data [present (1) or absent (0)] for each strain. Phylogenetic cluster analyses of the binary matrix were performed using the software NTSYSpc version 2.02 (Exeter Software, Setauket, NY, USA) by the UPGMA with Jaccard's similarity. The matrix was also analyzed by simple matching (SM) and Yule (Y) similarity coefficients in NTSYS.

2.6. Metabolite extraction

Metabolite extractions were performed on the 14-days-old DRYES cultures. The extraction protocol used was a micro-scale extraction method modified for *Alternaria* metabolites (Andersen et al., 2005). Three agar plugs (6 mm in diameter) were cut from the center of the three colonies and the nine plugs were placed in a 2-ml-screw-top vial. Then 1.0 ml ethyl acetate containing 1% formic acid (vol/vol) was added to each vial and the vial was placed at −18 °C for a minimum of 12 h. The plugs were extracted within ultrasound for 60 min in a Branson 3210 water bath (Ultrasonics Corporation, Danbury, CT, USA). The ethyl acetate extract was transferred to a clean 2-ml vial, evaporated to dryness in a rotary vacuum concentrator (Christ, Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), re-dissolved ultrasonically in 400 µl methanol for 15 min, and filtered through a 0.45-µm filter (National Scientific Company, Rockwood, TN, USA) into a clean 2-ml vial prior to HPLC analysis. Keep the vials in the freezer (−18 °C) until HPLC analysis.

2.7. HPLC-UV–VIS analysis

The HPLC analyses were performed on a HP-1100 high performance liquid chromatograph (Agilent, Waldbronn Germany) equipped with an auto sampler injecting 3.0 µl sample^{-1} and a diode array detector collecting 2 ultraviolet–visible (UV–VIS) spectra s^{-1} from 200 to 600 nm. Separations were done on a 100×2 mm Luna 3 µm C18(2) 100 Å column (Phenomenex, Torrance, CA, USA) with a 4×2 mm C18 (2) guard column (Phenomenex, Torrance, CA, USA). The column temperature was 40 °C. The mobile phase consisted of a linear gradient starting at 85% water and 15% acetonitrile reaching 100% acetonitrile in 20 min. 100% acetonitrile was maintained for 5 min. Thereafter, the gradient was returned to 15% acetonitrile in 3 min and allowed to equilibrate for 5 min before the next analysis. Both eluents contained 50 ppm trifluoroacetic acid. The flow rate was 0.4 ml min^{-1}. A homologous series of alkylphenones was analyzed as external retention time references and used to calculate a bracketed retention index (RI) for each detected peak (Frisvad and Thrane, 1987). Origin and preparation of fungal standards, such as altenuene, alternariols, altersolanol A, altertoxin I, erythroglaucin, tentoxin and tenuazonic acid, are given in Nielsen and Smedsgaard (2003). All solvents were HPLC grade, chemicals were analytical grade and the water was double distilled.

2.8. Data treatment of metabolite profiles

The raw HPLC data files, which are quantitative 2-D matrices (x-axis: time, Y-axis: wavelength, value in matrix: UV–VIS absorbance), were transferred from the HPLC to a standard PC and analyzed by an in-house written chemical image analysis (CIA) program (Hansen, 2003). No manipulations or peak selections were made before processing. Each HPLC file was processed first by a $\log_{10}$ scaling (to account for concentration differences among extracts), then a baseline correction and finally an alignment (to account for drift in baseline and retention time among identical metabolites in different runs) (Hansen, 2003). Each HPLC file was then compared to the other 29 HPLC files, pair-wise, using an algorithm described by Hansen (2003) giving a similarity value for each pair, which was entered into a new matrix. The resulting 30×30 similarity matrix was then used to calculate a dendrogram using WARD clustering method. Based on the result of the CIA, *a* binary matrix was made manually by scoring each metabolite as binary data [present (1) or absent (0)] from the printed chromatograms and subjected to multivariate statistics using The Unscrambler version 9.2 (CAMO ASA, Oslo, Norway). This full manual metabolite matrix consisted of 61 X-variables (known and unknown metabolites) and 3 Y-variables (3 species names) for the 30 objects (fungal strains with confirmed ID and clustering according to ID). The matrix was analysed using Partial Least Squares Regression (PLS-R), which relates the variations in response variables (Y-matrix) to the variations of several predictors (X-matrix) (Wold et al., 2001).

2.9. Cluster analysis of metabolite data

A manual metabolite matrix was made and contained consistently produced metabolites with both known and unknown chemical structures. The binary matrix, consisting of 30 strains and 61 metabolites, was subjected to cluster analysis using NTSYS-pc version 2.11 N without standardization using Yule (Y) as correlation coefficient and UPGMA as clustering method. The matrix was also analyzed by simple matching (SM) and Jaccard (J) similarity coefficients in NTSYS.

2.10. Cluster analysis of all data

A binary matrix was made by pooling morphological data (20 characters), molecular data (73 RAPD bands) and chemical data (53 metabolites) for the 30 *Alternaria* strains. The matrix was subjected to cluster analysis using NTSYS-pc version 2.11 N without standardization using Yule (Y) as correlation coefficient and UPGMA as clustering method. The matrix was also analyzed by simple matching (SM) and Jaccard (J) similarity coefficients in NTSYS.

Each experiment was performed at least two times and data are expressed as the means (SE).

3. Results

3.1. Morphology and cultural characterization

When grown on PCA under standardized conditions, the 20 endophytic strains showed two different sporulation patterns that corresponded to the representative strains: one similar to the *A. arborescens* sporulation pattern (sporulation group 3, Simmons and Roberts, 1993) and one similar to *A. tenuissima* sporulation pattern (sporulation group 5, Simmons and Roberts, 1993) (Table 1 and Figs. 1 and 2, respectively).

None of the endophytic strains gave a sporulation pattern similar to that of *A. alternata*, characterized by short primary conidiophores and chains that mainly branch from the conidial body by means of short secondary conidiophores. The color of *A. alternata* colonies on DRYES are primarily dark green. Neither did any of the endophytic strains yield sporulation patterns similar to those of the *A. infectoria* species-group, characterized by short primary conidiophores and conidia in branched chains with long secondary conidiophores. The color of the *A. infectoria* species-group colonies on DRYES were white to grayish white.

Nine endophytic strains had sporulation patterns similar to that of the *A. arborescens* species-group, and were characterized by long (up to 200 μm) primary conidiophores bearing between 15 and 20 conidia in branched chains at the top (Fig. 1). The branching predominately occurred from the conidial apex and the primary conidium could often be twice as long (30–50 μm) as the subsequent conidia and having geniculate secondary conidiophore (up to 30 μm) with several loci. On PCA, the nine strains of the *A. arborescens* species-group showed the characteristic alternating heights of growth rings, while on DRYES the color of the colonies varied from dark green to grayish green. Strains P85B, P85C, P85D, R42A and T50C1 were very similar, while A-MELO and P85A were not as branched as the first. The last two strains (T65A and R89A) deviated in both micro- and macro-morphological characteristics from the representative strains. Strain R89A produced fewer conidia per primary conidiophore and colonies on DRYES were sulcate and lighter green in color. Strain T65A had a higher percentage of its secondary conidiophores originating from the conidium body, darker conidial walls and colonies were dark green and heavily wrinkled on DRYES.

Eleven endophytic strains identified as belonging to the *A. tenuissima* species-group had conidia in unbranching chains, borne on short primary conidiophores (Fig. 2). If branching occurred in these strains, short (6–12 μm) simple secondary conidiophores would usually originate from the conidial body and not from the apex. The endophytic strains of the *A. tenuissima* species-group had mostly green or light green colonies on DRYES. Strains M44B, R68B, T65B, VP13B2B and VP13B2B2 looked similar with more stubby conidia compared to strains R94A2, VP12B1A, VP12B2A and VP12A5B that have more elongated conidia. However, two strains, M44C and T65D, differed more in micromorphological characteristics. Strain M44C sometimes produced long (up to 40 μm), but simple, apical secondary conidiophores. Strain T65D sometimes produced up to four short, simple secondary conidiophores from the central cells of a conidium giving rise to chains of mostly two new conidia.

3.2. Molecular RAPD characterization

Only 30 out of the 32 strains were subjected to RAPD analyses, excluding the two strains representing the *A. infectoria* species-groups. RAPD-PCR of the grapevine *Alternaria* endophytes as well as reference strains yielded a total of 77 reproducible RAPD fragments. Minor fragments (i.e., those less intensely stained by ethidium bromide) were not reproducible and were not scored. The RAPD fragment patterns showed a high degree of variation between strains of the *A. arborescens* and strains of the *A. tenuissima* species-group, including the reference strains. The RAPD analyses also showed a high degree of pattern similarity within the species or species-groups. Fig. 3 shows the result of the RAPD analysis with OPA 9 primer for all strains except the 2 strains belonging to the *A. infectoria* species-group.

Cluster analysis based on RAPD similarities showed two distinct groups of strains similar to the *A. tenuissima* species-group (cluster B) and the *A. arborescens* species-group (cluster A) (Fig. 4). These species-groups were well separated and supporting the morphological identification. The *A. arborescens* species-group showed 47% similarity (Fig. 4); only two endophytic strains (T65A and R89A) clustered

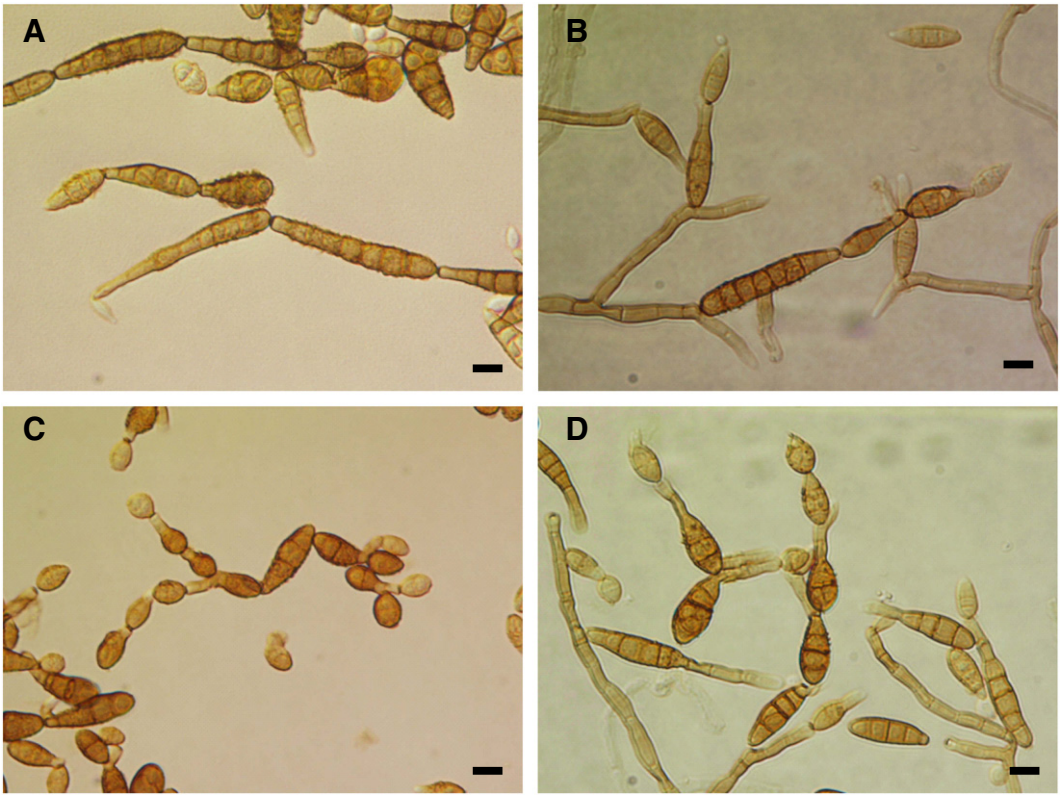

Fig. 1. Sporulation pattern on 7-day old PCA cultures of the *Alternaria arborescens* species-group. A and C: sporulation patterns of two endophytic strains belonging to *A. arborescens* species-group (P85A and T50C1, respectively); B and D: sporulation patterns of two representative strains of the *A. arborescens* species-group (BA1343 and BA1382, respectively). Bars correspond to 10 μm.

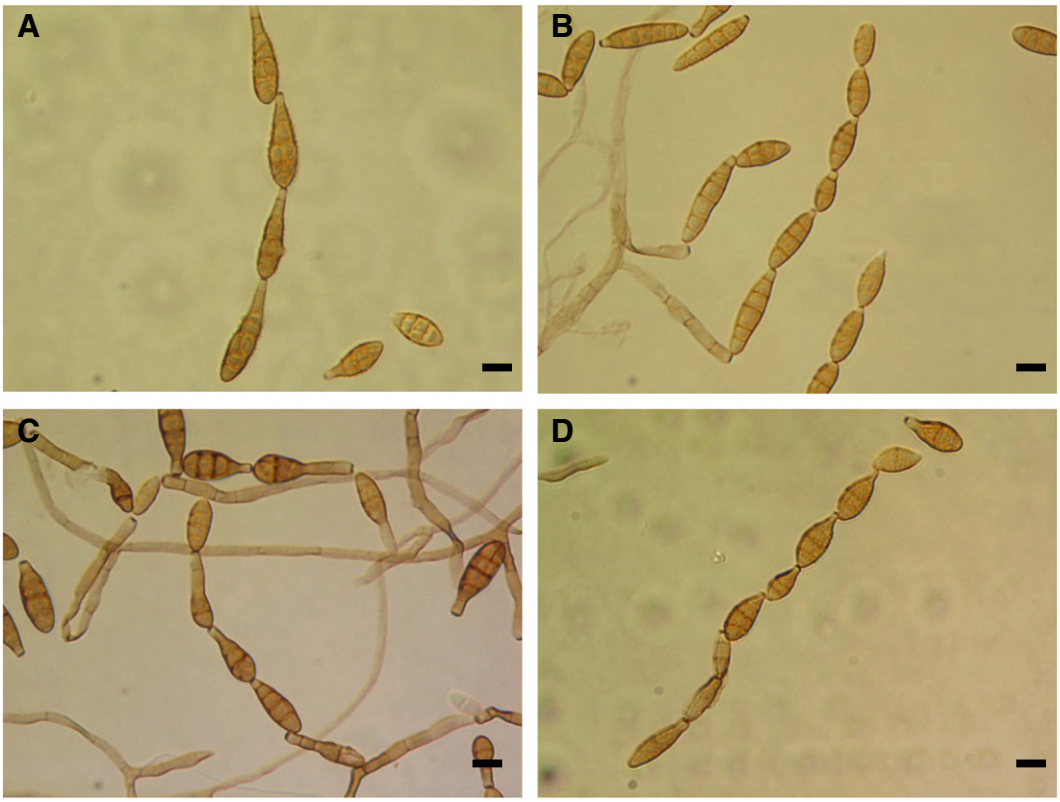

Fig. 2. Sporulation pattern on 7-day old PCA cultures of the *Alternaria tenuissima* species-group. A and C: sporulation patterns of two endophytic strains belonging to *A. tenuissima* species-group (R94A2 and VP12B2A); B and D: sporulation patterns of two representative strains of the *A. tenuissima* species-group (BA853 and BA925, respectively). Bars correspond to 10 μm.

separately and this result is correlated with morphological observations. Strains of the *A. alternata* and *A. tenuissima* species-groups clustered together with 52% similarity in RAPD fragment patterns (Fig. 4). Within cluster B, the *A. alternata* strains clustered together, but separately (cluster C) from the majority endophytic *A. tenuissima* strains at 65% similarity with the exception of the representative strains BA925 (*A. tenuissima* species-group), which clustered with the *A. alternata* group. In the *A. tenuissima* species-group, endophytic strains and representative strain BA953 clustered at 65% similarity, except representative strains BA879 (*A. tenuissima* species-group), which clustered outside the main cluster. The endophytic strains (M44C and T65D) clustered separately from *tenuissima* species-group in agreement with morphological results.

3.3. Chemical characterization

The result of the automated and unbiased CIA of 30 *Alternaria* extracts (excluding the strains in the *A. infectoria* species-group) is shown in Fig. 5. The analysis is based on chromatograms (HPLC files) of raw fungal extracts that contained compounds from the growth media (e.g., chloramphenicol and dichloran), impurities from extraction solvents, fungal-specific (e.g., ergosterol), species-specific, and strain-specific metabolites. The dendrogram shows two main cluster, A and B. Cluster B contains all but one of the *A. tenuissima* species-group strains and one *A. arborescens* species-group strain (P85A), but only one of the representative *A. tenuissima* strains (BA853). Cluster A contains one minor cluster (A1) with two *A.*

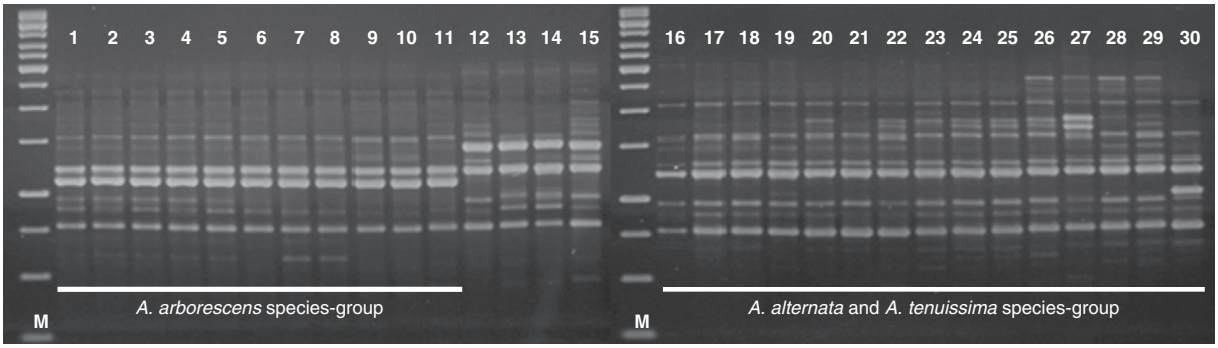

Fig. 3. DNA banding patterns from random amplified polymorphisms DNA (RAPD) analysis with primer OPA9 of 20 endophytic *Alternaria* isolates collected from grapevine and 10 representative *A. arborescens*, *A. tenuissima*, and *A. alternata* strains. Lanes 1–11: *A. arborescens* species-group (P85A, T50C1, R42A, BA1343, BA1422, P85B, P85C, P85D, A.MELO, BA961, and BA1382, respectively); lanes 16, 17, and 20: *A. alternata* (BA923, BA924, and BA922, respectively); lanes 18, 19, and 21–30: *A. tenuissima* species-group (BA925, BA879, T65B, BA853, R68B, VP13B2B, VP13B2B2, M44B, VP12B2A, R94A2, VP12A5B, and VP12B1A, respectively). Lanes 12–15: M44C, T65A, R89A and T65D (strains with deviant morphological characters). M: 1 Kb DNA Ladder (Fermentas, Lituania).

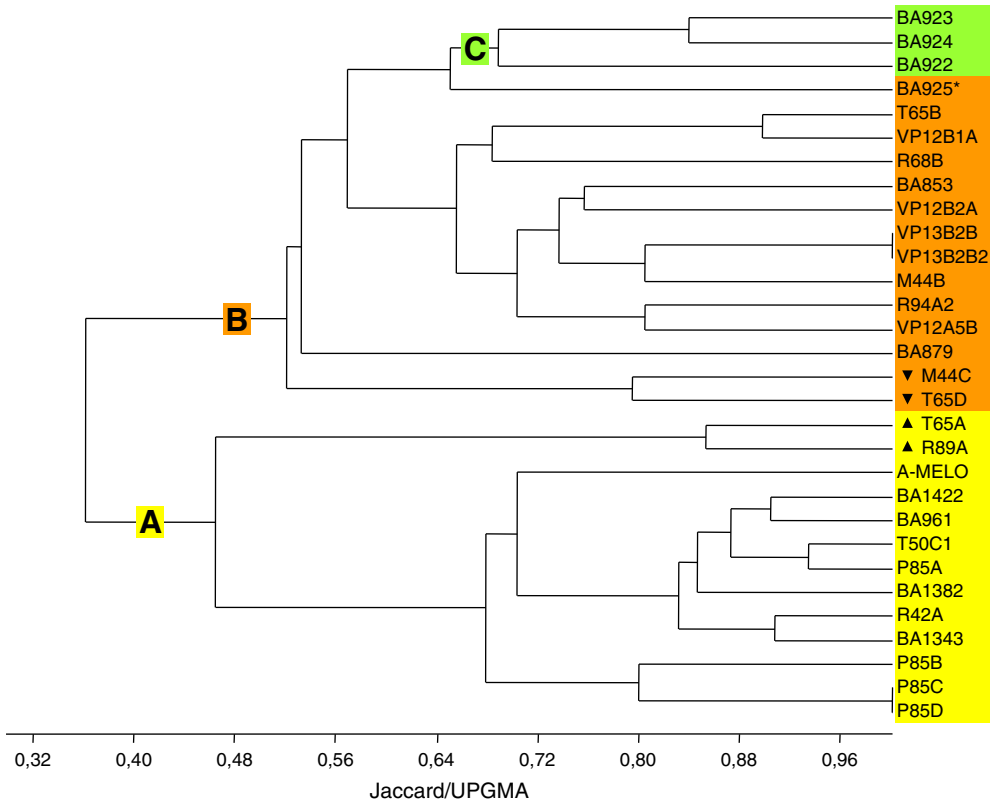

Fig. 4. Dendrogram based on RAPD profiles of 20 endophytic *Alternaria* strains and 10 strains of representative *Alternaria* species-groups using Jaccard similarity coefficient and UPGMA analysis. The primers OPA9, OPA13, OPR-02 and OPR-12 used were. *A. alternata* strains (cluster C in green), strains belonging to the *A. tenuissima* species-group (cluster B in orange) and strains belonging to the *A. arborescens* species-group (cluster C in yellow). Strains marked with ▲ or ▼ are atypical compared to their species group.

arborescens species-group strains (P85B and P85C) and one the *A. tenuissima* species-group strain (M44B) and one major cluster (A2) with all but one of *A. arborescens* species-group strains, all the *A. alternaria* strains and the remaining two representative *A. tenuissima* strains (BA879 and BA925).

A Partial Least Squares Regression (PLS-R) was made to select species-specific metabolites and deselect common and inconsistently produced metabolites. The matrix was constructed from the 30 HPLC chromatograms as a binary matrix based on the presence or absence of 61 selected metabolites that were produced in high concentration and that had distinct UV–VIS spectra. The plot in Fig. 6 shows the calculated model, which explains 52% of the fungal identity (y-data) and 26% of the chemical diversity (x-data) on the first two axes out of the four axes that constituted the model. The 61 metabolites were represented in the plot by metabolite name or their RI values when the metabolite is of unknown structure. The model in Fig. 6 gave the group-specific metabolites by arranging them along a line (arrow) from the center of the coordinate system to the given species-group. By plotting the PLS components one can view main associations between X-variables (metabolites) and Y-variables (strain ID), and also inter-relationships within X- and Y-data. The closer a metabolite is to the arrowhead and the species-group name, the more specific it is and the larger a percentage of the strains in the given species produce it. For example, all the *A. alternata* strains produced the metabolites with RI value 798 and 903. Metabolites that are located between two arrows are common to both species-groups, whereas metabolites located diametrically opposite a arrow are not produced by the given species-group. Also, alternariol (852), alternariol monomethyl ether (987) and metabolite 667 are commonly produced by both *A. alternata* and strains belonging to the *A. arborescens* species-group, but produced more scarcely by *A. tenuissima* species-group strains. Altersetin (1374) and 722, on the other hand, are common to both *A. arborescens* and *A. tenuissima* species-groups, but not found in *A. alternata*. Metabolites that are either produced by all strains or produced by few strains would be located in the center of the coordinate system.

3.4. Endophyte-specific metabolites

No metabolites specific to all the 20 endophytic strains were found among the 61 selected metabolites. However, some metabolites (marked in bold green in Fig. 6) were found to be produced only by the endophytic strain, though inconsistently. The majority of the metabolites could be identified with known chemical structure, based on their UV–VIS spectra and compared to the metabolite standards (Nielsen and Smedsgaard, 2003). In particular, none of the representative *A. alternata* strains produce tenuazonic acid (TeA), while the *A. tenuissima* and *A. arborescens* strains produce it consistently (100% and 78%, respectively). Furthermore, the two species-groups are characterized by a broad variety of *Alternaria* known metabolites (e.g., altenuene, alternariol, alternariol monomethyl ether), new metabolite, such as altersetin, extracted from endophytic *Alternaria* strains, and unknown metabolite. None of the 30 *Alternaria* strains were found to produce diketopiperazines on the media used in this study.

3.5. Polyphasic characterisation

Fig. 7 shows the resulting dendrogram of a cluster analysis made on the pooled binary matrix of morphological, molecular and chemical data. The dendrogram shows *A. alternata* (cluster C) is a tight group with approximate 91% overall similarity. One representative strain of the *A. tenuissima* species-group (BA879) is an outlier to this cluster. The strains belonging to the *A. arborescens* species-group (cluster A) represent a variable group with about 70%

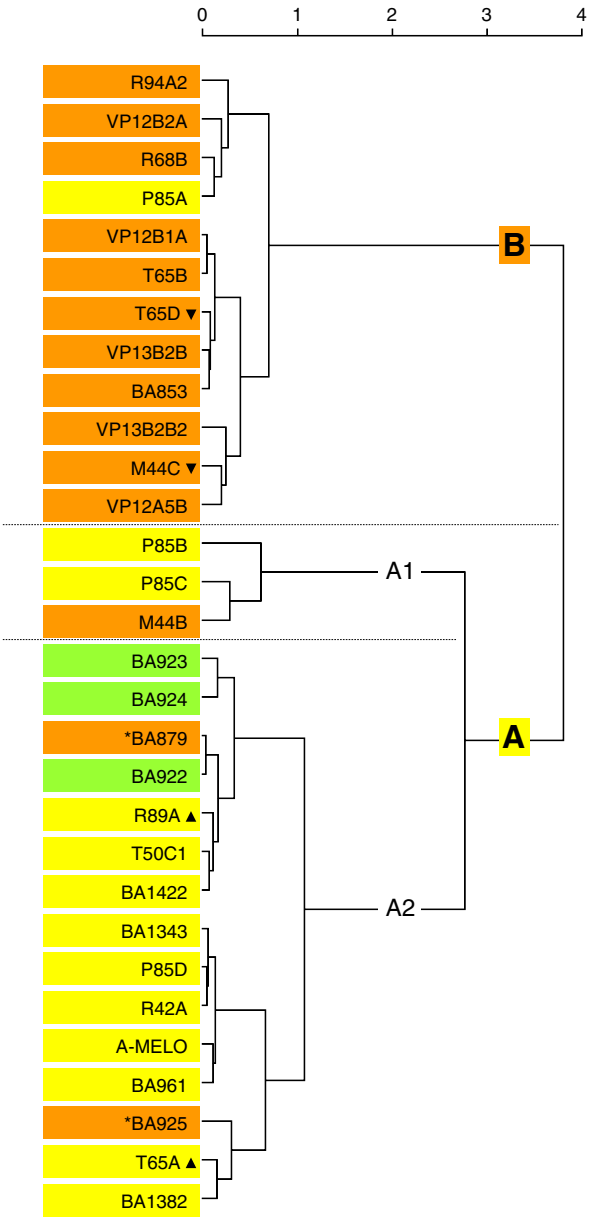

Fig. 5. Dendrogram based on an automated CIA of all metabolites of 20 endophytic *Alternaria* strains and 10 strains of representative *Alternaria* species-groups using the Ward clustering method. Arbitrary scale. *A. alternata* strains (cluster C in green), strains belonging to the *A. tenuissima* species-group (cluster B in orange) and strains belonging to the *A. arborescens* species-group (cluster C in yellow). Strains marked with ▲ or ▼ are atypical compared to their species group.

similarity. Two endophyte strains (R89A and T65A) stay together as a sister cluster to the main *A. arborescens* cluster. Strains belonging to the *A. tenuissima* species-group (cluster B) are a more variable group with an overall similarity of 70%. Three endophyte strains (M44C and T65D) stay together as a sister cluster to the main *A. tenuissima* cluster with M44B as an outlier.

4. Discussion

Most studies on identification of endophytic fungi have been conducted by using a combination of morphological and molecular methods. Since endophytes are said to produce additional bioactive metabolites (Tan and Zou, 2001), a polyphasic approach including metabolite profiling should give a good basis to classify this type of fungi (Frisvad et al., 2008).

In this work, a polyphasic approach has been set up for the characterization of 20 grapevine endophytic strains belonging to the genus *Alternaria*.

Regarding the first objective of this research, i.e., the evaluation of *Alternaria* endophytes by micro-morphological traits, the use of PCA incubated under standardized conditions and the comparison to representative strains, showed that the 20 endophytic strains belonged to either the *A. arborescens* or the *A. tenuissima* species-group (Andersen and Thrane, 1996; Andersen et al., 2001) according to the sporulation patterns proposed by Simmons and Roberts (1993). The use of different culture media (DRYES, MEA, PDA, DG18) and the evaluation of the macro-morphological characteristics, already used to discriminate between pathogen *Alternaria* species (Andersen et al., 2005), proved to have taxonomic value also for endophytes, corroborate the results obtained by micro-morphological observations. By means of the association of micro- and macro-morphological data, seven endophyte strains could be grouped with the *A. arborescens* species-group and nine belonged to the *A. tenuissima* species-group. None of the endophytic strains recovered from grapevine produced sporulation patterns or metabolite profiles identical to those of *A. alternata* or the *A. infectoria* species-group. The results showed that most of the endophytic strains grouped with known *Alternaria* taxa isolated as saprophytes from other substrates than grapevines. Only four endophytic strains showed characteristics of their own. The couples T65A–R89A (▲ in Figs. 4, 5 and 7) and M44C–T65D (▼in Figs. 4, 5 and 7) each showed different micro- and macro-morphological characteristics and may represent distinct and more distant taxa, one in each species-group.

The second part of the polyphasic characterization of *Alternaria* endophytes regarded the individuation of an appropriate molecular method to differentiate the strains, complementing morphological observations. To find suitable genes to analyze and compare sequences of 'small spored' *Alternaria* is not simple (Bruns et al., 1991). It is known that the analysis of the sequences coding for ITS1 and ITS2 regions is not sufficiently variable to estimate a phylogeny among the 'small spored' *Alternaria* (Kusaba and Tsuge, 1995) as well as the use of EF-1α, CAL, CHS, and THN sequences (Roberts et al., 2000; Pryor and Michailides, 2002; Hong et al., 2006; Andrew et al., 2009). Moreover, as MtLSU sequence data resulted satisfactorily variable to differentiate the 'large-spored' species of *Alternaria* from the 'small-spored' ones, the analysis of this gene region do not allow to segregate among 'small-spored' strains (Peever et al., 2004). However, RAPD technique, which characterizes random priming sites across the entire genome, consents to evidence a high genetic variability among 'small-spored' *Alternaria*; such variability is coherent with morphological, physiological and chemical observations (Roberts et al., 2000). In the present study, RAPD fingerprint pattern analysis, allowed to demonstrate that grapevine endophytes belonging to the *A. arborescens* species-group are molecularly distinct from endophytes belonging to the *A. tenuissima* species-group (Fig. 4). These results are in agreement with those already reported by Roberts et al. (2000) and by Pryor and Michailides (2002). Moreover, cluster analysis of RAPD profiles permitted to discriminate, into *A. tenuissima* species-groups, representative strains belonging to *A. alternata*, whereas they did not separate in the study by Pryor and Michailides (2002). Results achieved by molecular methods also confirm the low similarity of the endophytic strains marked with ▲ (T65A–R89A) and ▼ (M44C–T65D) within their species group.

To complete the endophyte fungal characterisation, chemotaxonomy based on secondary metabolite profiles was employed. Metabolite profiling is a relevant tool for characterization of endophytic *Alternaria* spp., because they are supposed to produce chemically different and bioactive compounds (Quiao et al., 2007; Aly et al., 2008). The role of these compounds, e.g. altersetin and altenuisin, antibiotics from endophytic *Alternaria* spp. (Hellwig et al., 2002; Cota et al., 2008) and others of yet chemically unknown structure, is waiting to

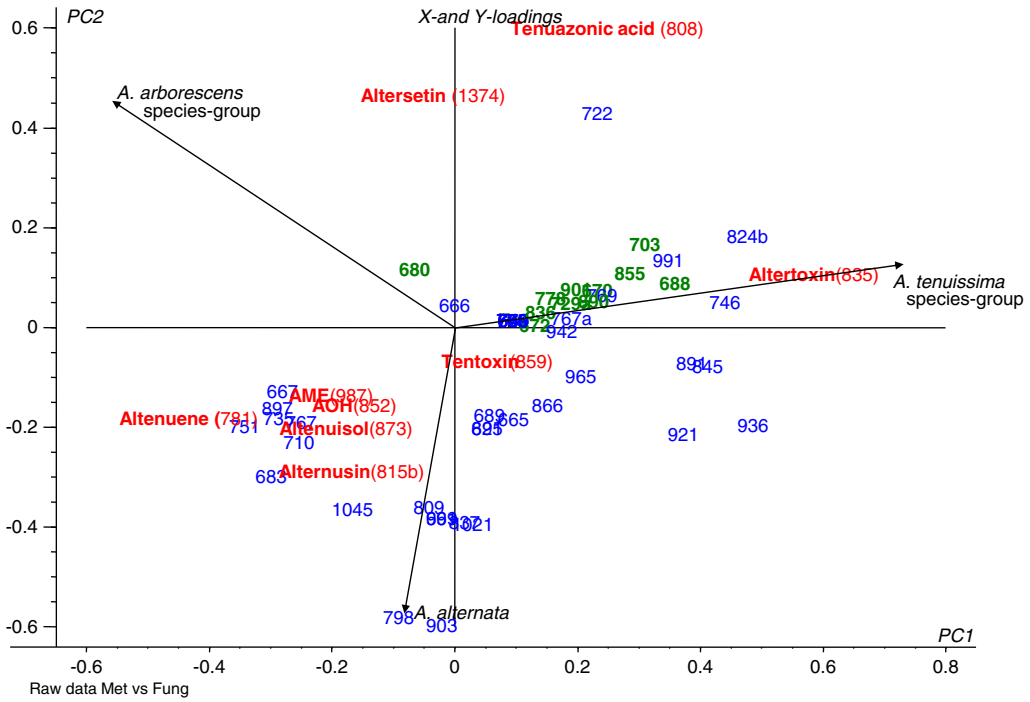

Fig. 6. Loadings plot based on a PLS-R of the manual metabolite matrix of 61 metabolites of 20 endophytic *Alternaria* strains and 10 strains of representative *Alternaria* species-groups. Known metabolites are given by their name and RI value (in bold red) and unknown metabolites are given only by their RI value (in blue). Endophyte-specific metabolites are marked in bold green. Axes are principal components 1 and 2.

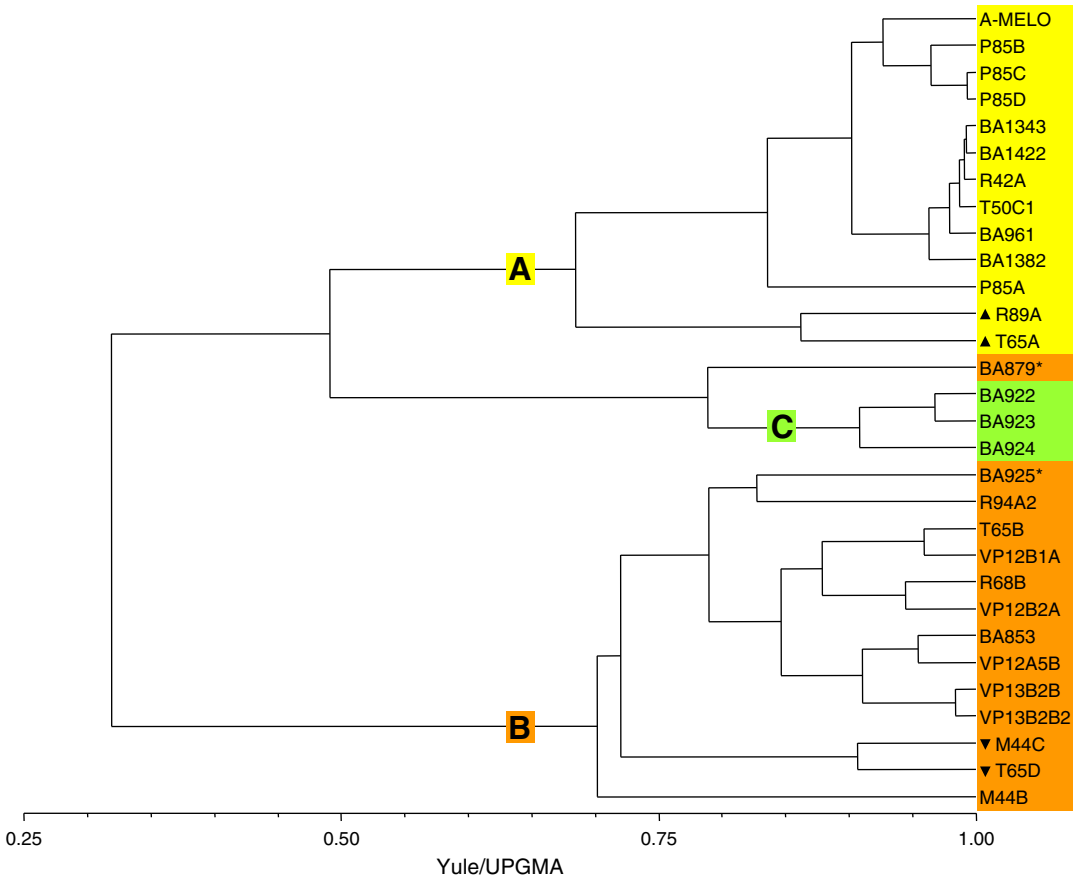

Fig. 7. Dendrogram based on morphological characters, metabolite profiles and RAPD profiles of 20 endophytic *Alternaria* strains and 10 strains of representative *Alternaria* species-groups using Yule similarity coefficient and UPGMA analysis. *A. alternata* strains (cluster C in green), strains belonging to the *A. tenuissima* species-group (cluster B in orange) and strains belonging to the *A. arborescens* species-group (cluster C in yellow). Strains marked with ▲ or ▼ are atypical compared to their species group.

be investigated. In our study, metabolite profile analyses resulted species-characteristic and permitted to assign the 20 grapevine endophytes in two main groups, the first having metabolic profile similar to *A. arborescens* species-group, the second having metabolic profile similar to *A. tenuissima* species-group, also confirming the presence of atypical strains (▲ and ▲). Endophytic strains belonging to these two groups revealed a more complex metabolite profile characterized by more metabolites than those produced by *A. alternata*. Generally, endophytes belonging to *A. arborescens* and *A. tenuissima* species-groups produced the same known metabolites typical of these two groups. Especially, the production of tenuazonic acid differentiated them from other 'small-spored' *Alternaria*, such as *A. alternata* and the *A. infectoria* species-group (Andersen et al., 2002) that typically produces altenuene, alternariol, alternariol monomethyl ether and altertoxin I (Bottalico and Logrieco, 1992). It has been indicated that tenuazonic acid, and other known secondary metabolites produced by *Alternaria* spp., demonstrated severe growth-inhibiting activity and alteration of cytoplasmatic structurals of plant host (Bottalico and Logrieco, 1992). Nevertheless, particular toxicological risks are caused in relatively few plant products: tomatoes, apples, citrus and related processed products (Bottalico and Logrieco, 1992).

Among the secondary metabolites produced by the 20 *Alternaria* endophytes, many are of unknown chemical structure. Considering that literature reported about the effectiveness of *Alternaria* endophytes against important grapevine pathogens (Musetti et al., 2006; 2007), it should be interesting to elucidate the chemical structure of *Alternaria* unknown metabolites and to evaluate them as new biological method in the control of grapevine diseases. Moreover, the complete knowledge of endophyte chemical characteristics could be useful as chemical marker for a more precise segregation of endophytic and pathogenic *Alternaria* strains.

Results obtained by the polyphasic approach described in this work, indicate that the majority of grapevine endophytic strains clearly belongs to the representative *Alternaria* species-groups, with the exception of the strains M44C and T65D (▼) and R89A and T65A (▲) that resulted distant; so they could be considered as different taxa.

Concluding, our data demonstrate that grapevine is associated with a different *Alternaria* endophyte community. The combination of microscopy, isolation of pure cultures, molecular and chemical analyses, yielded comprehensive information about the identity, diversity, and phylogeny of these microorganisms, confirming that *Alternaria* endophytes, may constitute the dominant fungal consortium in grapevine tissues.

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