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RESEARCH PAPERS

Identification of fungi associated with grapevine decline in Castilla y León (Spain)

María Teresa MARTIN and Rebeca COBOS

Dept. Viticultura, ITACYL, Ctra. de Burgos Km 119, 47071 Valladolid, Spain

Summary. A number of phytopathogenic fungal species are associated with grapevine decline. Esca, eutypa dieback, black dead arm, and other grapevine decline diseases affecting vine wood have a worldwide distribution. The external symptoms of these diseases, however, can be erratic; even asymptomatic infections are known. Grapevine decline causes economic losses, the size of which depends on factors that still remain unclear, but in all cases the productive life of affected plants is shortened. Grapevine decline is present throughout Castilla y León (Spain). In the present work, the fungi potentially associated with grapevine decline were isolated and identified. *Botryosphaeria*-like spp., *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. were common. *Cylindrocarpon* spp. were found mainly in young plants, while *Phomopsis viticola*, *Fomitiporia mediterranea*, *Eutypa lata* and *Stereum hirsutum* were found only occasionally. Particular attention was given to the identification of the *Botryosphaeria*-like species, of which several were found. By comparing restriction endonuclease patterns (ITS1-NL4) and the sequences of the internal transcribed spacer fragments (ITS4-ITS5), *Diplodia seriata* (=*B. obtusa*), *Diplodia mutila* (=*B. stevensii*), *Neofusicoccum parvum* (=*B. parva*), *B. dothidea*, *Dothiorella iberica* (=*B. iberica*) and *Dothiorella sarmentorum* (=*Diplodia sarmentorum*) were all identified. *D. seriata* and *D. mutila* were identified on plants older than five years. The incidence of infection by "*Botryosphaeria*" species in young plants was very variable.

Key words: *Botryosphaeria, Diplodia*, *Dothiorella*, *Neofusicoccum*, *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum*

Introduction

Grapevine decline causes serious economic losses to the wine industry worldwide. Over the last two decades, new vineyards in Castilla y León (Spain) have shown an increasing percentage of plants with symptoms of wood decay. So far, despite research efforts to control the decay fungi, no effective treatment has been found. Prevention is therefore of great importance. The general symptoms of grapevine decline include deformed, chlorotic leaves, precocious fading, lack of vigour, plugging of the xylem vessels and trunk dieback due to the formation of cankers in the vascular tissue. The impact of grapevine decline is particularly significant in older vineyards; especially when the vines are affected by esca, black dead arm or eutypa dieback.

The diseases of grapevine decline are associated with certain *Botryosphaeria*-like species and *Cylindrocarpon*, and sometimes with one or both of *Phaeomoniella chlamydospora* and *Phaeoacremo-*

Corresponding author: M.T. Martin
Fax: +34 983 414780
E-mail: marvilte@itacyl.es

nium aleophilum which have both been described as causal agents of Petri decline (Morton, 1999; Crous and Gams, 2000). These species, in association with *Fomitiporia mediterranea*, are frequently reported as being the cause of esca (Surico *et al*., 2006). In addition, other fungi such as *Stereum hirsutum*, *Cylindrocarpon* spp., *Diplodia seriata* (=*Botryosphaeria obtusa*) and *B. dothidea* (Armengol *et al*., 2001) have often been reported as associated with these symptoms. *Cylindrocarpon* spp. are also thought to cause black foot disease (Maluta and Larignon, 1991). Since 1930, "*Botryosphaeria*" spp. have also been associated with the disease currently known as black dead arm. Shoemaker (1964), Lehoczky (1974) and other authors later described a number of the *Botryosphaeria* species involved in black dead arm. Finally, Eutypa dieback, a fungal disease of vine wood found in most grape-growing areas, and which usually causes greater crop losses in areas of high rainfall than in high-yielding inland vineyards, is caused by *Eutypa lata*.

Colony morphology, chromogenicity and mycelial growth rates provide the first indications of the type of fungi infecting wood samples. Species identification is then based mainly on morphological characteristics such as the size, shape, colour, septation, wall thickness and the texture of the conidia. Nevertheless, such features can show a degree of plasticity. Therefore, in this work, molecular analyses were used to confirm the identity of the different fungi (particularly in the case of *Botryosphaeria*-like spp., which were identified using two complementary methods). Nucleotide sequences of the ribosomal DNA (rDNA) of fungi contains conserved and variable regions that can be used to differentiate these organisms at different taxonomic levels. Based on PCR amplification of the 5.8s ribosomal RNA gene, the flanking internal transcribed spacers (ITS) and the D1/D2 variable domain of 28S rDNA and the generation of restriction fingerprints, 10 "*Botryosphaeria*" species were identified by Alves *et al*. (2005). The present work involved a similar analysis. The sequences of the ITS amplicons produced with the ITS4 and the ITS5 primers (White *et al.,* 1990) were also determined to help to identify the different species.

The aim of this work was to identify the different fungi associated with grapevine decline in young and mature grapevines from Castilla y León (Spain). The samples studied were complete vines, branches from symptomatic grapevines, and complete young plants from nurseries.

Materials and methods

Sample analysis

Fungi associated with grapevine decline were obtained from 84 vine branches, most of them with eutypa foliar symptoms and from 22 complete vines with esca or apoplexy foliar symptoms, collected at different sites in the main Appellation area of Castilla y León. Varieties analysed were Tempranillo, Verdejo, Garnacha, Mencia, Viura, Prieto picudo and Sauvignon. Seventy-four samples were collected from young symptomatic plants or asymptomatic vines provided by nurseries. The young plants were up to five years old; plants over five years of age were considered adult or mature plants.

A total of 180 samples was analysed; 124 harboured at least one type of fungal species associated with grapevine decline. For the identification of *Botryosphaeria*-like species (Alves *et al*., 2005) isolates (*D. seriata* as *B. obtusa* CBS 719.85 and 112555; *B. dothidea* CBS 113190; *D. mutila* as *B. stevensii* CBS 43182; *N. parvum* as *B. parva* CBS 110301; *N. ribis* as *B. ribis* CBS 637.77; *Lasiodiplodia theobromae* as *B. rhodina* CBS 11011; *N. luteum* as *B. lutea* CBS110299 and *Do. sarmentorum* as *Diplodia sarmentorum* CBS120.41) were obtained from the CBS fungal collection (Utrecht, The Netherlands) and used as references for the comparison of endonuclease restriction patterns.

Isolation of fungi and morphological identification

Different cutting were made from vine branches. From the complete vines wood tissue was sampled from roots, rootstock, graft union, graft and branches. In all cases small pieces of tissue were sampled from wood with or without internal symptoms and surface-sterilised. Seven tissue pieces per explant were placed on malt extract agar with 0.25 mg ml^{-1} chloramphenicol in Petri dishes and incubated at 25ºC in the dark. The colonies that developed were transferred to potato dextrose agar (PDA) for identification. They were exposed to UV light until sporulation. Isolates were identified to genus or species level by their morphological, culture and sporulation characteristics (Armengol *et al*., 2001).

Molecular identification

Restriction patterns

Genomic DNA was isolated and amplified from fresh mycelium using the REDExtract-N-Amp Kit (XNAP) (Sigma, St. Louis, Missouri, USA) following manufacturer's instruction, or the method described by Alves *et al*. (2005). All PCR amplifications were performed using a PerkinElmer thermocycler; the primers used were ITS1 and NL4 (IZASA, Barcelona Spain). An amplicon of about 1200 bp was detected for each of the isolates tested.

The amplification products (30 μl) were then digested with the endonucleases TaqI, BsuRI, SauI (Bioron Gmbh, Ludwingshagen, Germany) and AluI (Biotools Biotechnological and Medical Laboratories S.A, Madrid, Spain) following manufacturer's instructions. The DNA fragments were separated electrophoretically on 3% agarose (Sigma) gels in 1×TBE buffer using a constant voltage of 90 V for 3 h. The gels were stained with ethidium bromide and visualized with a UV transilluminator. The specific restriction patterns for the different species were recorded.

Sequence analysis

DNA extractions were obtained as described above. PCR amplification of the nuclear 5.8S ribosomal RNA gene and its flanking ITS regions was performed using the XNAP Kit as above, following manufacturer's instructions. All amplifications were performed using a PerkinElmer thermocycler. The primers used were ITS4 and ITS5 (White *et al*., 1990) (IZASA). The amplification conditions were: initial denaturation for 5 min at 95ºC, followed by 25 cycles of 1 min at 94ºC, 1 min at 58ºC, 1 min at 72ºC, and a final extension step of 10 min at 72ºC. After amplification, 5 μl of each PCR product was separated by electrophoresis on 1% agarose gels in 1×TBE. The gels were stained with ethidium bromide and visualized using a UV transilluminator. The amplified PCR fragments were purified using a GFX PCR DNA Gel Band Purification Kit (Amersham, Buckinghamshire, UK) before sequencing.

DNA sequencing

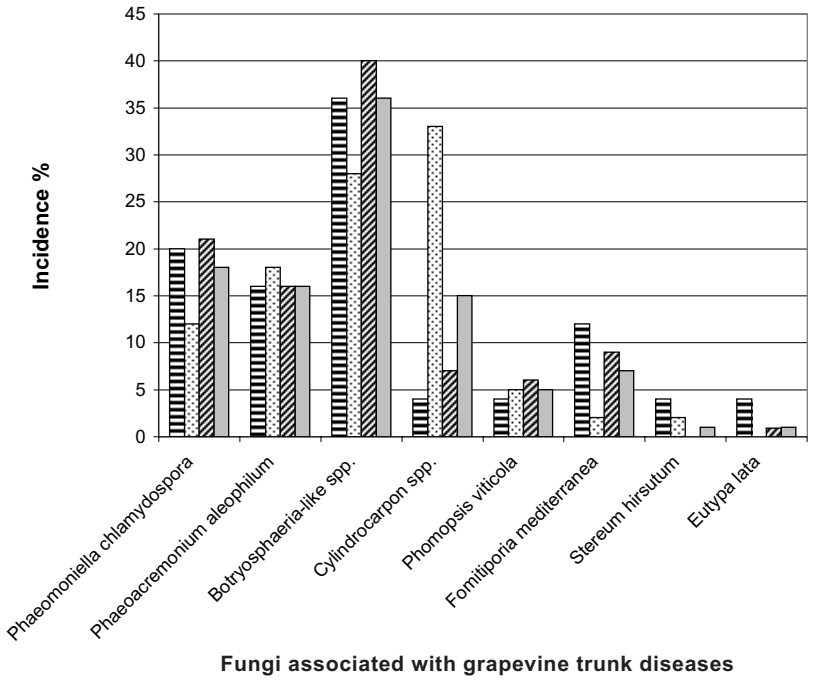
PCR fragments were sequenced by the Instrumental Techniques Laboratory of the University of León. The DYEnamic ET Dye Terminator kit (MegaBACE, Amersham) was used for the sequencing reaction; amplification was performed using a MJ Research PTC-200 thermocycler. The DNA sequences were analysed in a MegaBACE 500 sequencer (Amersham). The complete sequence of the ITS region was read and edited using Chromas v.1.45 software.

Results

Most of the fungi isolated on PDA could be identified based on their morphology in culture (colour and texture of the mycelium and the characteristics of the conidia). These identifications were confirmed by molecular analysis. Fig. 1 shows the incidence of the different fungi found. The 180 samples analysed (divided into the categories of complete adult plants, complete young samples and branches of adult plants) gave 194 isolates of interest. Fig. 1 shows the results for each fungus in each category, and the means of all categories. Young plants were less commonly infected with *P. chlamydospora* mean incidence (12%) than were adult plants (18%). The incidence of *P. aleophilum* was the same in samples from all categories (16%). *Botryosphaeria*-like species were found mainly in the branches (40%), but less often in young samples (28%); the mean for the three categories was 36%. *Cylindrocarpon* spp. were uncommon in adult plants (around 5%); however, they were isolated from 33% of young samples (mean incidence for all categories = 15%). *P. viticola*, *F. mediterranea*, *S. hirsutum* and *E. lata* were found rarely, and mostly on adult plants. The incidence of these last four fungi together was only 14%; with *P. viticola* and *F. mediterranea,* the most common, accounting for 5% and 7% respectively.

Botryosphaeria-like species were common and molecular tools were used to distinguish them. Using 10 reference cultures from CBS, typical restriction enzyme banding patterns were compared with those of the different isolates. The TaqI endonuclease pattern distinguished between *D. seriata*, *D. mutila*, *B. dothidea* and *N. parvum*, which showed specific bands of 124 bp, 173 bp, 243 bp and 117 bp respectively (Fig. 2).

With respect to the BsuRI endonuclease patterns, *D. seriata* had a specific band of 113 bp, the *D. mutila* pattern had no such band (Fig. 2), while *B. dothidea* and *N. parvum* had specific bands of 716 bp and 268 bp respectively. Other patterns



	Number of samples	Number of isolations
Complete young plants (<5 years)	74	60
Complete adult plants (> 5 years)	22	25
Complete plants	96	85
Branches	84	109
Total	**180**	**194**

Fig. 1. Incidence of fungi isolated from grapevine. The samples were divided into three categories depending on wether they were taken from: complete adult vines, complete young vines, and adult vine branches. Means of these categories are also given.

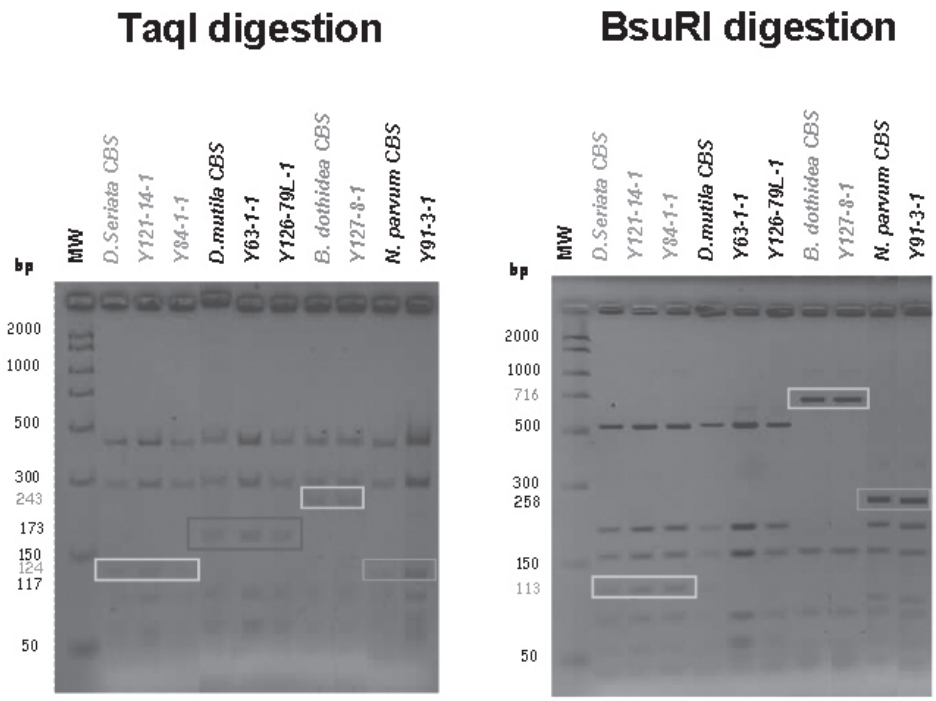


Fig. 2. Endonuclease restriction patterns TaqI, and BsuRI, for distinguishing between different *Botryosphaeria*-like species.

were obtained that allowed differentiation between *Do. sarmentorum*, *N. ribis*, *L. theobromae* and *N. luteum*. However, *Do. iberica* and *Do. sarmentorum* could not be distinguished from one another. Neither *N. ribis*, *L. theobromae* nor *N. luteum* were isolated from any of the present samples and this was confirmed by the ITS sequence data.

DNA extracted from *Botryosphaeria*-like species were amplified by PCR using ITS4 and ITS5 primers. The sequences of the ITS4-ITS5 amplicons (around 650 bp) obtained were compared with the sequences of the GenBank data bases. The results confirmed the absence of *N. ribis*, *L. theobromae* and *N. luteum* and the presence of *Do. iberica* and

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B.dothidea          CTCCGGCTCGACTCTCCCACCCTATGTGTACCTACCTCTGTTGCTTTGGCGGGCC-GCGG 59
N.parvum            CTCCGGCTCGACTCTCCCACCCAATGTGTACCTACCTCTGTTGCTTTGGCGGGCC-GCGG 59
Do.iberica          CTCCGGCTCGACTCTCCCACCCTTTGTGTACCTACCTCTGTTGCTTTGGCGGGCCCGCGG 60
Do.sarmentorum      CTCCGGCTCGACTCTCCCACCCTTTGTGTACCTACCTCTGTTGCTTTGGCGGGCCCGCGG 60
D.mutila            CTTCGGCTCGAATCTCCCACCCTTTGTGAACATACCTCTGTTGCTTTGGCGG-CTCTTTG 59
D.seriata           CTTCGGCTCGAATCTCCCACCCTTTGTGAACGTACCTCTGTTGCTTTGGCGGGCTCTTTG 60
                    ** ******** **********  **** ** ********************* *     *

B.dothidea          TCCTCCGCACCGGCCCCCCTTCGGGGGGCTGGCCAGCGCCCGCCAGAGGACCACAAAACT 119
N.parvum            TCCTCCGCACCGGCGCCC-TTCGAGGGGCTGGCCAGCGCCCGCCAGAGGACCATAAAACT 118
Do.iberica          TC---------GGCCTC--------GTGCCGTCCAGCACCCGCCAGAGGACCACAAAACT 103
Do.sarmentorum      TC---------GGCCTC--------GTGCCGTCCAGCACCCGCCAGAGGACCACAAAACT 103
D.mutila            CC--GCGAGGAGGCCCTCGCGGGCCCCCCCCGCGCGCTTTCCGCCAGAGGACCTTCAAACT 117
D.seriata           CC--GCGAGGAGGCCCTCGCGGGCCCCCCCCGCGCGCTTTCCGCCAGAGGACCTTCAAACT 118
                     *         ***              * *  *     *************   *****

B.dothidea          CCAGTCAGCAAACGTCGCAGTCTGAAAAACAAGTTAATAAACTAAAACTTTCAACAACGG 179
N.parvum            CCAGTCAGTGAACTTCGCAGTCTGAAAAACAAGTTAATAAACTAAAACTTTCAACAACGG 178
Do.iberica          CCAGTCAGTAAACGTCTCAGTCTGAAAAACAAGTTAATAAACTAAAACTTTCAACAACGG 163
Do.sarmentorum      CCAGTCAGTAAACGTCTCAGTCTGAAAAACAAGTTAATAAACTAAAACTTTCAACAACGG 163
D.mutila            CCAGTCAGTAAACGTCGACGTCTGATAAACAAGTTAATAAACTAAAACTTTCAACAACGG 177
D.seriata           CCAGTCAGTAAACGTCGACGTCTGATAAACAAGTTAATAAACTAAAACTTTCAACAACGG 178
                    ********  *** **   ****** ************************************

B.dothidea          ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC 239
N.parvum            ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC 238
Do.iberica          ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC 223
Do.sarmentorum      ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC 223
D.mutila            ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC 237
D.seriata           ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC 238
                    ************************************************************

B.dothidea          AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGG 299
N.parvum            AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGG 298
Do.iberica          AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGG 283
Do.sarmentorum      AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGG 283
D.mutila            AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGG 297
D.seriata           AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGG 298
                    ******************************************** *** ****** ****

B.dothidea          CATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCTCCGTCCTC 359
N.parvum            CATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCCCCGTCCTC 358
Do.iberica          CATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCTCCGTCCTC 343
Do.sarmentorum      CATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCTCCGTCCTC 343
D.mutila            CATGCCTGTTCGAGCGTCATTACAACCCTCAAGCTCTGCTTGGTATTGGGCGCCGTCCTC 357
D.seriata           CATGCCTGTTCGAGCGTCATTACAACCCTCAAGCTCTGCTTGGTATTGGGCGCCGTCCTC 358
                    ********************* ****************************** ********

B.dothidea          C--ACGGACGCGCCTCGAAGACCTCGGCGGTGGCGTCTTG--CCTCGAGCGTAGTAGAAA 415
N.parvum            C--ACGGACGCGCCTTAAAGACCTCGGCGGTGGCGTCTTG--CCTCAAGCGTAGTAGAAA 414
Do.iberica          ---AGGGACGCGCCTCAAAGACCTCGGCGGTGGCGTCTTG--CCTCAAGCGTAGTAGAAA 398
Do.sarmentorum      ---AGGGACGCGCCTCAAAGACCTCGGCGGTGGCGTCTTG--CCTCAAGCGTAGTAGAAA 398
D.mutila            TCTGCGGACGCGCCTTAAAGACCTCGGCGGTGGCTGTTCAGCCCTCAAGCGTAGTAGAAT 417
D.seriata           TCTGCGGACGCGCCTTAAAGACCTCGGCGGTGGCTGTTCAGCCCTCAAGCGTAGTAGAAT 418
                         **********  *****************   *    **** ************

B.dothidea          ACACCTCGCTTTGGAGCGCACGGCGTCGCCCGCCGGACGAACC 458
N.parvum            ACACCTCGCTTTGGAGCGCACGGCGTCGCCCGCCGGACGAACC 457
Do.iberica          ACACCTCGCTTTGGAGTGCATGGCGTCGCCCGCCGGACGAACC 441
Do.sarmentorum      ACACCTCGCTTTGGAGTGCATGGCGTCGCCCGCCGGACGAACC 441
D.mutila            ACACCTCGCTTTGGAGCGGTTGGCGTCGCCCGCCGGACGAACC 460
D.seriata           ACACCTCGCTTTGGAGCGGTTGGCGTCGCCCGCCGGACGAACC 461
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Fig. 3. Multiple alignments obtained with ITS4-ITS5 primers that amplify the nuclear 5.8S rDNA, and its flanking ITS regions (650bp) of *Botryosphaeria*-like species. In the aligned sequences, asterisk = match, dash = gap.

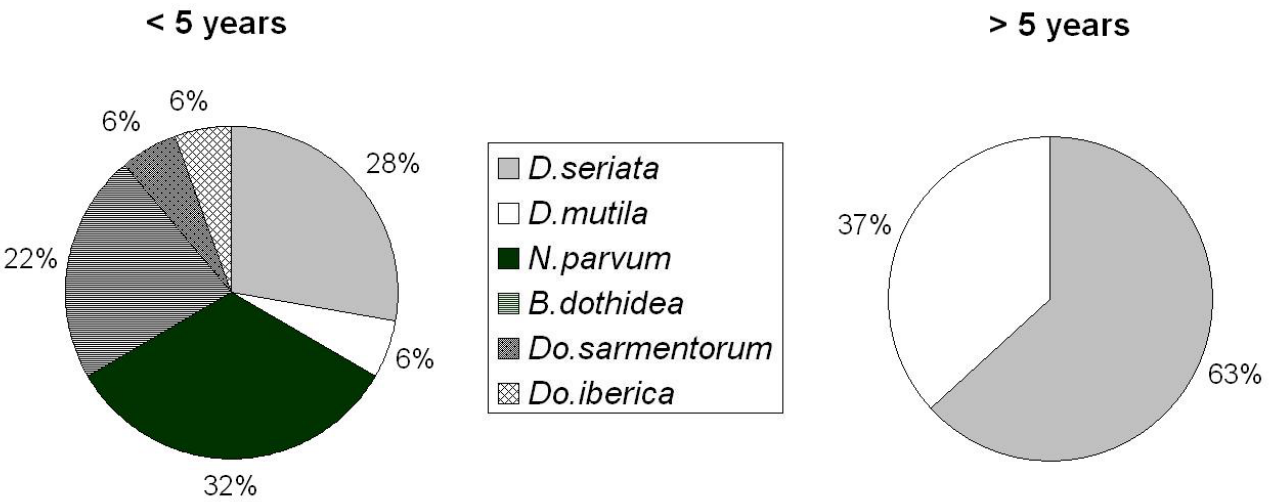

Fig. 4. Percentages of *Botryosphaeria*-like species identified in young and adult vines.

Do. sarmentorum. Sequence comparison of the six species found showed the three well-known regions of homologies. The analysis revealed that these six species formed three homology groups: i) *Do. iberica* with *Do. sarmentorum*, ii) *N. parvum* with *B. dothidea* and iii) *D. seriata* with *D. mutila* (Fig. 3).

Figure 4 shows the results for young and adult vine samples. Only two species of "*Botryosphaeria*", *D. seriata* and *D. mutila*, were identified on adult samples. In contrast, six species were identified on young vines, namely *D. seriata* and *D. mutila* (as on adult samples) together with *N. parvum, B. dothidea, Do. iberica* and *Do. sarmentorum*. *D. seriata* was found in both young and adult vines (28% and 63% respectively). *D. mutila* occurred mainly on adult plants. *Neofusicoccum parvum* and *B. dothidea* were present, although commonly, only on young vines (32% and 22% respectively), while the incidence of both *Do. iberica* and *Do. sarmentorum* was low (6% each).

Discussion

Annual surveys of the vineyards of Castilla y León have been undertaken since 2001; by 2006 the incidence of grapevine decline had reached 7% (data not shown). Grapevine decline was increasing in Castilla y León. The Trentino area in Italy seems to be suffering the same fate (Michelon *et al*., 2006). The increasing incidence of grapevine decline require a continuous surveying over the following years to confirm this trend.

The most commonly identified fungi associated with grapevine decline were *P. chlamydospora*, *P. aleophilum*, *D. seriata* and *D. mutila*. *P. viticola*, *F. mediterranea*, *S. hirsutum* and *E. lata* were identified on only a few occasions, perhaps because of the relatively low number of complete adult vines analysed. However, these figures agree with those of Armengol *et al*. (2001) who based themselves on samples from different areas of Spain. Data from other countries show different distributions. For instance, in French vineyards *E. lata* is common (Dubos *et al*., 1980) and shows variation in aggressiveness and genetic structure (Peros and Berger, 2003). Further investigation showed the existence of several species within the genus *Eutypa* (Rolshausen, 2004). In addition, *F. mediterranea* has often been cited as being involved in esca, along with *P. aleophilum* and *P. chlamydospora* (Surico *et al*., 2006). However, our results indicated that *F. mediterranea* was found only rarely, as were *S. hirsutum* and *P. viticola*.

Cylindrocarpon spp. were mainly identified on young vines. This is consistent with studies showing that infections with different *Cylindrocarpon* spp. occur in nursery soil. At the time of planting, the susceptible basal ends of most nursery cuttings are exposed to fungal contamination. The first infections occur in the roots and then spread to oth-

er organs during the course of the growing season (Halleen *et al*., 2004, 2006).

Since *Botryosphaeria*-like species grow quickly and spore formation is late, it is more efficient to identify these species by molecular techniques. Two methods based on PCR were used in the present study: 1) a rapid method involving the analysis of the banding obtained by PCR amplification of the ITS1-NL4 region and endonuclease digestion of the products, as described by Alves *et al*. (2005), and 2) the sequencing of the amplicon obtained by PCR using the ITS4 and ITS5 primers. Sequence homologies indicated that *D. seriata* and *D. mutila* formed a group, *Do. iberica* and *Do. sarmentorum* formed a second group; but that the *B. dothidea* and *N. parvum* sequence homology was less. These results were in agreement with the recently revised *Botryosphaeria* genus (Crous *et al*., 2006; Phillips *et al*., 2007) where *D. seriata* and *D. mutila* are included in the *Diplodia* lineages and *Do. iberica* and *Do. sarmentorum* are included in the *Dothiorella* lineages, while *Botryosphaeria dothidea* and *Neofusicoccum parvum* are included in *Botryosphaeria* and *Neofusicoccum* lineages respectively.

The sequence data and endonuclease patterns showed that the young vines were home to a wide range of *Botryosphaeria*-like species (*D. seriata*, *N. parvum*, *B. dothidea*, *D. mutila, Do. iberica* and *Do. sarmentorum*) while the mature vines harboured only *D. seriata* and *D. mutila*. Urbez-Torres *et al*. (2006) identified only *D. seriata*, *N. parvum* and *B. dothidea* from vines collected from similar areas of Castilla y León, where new potential pathogenic *Botryosphaeria*-like species could be introduced with young vines. The present work suggests six fungi (*D. seriata*, *N. parvum*, *B. dothidea*, *D. mutila, Do. iberica* and *Do. sarmentorum*) are associated with the growing problem of grapevine decline in Castilla y León.

Botryosphaeria-like species are ubiquitous wherever grapevines are grown (van Niekerk *et al*., 2006) and different species are frequently isolated from the same vines as we identified *P. chlamydospora* and *Phaeoacremonium* spp., fungi associated with esca. The analysis of more than 22 complete adult vines will make it possible to establish a classification into categories of symptoms observed on the herbaceous parts and the wood parts of roots, rootstock, graft union, graft and branches. Further studies are needed to investigate the pathogenicity of the different *Botryosphaeria*-like species found in vines.

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