

Fungi associated to grapevine trunk diseases in young plants in Asturias (Northern Spain)

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Abstract

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Dark discolorations of the vascular vessels of 3-year-old potted plants of Asturian grapevine cultivar (Northern Spain), were observed during pruning. These symptoms can be associated to fungal trunk diseases that, in the last decades, are affecting young vineyards all over the world. Cross sections from root, trunk and canes of 19 young grapevine plants were analyzed for the presence of pathogenic fungi associated with these diseases. Non-pathogenic fungi were isolated from both asymptomatic and symptomatic samples, showing that dark discolorations, in some cases, were a consequence of abiotic causes. Regarding pathogenic fungi, *Cylindrocarpon* spp. colonies were the most frequent, isolated mainly from roots and from asymptomatic tissue. *Botryosphaeria* spp. colonies were mostly isolated from trunk and from sections with dark discolorations generated by pruning. *Phaeoacremonium* spp. was isolated from all the organs (roots, trunk and canes). Only one colony of *Libertella* spp. was isolated. These results suggest the need of a standard protocol, combining treatments and management activities, to be performed in nurseries to limit the spread of these diseases.

Keywords: grapevine decays; young vines; fungal pathogens; *Vitis vinifera* L.

Trunk diseases cause a slow decline of grapevines due to internal alterations of wood, reducing yield and quality of grapes, even leading to partial or total death of the plant. Traditionally, these diseases were associated with pathologies present in adult grapevines, over 10-year-old, such as esca and eutypiosis; however, in recent years an increase in the incidence of these diseases in young plants (one to four years) was recorded worldwide (BERTELLI et al. 1998; GIMÉNEZ-JAIME et al. 2006; DUBROVSKY, FABRITIUS 2007), generating major economic losses in new plantations. This increase could be a conse-

quence of the prohibition of using sodium arsenite, the only treatment effective against these diseases. Petri disease, black foot and black dead arm (BDA) are trunk diseases present in young grapevines for which an association between symptoms and etiological fungal agents was established. *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* were associated with Petri disease (GARCÍA-JIMÉNEZ et al. 2002; FOURIE, HALLEEN 2004). *Cylindrocarpon* spp. fungi were identified in plants with black foot (PETIT, GUBLER 2005; ALANIZ et al. 2007), and sometimes appear associated to Petri disease as

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well (GARCÍA-JIMÉNEZ et al. 2002). *Botryosphaeria* spp. were associated with BDA (LARIGNON et al. 2001). Young vines affected by these diseases are characterized by poor development, stunted shoots and small and chlorotic leaves and, in the case of BDA, foliar symptoms are very similar to those of esca. Cross sections of plants with decay symptoms show dark discolorations of the vascular vessels (sector-shaped or spots or dark discoloration areas concentric to the pith). While the omission of certain cultural practices during pruning contributes to the transmission of these diseases (disinfection of pruning tools between plants, protection of the cuts with fungicide, pruning until reaching healthy tissue, burning of pruning wood debris, application of a fungicide treatment immediately after pruning), the greatest risk of transmission is due to the use of infected propagation material, making rigorous control of these diseases in nurseries necessary (GIMÉNEZ-JAIME et al. 2006; DUBROVSKÝ, FABRI-TIUS 2007; REGO et al. 2009).

Several screenings were performed in Spanish nurseries. RAPOSO and AROCA (2005) found a 51.1% of the total plants analyzed infected, and *Botryosphaeria* as the most extended genera. GIMÉNEZ-JAIME et al. (2006) found *Botryosphaeria* and *Cylindrocarpon* at the first stages of production in nursery, and *Phaeomoniella* and *Phaeoacremonium* as the most frequently isolated fungi prior to planting in field. These authors studied young vineyards too, finding a 76.4% of them affected with trunk pathogens. *P. chlamydospora* was the most frequently isolated species, followed by *P. aleophilum*, *Botryosphaeria* spp., *Cylindrocarpon* spp., and *B. obtusa*.

The SERIDA (Regional Service of Agri-Food Research and Development of Asturias, Villaviciosa, Spain) is carrying out, since 2003, the process of clonal selection of grapevine cultivars (LOUREIRO et al. 2011). Since 2006, plant material of the candidate clones was collected from field-grown mother plants that showed no apparent disease problems, planted and kept in the conditions required by the clonal selection protocol. At pruning time, extreme care is taken in disinfecting the pruning tools after pruning each plant and in applying fungicide treatments. During the pruning of 2009, symptoms of fungal trunk diseases were observed on transverse sections of cuttings of various 3-year-old plants, which did not show any external symptom of decay and whose development was normal. Several of them were selected for analysis in order to en-

able the identification of possible fungal agents causing the symptoms observed. The aim of this work was to determine the presence of pathogenic fungi causing trunk diseases in these plants in order to decide the sanitation procedures to apply to the plant material, if needed. This is of great importance because these plants are intended for the production of certified clones of grapevine.

MATERIAL AND METHODS

Plant material. Cuttings from 19 different candidate clones from five Asturian grapevine cultivars (two clones of cv. Albarín Blanco, five of cv. Albarín Tinto, three of cv. Carrasquín, four of cv. Mencía and five of cv. Verdejo Tinto) were planted in pots with commercial planting substrate. After three years of cultivation, plants showed dark discolorations of wood, so they were up-rooted and analyzed. All the analyses were performed in the laboratories of the CRA-VIT, Experimental Institute for Viticulture of Conegliano, Conegliano, Italy.

Preparation of samples. Each part of the plant (roots, trunk and canes) was weighed separately. The length and the basal, middle and apical diameter of the trunk and canes of each plant were recorded. The number of roots with a diameter larger or smaller than 2 mm was counted, and the good, medium or poor root development was also scored. Two roots > 2 mm and two < 2 mm in diameter from each plant were selected for the analysis. Trunk and canes were cut at each node generating as many pieces as internodes; pieces were then numbered according to their position in the plant from the base to the apex. The basal and apical sides of each piece were observed, scoring the percentage of the cut surface affected by dark discoloration. The pieces of each cane that showed some symptoms were selected for the analysis; in case that no symptom was present, wood pieces with alternate positions within the same cane were selected for analysis. Wood pieces were peeled, just leaving some of them with part of the bark to be analyzed, when it did not have a healthy-looking appearance. After selecting the wood pieces to be analyzed, isolation of fungi was performed.

Isolation of fungi. All selected pieces from root, trunk and canes were flame-sterilized. Sections of 1 mm thick from basal and apical sides of each piece were cultured on Petri dishes containing Malt Agar (MA) (1.5% malt extract agar, 1.25%

Table 1. Abbreviation codes and description of the observed wood symptoms, number and percentage of samples isolated

Code	Symptom description	Samples	
		No.	%
A1	sector-shaped dark discoloration	3	0.4
A2	halo dark discoloration	5	0.6
A5	dark discoloration generated by an old pruning wound which is now embedded on the wood tissue	13	1.5
AAL	dark discoloration around the pith	30	3.5
AT	peripheral dark discoloration caused by recent pruning wounds	45	5.3
D	brown spots	59	6.9
DN	black spots	33	3.9
Str	streak observed when a longitudinal section is made	66	7.8
Bk	symptoms on the bark (injuries, spots, superficial cracks, alterations of the colour)	27	3.2
F	dark discoloration on roots	12	1.4
Dry	dry wood	5	0.6
S	asymptomatic	550	64.7
AAL + DN	presence of both symptoms: AAL and DN	2	0.2

agar in distilled water) supplemented with 0.01% of a chloramphenicol solution of 25 mg/ml in ethanol. In the cases where the same side of a piece clearly showed different symptoms, a piece of each of them was isolated and cultured separately. A longitudinal section of each piece was also performed and, if dark streaks were present, a sample of them was cultured. An apparent healthy section (asymptomatic) of each piece was also cultured as a control. The vine of origin and the position of the piece on the plant from which the section was isolated were recorded, as well as the symptoms present on the section (Table 1). Dishes were incubated at 25°C in the dark and, after 15 days, growth was checked every week. Once the mycelium was developed, identification of fungi, at a genus level, was performed morphologically by direct observation of the colonies under the stereomicroscope and the optical microscope. Petri dishes with no mycelium growth were left in incubation for at least two months for further verification.

Statistical analyses. The frequency of the diverse kind of symptoms analyzed was evaluated, as well as the frequency of isolation of the main fungal genera in relation to the part of the plant (roots, trunk and canes). Analysis of the variance (ANOVA) and comparison between the average values were performed according to the Student Newman-Keuls

test ($P \leq 0.05$). The analyses were performed with the statistic package Costat New 5.034 [Cohort Software (www.cohort.com)].

RESULTS AND DISCUSSION

All vines analysed had a healthy external appearance and good growth. In addition, the state of the roots was good for all of them, except for a plant of cv. Mencía, which had a medium development (data not shown). About 75% of the symptomatic sections were isolated from the trunk, while almost all sections isolated from canes and roots showed a healthy appearance.

A total of 850 samples were isolated and analysed *in vitro* for fungal identification; 550 of them (64.7%) were asymptomatic, while the rest showed different symptoms, mostly streaks (7.8%), but also brown spots (6.9%), peripheral dark discoloration caused by recent pruning wounds (5.3%) and other symptoms that represented less than 4% each one (Table 1). On the other hand, 369 samples (43.4% of the total number of samples) showed no mycelial growth; however, not all of them corresponded to asymptomatic sections, but 77 (20.8%) displayed some symptoms (data not shown). The lack of fungal growth highlighted that dark discolorations can

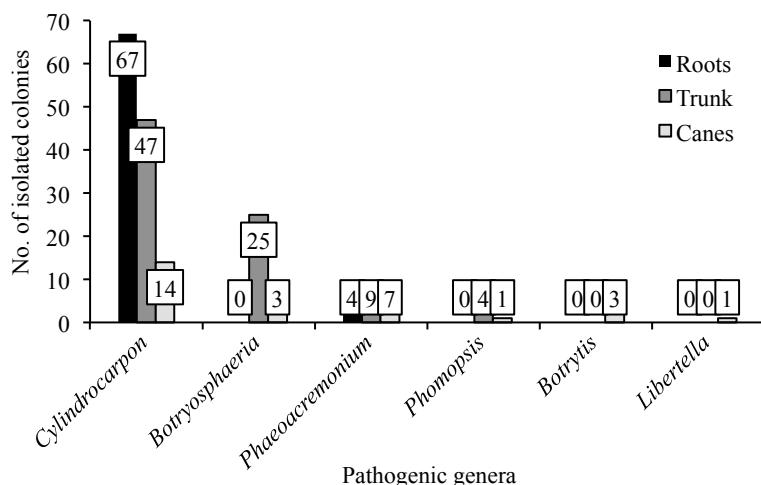


Fig. 1. Number of colonies of each pathogenic genera isolated from each organ

also be a consequence of abiotic factors (ZANZOTTO et al. 2007). In some of the samples with mycelial growth, multiple colonies of different genera grew, so each one was isolated for identification. A total of 597 isolates were obtained, of which 31% of the colonies were identified as pathogenic, 55% as non-pathogenic fungi and 14% could not be identified. Just over half of the non-pathogenic colonies were isolated from canes (52.9%); in the case of pathogenic ones, only 15.7% came from that organ, while 84.3% from trunk and roots (45.9% and 38.4%, respectively). In both cases, more than 40% of the colonies were isolated from asymptomatic samples.

Among the 329 colonies isolated belonging to trunk wood non-pathogenic taxa, 20 different genera were identified. The most frequently isolated were *Cladosporium* spp., *Penicillium* spp., *Fusarium* spp. and *Trichoderma* spp., which accounted for 72.9% of the total. HUERTAS-NEBREDA et al. (2010) also found the genera *Penicillium* and *Fusarium* at a high frequency in Spanish grapevines. Other genera isolated were: *Alternaria* spp., *Gliocladium* spp., *Acremonium* spp., *Epicoccum* spp., *Aureobasidium* spp., *Arthrinium* spp., *Aspergillus* spp., *Ulocladium* spp., *Phialophora* spp., *Sporothrix* spp., *Nigrospora* spp., *Mucor* spp., and different genera of the Coelomycetes group (*Pestalotia*, *Monochaetia*).

Regarding pathogenic fungi, a total of 185 colonies were isolated, corresponding to six different genera (Fig. 1). In descending order, *Cylindrocarpon*, *Botryosphaeria*, and *Phaeoacremonium* were the genera of pathogenic fungi most commonly isolated in the plant material analyzed, and they were isolated in 18, 6 and 11 plants out of the 19 analyzed, respectively. These three genera were also identified in other studies as the aetiological agents of the de-

cline of young vines in Spain (GARCÍA-JIMÉNEZ et al. 2002; AROCA et al. 2006; HUERTAS-NEBREDA et al. 2010). It is noteworthy that no colony of *Phaeoacremonium chlamydospora*, the species related with esca disease, was isolated. Only a single colony of *Libertella* (anamorph of *Eutypa lata*, which causes eutypiosis) was isolated from the bark of a cv. Carrasquín vine. It is generally believed that *Eutypa lata* is widespread in Spanish vineyards, but this result supports several recent studies where it was found

Table 2. Isolation frequencies of the most present fungal genera in the different part of the plant (root, trunk, canes)

Pathogenic genera	Roots	Trunk	Canes
<i>Acremonium</i>	1.5	2.3	1.1
<i>Alternaria</i>	0.0	2.4	2.4
<i>Botryosphaeria</i>	0.0	7.7	3.9
<i>Cladosporium</i>	5.4 ^b	5.3 ^b	27.9 ^a
<i>Cylindrocarpon</i>	46.2 ^a	19.1 ^a	7.0 ^b
<i>Fusarium</i>	11.4	12.8	4.5
<i>Gliocladium</i>	0.9	2.0	1.7
<i>Coelomycetes</i> group	0.0 ^b	4.3 ^a	0.9 ^b
<i>Libertella</i>	0.0	0.0	0.8
<i>Sterile mycelium</i>	4.0 ^b	13.3 ^a	9.3 ^{ab}
<i>Penicillium</i>	12.1	4.6	12.4
<i>Pestalotia</i>	0.0	1.7	2.8
<i>Phaeoacremonium</i>	3.0	5.7	5.6
<i>Phomopsis</i>	0.0	1.7	0.4
<i>Trichoderma</i>	0.8 ^b	7.4 ^a	8.4 ^a

values in rows with different letters are statistically different ($P > 0.05$) by Student Newman-Keuls test

Table 3. Isolation frequencies of the most frequent fungal genera from different symptomatic tissues

Fungal genus	Str	A1	A2	A5	AAL	AT	Bk	D	DN	F	S
<i>Acremonium</i>	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	1.8 ^b	0.0 ^b	0.0 ^b	0.0 ^b	2.6 ^b	2.5 ^a
<i>Alternaria</i>	0.9 ^b	0.0 ^b	0.0 ^b	3.5 ^b	0.0 ^b	4.0 ^{ab}	4.3 ^a	0.0 ^b	0.0 ^b	0.0 ^b	0.4 ^b
<i>Botryosphaeria</i>	6.6	0.0	5.3	1.8	0.0	7.5	5.3	4.1	5.3	0.0	0.7
<i>Cladosporium</i>	2.6 ^b	0.0 ^b	0.0 ^b	5.3 ^b	4.1 ^b	2.9 ^b	23.4 ^a	10.9 ^b	0.0 ^b	0.0 ^b	16.0 ^a
<i>Cylindrocarpon</i>	18.9 ^{bcd}	0.0 ^d	0.0 ^d	4.4 ^d	41.8 ^a	7.9 ^{cd}	8.4 ^{cd}	1.5 ^d	36.8 ^{ab}	15.4 ^{cd}	28.5 ^{abc}
<i>Fusarium</i>	9.0	0.0	1.8	2.6	14.9	6.0	5.7	7.5	6.6	12.3	6.0
<i>Gliocladium</i>	1.8	0.0	1.8	0.0	0.0	0.0	1.5	2.6	2.6	0.0	1.2
Coelomycetes group	6.9	5.3	1.8	6.6	0.0	5.0	0.7	2.5	0.0	0.0	0.4
<i>Libertella</i>	0.0	0.0	0.0	0.0	0.0	0.0	2.6	0.0	0.0	0.0	0.0
<i>Sterile mycelium</i>	15.4	0.0	0.0	3.5	3.7	12.7	14.3	13.5	1.3	0.0	6.5
<i>Penicillium</i>	6.9	5.3	0.0	1.8	2.6	14.0	6.3	1.6	0.0	3.9	13.8
<i>Phaeoacremonium</i>	1.8	0.0	0.0	5.3	0.0	5.0	0.0	1.5	10.5	0.0	4.0
<i>Trichoderma</i>	14.4	0.0	0.0	3.9	2.6	6.9	8.8	1.3	2.6	0.0	4.2

values in rows with different letters are statistically different ($P > 0.05$), Student Newman-Keuls test. For abbreviations, see Table 1

that this species is not present, or its frequency is very low (TORRES-CHICA et al. 2009; HUERTAS-NEBREDA et al. 2010). The other pathogenic fungi isolated were *Phomopsis*, associated to excoriosis, and *Botrytis*, causal agent of grey mould.

Cylindrocarpon was the most frequent trunk wood pathogen, with 128 colonies isolated (21.4% of the total number of colonies and 69.2% of the total number of pathogens), while *Botryosphaeria* and *Phaeoacremonium* spp. colonies were identified in 15.1% and 10.8% of the total pathogens' isolates, respectively, which corresponds to 4.7% and 3.3% of the total number of colonies.

No differences were apparent between cultivars for each specific symptom, but, taking into account

only the samples for which there was pathogenic fungal growth, differences were noted between the frequency of symptoms regardless of the variety (data not shown). In this case the most frequent symptoms were S, followed by Bk and Str (for abbreviations see Table 1).

The isolation frequencies according to the part of the plant from where the different genera were isolated are shown in Table 2. Most fungi did not show differences in the occurrence on the diverse part of the plant (root, trunk, canes). *Cylindrocarpon* spp. were mainly isolated from roots and trunk; yet more than half of the colonies were isolated from the roots (Fig. 1). However, the root samples analyzed were mostly asymptomatic (92.5%), and there

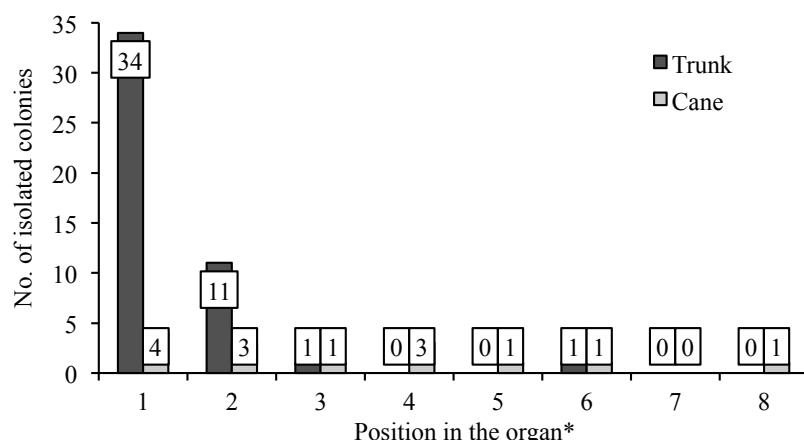


Fig. 2. Distribution pattern of *Cylindrocarpon* spp. isolates on the plant

*from the base (1) to the apex (8) of the plants

was no difference between the number of colonies isolated from the proximal and the distal part (33 and 34 colonies, respectively). No difference was observed in the occurrence of *Phaeoacremonium* and *Botryosphaeria* spp. Coelomycetes and the sterile mycelium were mainly collected from trunk, *Cladosporium* spp. from canes and *Trichoderma* spp. from canes and trunk (Table 2). The distribution on the plant of the pathogenic genera isolated is in accordance with the dispersal mechanisms of each one: *Cylindrocarpon* spp., a soil fungus, was mainly isolated from roots, in agreement with other studies (GARCÍA-JIMÉNEZ et al. 2002; HUERTAS-NEBREDA et al. 2010). In fact, when the distribution of the colonies was analyzed according to the position of the samples in the plant, it was clearly observed that the number of colonies of *Cylindrocarpon* decreased as the samples were taken further from the root (Fig. 2). *Botryosphaeria*, which has an air dispersal mechanism infecting through the pruning wounds, was absent in roots and chiefly present on the trunk, and *Phaeoacremonium*, which is spread by air and soil, was isolated from all the organs (root, trunk and canes). As far as distribution of the symptoms is concerned, the low incidence of symptoms in sections from canes can be due to the fact that they are regenerated with pruning each year (ZANZOTTO et al. 2007).

Considering the different wood symptoms observed, and with regard to the trunk wood pathogens, only *Cylindrocarpon* spp. showed differences in the isolation frequencies, being present especially in AAL, DN and S. More than half of *Cylindrocarpon* colonies were isolated from apparently healthy tissue. This can be explained by the fact that *Cylindrocarpon* is an opportunistic pathogen, so usually it produces damages to the tissues when plants are under stress. DUBROSKY and FABRITIUS (2007) also found *Cylindrocarpon* as the most frequent pathogenic fungi isolated from asymptomatic nursery vines. *Botryosphaeria* colonies were mainly isolated from samples with AT, D/DN and Str, but no difference was observed in its distribution between symptoms. Concerning *Phaeoacremonium* spp., half of the colonies were isolated from AT/A5 and D/DN, even though a noticeable number of them came from asymptomatic tissue (Table 3). ZANZOTTO et al. (2007) recorded the higher isolation percentages of *Phaeoacremonium* spp. on streaks, spots and brown-red halo symptoms; SIDOTI et al. (2000) found this species in spots and streaks.

Based on the results of the present study, it is clear that neither the presence of dark discolora-

tions on wood tissue is a confirmation of fungal problems, nor the absence of them is a guarantee of having healthy plant material. Thus, in addition to the prophylactic measures recommended during pruning, and to the special care to take with maintenance activities that cause some kind of wound to the vines, it is necessary to operate from nurseries as well. While other types of diseases have a legal control, with regard to fungal diseases there is no legislation about a protocol that guarantees a minimum of quality. Some countries, like Portugal, started to take legal decisions in this respect, allowing only one year of continuous grapevine planting in grapevine nurseries (REGO et al. 2009). There exist different methods that allow to reduce the incidence and severity of these pathogens like fungicide and/or hot water treatments or combination of them with a biological control agent such as *Trichoderma* (HALLEEN et al. 2006; GRAMAJE et al. 2009; REGO et al. 2009). Soil also plays an important role on the transmission of some pathogens, so regular (annually) controls of the field soil and planting substrates used by nurseries, as well as of the propagation material (by analyzing pieces of canes collected at pruning) should be considered.

Young vine samples were also analyzed by the Laboratory of Plant Health of Asturias (Oviedo, Spain) and they also isolated colonies mainly of *Cylindrocarpon* and, in a lesser extent, of *Botryosphaeria*, *Phaeoacremonium aleophilum*, and *Phaemoniella chlamydospora* (E. Landeras, pers. comm.). In that case, the analyzed vines came from field, were grafted, and roots were not analyzed. Most of the isolated colonies came from rootstock sections.

CONCLUSION

In conclusion, trunk diseases pathogens were isolated in grapevine material from Asturias, especially *Cylindrocarpon* although mainly from asymptomatic tissue. A protocol with a combination of treatments and/or management activities that helps to reduce the incidence of trunk diseases fungi should be created and performed in nurseries to limit the spread of these diseases.

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